University of Reading

# A Nutrigenetic approach to examine the relationship between vitamin B12 status and metabolic traits in multiple ethnic groups

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**DECLARATION OF AUTHORSHIP**

I confirm that this is my own work and the use of all materials from other sources has been properly and fully acknowledged.

Shelini Surendran

## ABSTRACT

Low vitamin B12 concentrations have been shown to be risk factors for metabolic traits in numerous observational studies; however, the relationship has remained inconsistent. It is possible that certain genotypes might jointly contribute to obesity and vitamin B12 deficiency, and these may be modulated by lifestyle factors (dietary factors and physical activity levels) across different ethnic groups. The implementation of a genetic approach to establish the relationship between vitamin B12 and obesity could be a more desirable option over observational studies, as results are less prone to confounding factors. Hence, the main aims of this thesis were to examine for the first time the association of common vitamin B12-related single nucleotide polymorphisms (SNPs) and metabolic SNPs with vitamin B12 concentrations and metabolic outcomes in multiple ethnic groups. In addition, the interaction between these SNPs and dietary factors (protein, carbohydrate and fat) on vitamin B12 concentrations and metabolic traits was investigated. A total of five studies with different study designs were used. These studies included a case-control study (Chennai Urban Rural Study; CURES, Asian India, n=900), three cross-sectional cohort studies [Genetics of obesity and Diabetes study (GOOD study; Sinhalese Sri Lankan adults, n=109), The Minangkabau Indonesia Study on Nutrition and Genetics (MINANG study; Indonesian women; n=118) and Brazilian adolescents (n=113)] and a 16 week-dietary randomized, single-blind, parallel-group dietary intervention [Dietary Intervention and VAScular function (DIVAS study; British adults, n=119)]. Gene-diet interactions were observed in the Sri Lankan and Indonesian populations between the vitamin B12-related SNPs and protein energy intake (%) on markers of central obesity (waist circumference (P=0.002) and body fat percentage (P= 0.034), respectively). In the Brazilian adolescent population, the metabolic and vitamin B12 related SNPs showed a significant interaction with carbohydrate and protein intakes on oxidised low density lipoprotein cholesterol (*P*=0.005) and homocysteine concentrations (P = 0.007), respectively, which are

well-known independent risk factors for cardiovascular disease. Additionally, in the Indonesian population, an interaction was observed between vitamin B12-related SNPs and dietary fibre intake (g) on glycated haemoglobin levels (P =0.042), a marker of long-term glycaemic status. Furthermore, for the first time, a novel association between two obesity-related SNPs and vitamin B12 concentrations (P = 0.018) was observed in the Indian population. In summary, these studies in multiple ethnic groups show that the relationship between B12 deficiency and metabolic outcomes may be influenced by dietary factors such as protein and fibre intake. However, in the Indian population, we found that vitamin B12 concentrations may be influenced by a genetic predisposition to obesity, but without a dietary influence. Given the limited sample size in some of the cohorts, replication of the study findings is highly warranted.

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**ABBREVIATIONS**

**Brazilian adolescent study:** SNPs, single nucleotide polymorphisms; *MTRR*, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; *COMT*, catechol-O-methyltransferase; *MTR*, 5-methyltetrahydrofolate-homocysteine methyltransferase; *MTHFR*, methylene tetrahydrofolate reductase; *TCN2*, transcobalamin 2; *BHMT*, betaine-homocysteine methyltransferase; *FUT2*, fucosyltransferase 2; BMI, body mass index; GWA, genome-wide association; ox-LDL, oxidized-low density lipoprotein.

**Sri Lankan (GOOD) study**: SNPs, single nucleotide polymorphisms; Methylenetetrahydrofolate reductase (*MTHFR)*; Carbamoyl-phosphate synthase 1 (*CPS1)*; Cubulin (*CUBN)*; CD320 molecule (*CD320)*; Transcobalamin 2 (*TCN2)*; Citrate lyase beta like (*CLYBL)*; Fucosyltransferase 2 (*FUT2)*; Transcobalamin 1 (*TCN1);* Fucosyltransferase 6 (*FUT6)*; Methylmalonyl-CoA mutase (*MUT);* Calpain 10 (*CAP10)*; Potassium voltage-gated channel subfamily J member 11 (*KCNJ11)*; Transcription factor 7-like 2 (*TCF7L2)*; Fat mass and obesity-associated (*FTO)* and Melanocortin 4 Receptor (*MC4R) ;*BMI, body mass index; SD, indicates standard deviations; WC, waist circumference; WHR, waist to hip ratio

**Indian (CURES) study**: SNPs, single nucleotide polymorphisms; GRS, genetic risk score; Fat mass and obesity-associated (FTO); BMI, body mass index; SD, indicates standard deviations; WC, waist circumference; WHR, waist to hip ratio

**Indonesian (MINANG) study**: SNPs, single nucleotide polymorphisms; Methylenetetrahydrofolate reductase (*MTHFR)*; Carbamoyl-phosphate synthase 1 (*CPS1)*; Cubulin (*CUBN)*; CD320 molecule (*CD320)*; Transcobalamin 2 (*TCN2)*; Fucosyltransferase 2 (*FUT2)*; Transcobalamin 1 (*TCN1);* Fucosyltransferase 6 (*FUT6)*; Methylmalonyl-CoA mutase (*MUT);* Calpain 10 (*CAP10)*; Potassium voltage-gated channel subfamily J member 11 (*KCNJ11)*; Transcription factor 7-like 2 (*TCF7L2)*; Fat mass and obesity-associated (*FTO)* and

Melanocortin 4 Receptor (*MC4R) ;*BMI*,* body mass index; SD, indicates standard deviations; WC, waist circumference.

**British (DIVAS) study:** SNPs, single nucleotide polymorphisms; Fucosyltransferase 2 (*FUT2)*; Calpain 10 (*CAP10)*; Transcription factor 7-like 2 (*TCF7L2)*; Fat mass and obesity-associated (*FTO)* and Melanocortin 4 Receptor (*MC4R);* BMI*,* body mass index; SD, indicates standard deviations; WC, waist circumference. SFA, saturated fatty acid; MUFA, with either cis-monounsaturated fatty acids; n–6 PUFA, polyunsaturated fatty acid; TE, total energy; TAG, triacylglycerol; TC, Total Cholesterol; high-density lipoprotein, HDL; low-density lipoprotein (LDL)

# Chapter 1

## Introduction to the thesis

### 1.1 Introduction

Vitamin B12 is an essential water soluble micronutrient, which participates as a cofactor for the synthesis of DNA, fatty acids, and myelin [1]. Vitamin B12 deficiency was previously thought to be limited to populations with a low intake of vitamin B12-rich foods (mainly vegetarians) and older adults, due to their impaired absorption of the vitamin through food [2]. However, alarmingly high prevalence rates of low plasma vitamin B12 status have been recognized to exist in the Indian subcontinent, Mexico, Central and South America, and selected areas in Africa [3]. Symptoms of vitamin B12 deficiency include haematological and neurological impairment. Additionally, observational studies have shown that low vitamin B12 concentrations are accompanied by a wide range of chronic diseases and conditions, including obesity, insulin dysregulation and adverse cardiometabolic outcomes [4-10].

Metabolic diseases such as type 2 diabetes and obesity are world-wide health problems, which are now increasingly diagnosed earlier in life. The metabolic diseases are generally caused by the interaction between environmental factors (dietary factors and sedentary lifestyle) and a genetic predisposition to the development of metabolic diseases [11]. Whilst dietary factors are an important contributor to metabolic disorders, this relationship differs across countries, due to the variation in food consumed worldwide [12]. Studies have shown that the intrauterine imbalance of vitamin B12 and folate can affect DNA methylation and ‘programme’ the offspring to develop metabolic disorders later in life [13] providing evidence for interactions between genes and nutrients in the development of metabolic disease.

Many candidate genes have been studied in relation to their potential role in vitamin B12 metabolism, and an association between these genes and vitamin B12 concentrations have

been confirmed [14]. To date only two Mendelian Randomization studies (an analytical tool used to measure the causal relations between modifiable risk factors and a clinically relevant outcome, using measured variation in genes of known function [15]) have explored the relationship between a genetically determined decrease in serum vitamin B12 concentrations on body mass index (BMI) [16] and cardiometabolic risk [17] highlighting the need for more studies. Vitamin B12 levels, which are not a homogenous phenotype, are responsive to changes in diet and are dependent on the quality and consumption of animal protein [18]. Therefore, controlling diet is recommended in preventing vitamin B12 deficiency [19]. Given that the genetic make-up varies from individual to individual, it is vital to examine the interactive effects between dietary factors and genetics on vitamin B12 concentrations and metabolic traits, which will ultimately allow us to personalise diet according to each ethnic sub-group [12]. Furthermore, other modifiable factors (e.g. physical activity), which could interact with genetic factors should be taken into account.

The following chapter will (i) explain the nutritional aspects of vitamin B12 (ii) focus on the importance of maintaining adequate vitamin B12 concentrations (iii) describe the symptoms associated with vitamin B12 deficiency (iv) explain the role of genes in influencing circulating vitamin B12 concentrations and (vi) explain the need for a nutrigenetics approach to study the role of genes and diet in the development of vitamin B12 deficiency and metabolic traits.

## 1.2 Vitamin B12 function

Vitamin B12 in the body is crucial for normal erythropoiesis [20]. Both folate and vitamin B12 are required for DNA synthesis, which codes for the production of billions of erythrocytes daily. Deficiency of either folate or vitamin B12 leads to the inhibition of purine and thymidylate synthesis, which impairs DNA synthesis. As a result erythroblast apoptosis and anaemia persists [20]. Additionally, vitamin B12 is an essential co-factor important for cell

metabolism, thus a deficiency will have serious clinical consequences. The intracellular conversion of vitamin B12 into two active co-enzymes, methylcobalamin (cytoplasm) and adenosylcobalamin (mitochondria) is essential for the homeostasis of methionine and methylmalonic acid, respectively [21, 22].

In the cytoplasm, methylcobalamin participates as a co-factor for the enzyme methionine synthase; which converts homocysteine to methionine. This reaction also depends on folate, where the methyl group of methyltetrahydrofolate is transferred to homocysteine, to produce methionine and tetrahydrofolate (**Figure 1**) [23]. A deficiency of vitamin B12 may lead to the increase in homocysteine concentration, which is a known marker of cardiovascular disease (CVD) [24]. Furthermore, methionine synthase is important for purine and pyrimidine synthesis [23].

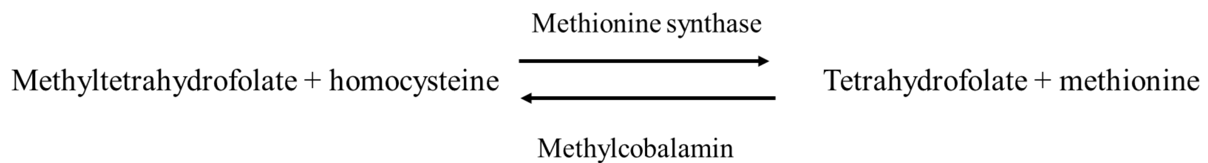


**Figure 1 Synthesis of methionine**

*This chemical reaction is catalysed by methionine synthase. Methylcobalamin is a co-factor which serves as an intermediate in the transfer of a methyl group from methyltetrahydrofolate to homocysteine.*

In mammals, the mitochondrial conversion of methylmalonyl-CoA to succinyl-CoA is catalysed by methylmalonyl-CoA mutase an enzyme which utilizes vitamin B12 (5-deoxy adenosyl cobalamin) as a co-enzyme [25, 26]. Subsequently, succinyl-CoA, which is important for lipid and carbohydrate synthesis, enters the Krebs cycle (**Figure 2**) [27]. A defect in the conversion of methylmalonyl-CoA to succinyl-CoA can cause the build-up of methylmalonyl-CoA which gets converts into methylmalonic acid (MMA), which has detrimental implications

on the nervous system. Accumulated MMA is thought to be a myelin destabiliser, where excessive MMA leads to the incorporation of abnormal fatty acids into the myelin sheath [28, 29].

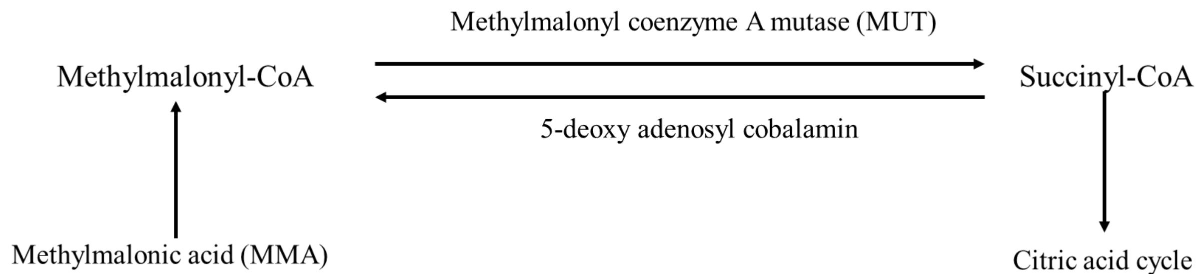


**Figure 2 Succinyl-CoA synthesis**

*This chemical reaction is catalysed by methylmalonyl coenzyme A mutase (MUT). 5-deoxy-adenosyl cobalamin is a co-factor which serves as an intermediate for the conversion of methylmalonyl-coA to succinyl-coA.*

## 1.3 Metabolism of vitamin B12

While ingesting food, the salivary and oesophageal glands release transcobalamin-I (TCN1, also known as haptocorrin), which binds strongly to vitamin B12. The function of TCN1 is to protect vitamin B12 from acid degradation in the stomach [30]. Once vitamin B12 reaches the duodenum, proteolytic enzymes from the pancreas release vitamin B12 from TCN1. Vitamin B12 then forms a new complex with intrinsic factor (IF), which is secreted by the gastric epithelium. The vitamin B12-IF complex interacts with the cubam receptor (consisting of cubilin and a receptor-associated protein) present on the apical surface of the distal ileal epithelium, at which the complex enters by endocytosis in the ileum [25]. Upon internalization, IF is degraded in enterocyte lysosomes, releasing the free vitamin B12 to the cytosol in the form of hydroxocobalamin [31]. Next, hydroxocobalamin is either transformed into methylcobalamin in the cytoplasm or to adenosylcobalamin in the mitochondria [32]. Alternatively, vitamin B12 is transported into portal circulation by the ABC drug transport

protein (ABCC1), also known as multidrug resistance protein (MRP1) [25, 33]. Vitamin B12 then binds to transcobalamin II (TC), which is then secreted into circulation and is transported as holotranscobalamin (holoTC) in serum and is distributed to tissues including the liver by receptor-mediated endocytosis [33].

In healthy adults, approximately 50-90% of vitamin B12 is stored in the liver as adenosylcobalamin (2000-5000 µg) [34, 35]. The remainder of vitamin B12 is stored in muscle, skin and blood plasma [36]. Approximately, 2-5 µg of vitamin B12 is lost daily as a result of cellular metabolism, irrespective of how much vitamin B12 is stored in the body [36]. Vitamin B12 is also excreted into bile (500 µg - 5000 µg) and is reabsorbed across the ileal enterocyte. Very small amounts of vitamin B12 absorption (1%–2% of an oral dose) occur by passive diffusion, and this route of absorption especially important for populations with limited or no intrinsic factor present (e.g., patients with gastric bypass surgery) [2].

## 1.4 Dietary sources and Bioavailability of vitamin B12

### 1.4.1 Bioavailability of vitamin B12

The intake of dietary vitamin B12 cannot be used as a sole measure of nutritional status. It is important to take into consideration how much of the vitamin B12 from the food source can be used systematically through normal body functions [37]. At present, the bioavailability of vitamin B12 is assumed to be between 40-50% for healthy adults with normal gastrointestinal functioning [37].

Absorption of vitamin B12 are traditionally assessed by measuring faecal extraction of radioactivity, after consuming 100g of a food item labelled with radioactive vitamin B12 [38]. In healthy humans, the absorption of vitamin B12 has shown to vary according to the type and quantity of protein consumed within the diet [19]. Studies assessing bioavailability of B12 from different food sources in healthy participants showed that the absorption of vitamin B12 was better in milk (65%) and chicken (61-65%), in comparison to eggs (24-36%) [38-41].

A further issue to take into consideration, when discussing bioavailability, is that IF-vitamin B12 receptors (present on the distal ileal epithelium), can be saturated and absorb a certain amount of vitamin B12 [38]. It is thought that approximately 1.5-2.0 µg of vitamin B12 can be absorbed from a meal, however other studies have reported higher absorption rates (up to 6 µg from a single meal) [37]. Bioavailability of vitamin B12 increases as the vitamin B12 content in food increases up to a certain point, and then it decreases if the vitamin B12 content is higher than the absorption capacity of the IF-vitamin B12 receptors [38].

Consuming processed food, improving hygiene and reheating cooked foods are some factors which reduce the bioavailability of vitamin B12 in foods [19]. Furthermore, the overgrowth of intestinal bacteria (because of poor dietary intake, antibiotics and stress), leads to the competitive uptake of vitamin B12 by bacteria and interferes with the bioavailability of vitamin B12 [42].

### 1.4.2 Nutritional Aspects of vitamin B12

Vitamin B12 is synthesized from bacteria growing in soil, sewage, water and the intestinal lumen of animals. Vitamin B12 enters animal tissues when animals ingest vitamin B12-producing bacteria present on legumes/roots or produced in the animal's rumen [38]. Although, micro-organisms in the human colon synthesize vitamin B12, humans cannot absorb it, as the majority of vitamin B12 is absorbed in the small intestine [43]. Consequently, the main sources of biologically active vitamin B12 vitamers are derived from animal products, such as milk, eggs, seafood and poultry [38]. Excellent sources of vitamin B12 include the livers of ruminant animals as well as shellfish, fish and fish roe (**Table 1**) [38, 43].

**Table 1**: **Contents of uncooked foods containing a high vitamin B12 content**

| Sources of B12 | Vitamin B12 content (µg/100 g) [a] |
|---|---|

| Beef liver | 60-122[b] |
|---|---|
| Shellfish[1] | 2-58 |
| Fish[2] | 3.0-8.0 |
| Fish roe[3] | 18 |

*The data has been extracted from a national Food composition data bank (The Danish National Food Institute, 2015). The data should be treated as an estimate, given that the food data base did not disclose how the levels of vitamin B12 were obtained.*

*[1] Clam, scallop, mussel, shrimp and oyster*

*[2] Salmon, trout, mackerel, and tuna*

*[3] Roe from Atlantic cod, lumpfish, and rainbow trout*

*[a]The adult UK Recommended Nutrient Intake (RNI) for vitamin B12 is 1.5 µg/day [44]*

*[b]The efficiency of absorption from liver is approximately 11% compared with 50% for other food [45].*

It is believed that individuals following a vegan/vegetarian diet are more susceptible to vitamin B12 deficiency [46]. Any vitamin B12 present on plant-derived products are usually because of bacterial contamination. However, some plants such as dried purple laver (nori), mushroom fruiting bodies fermented soybeans (Tempe), and tea leaves have been found to contain vitamin B12 [46, 47]. Most blue-green algae (cyanobacteria) and certain edible shellfish contain vitamin B12 analogues which are inactive in mammals and may inhibit cobalamin-dependent enzymes [48]. As a result, vegetarians and vegans are reliant on vitamin supplements containing vitamin B12, and foods such as breakfast cereals, soy milk and nutritional yeast products which are fortified with vitamin B12 [43].

### 1.4.3 Methods for the analysis of vitamin B12 in food

Several methods have been used to determine the vitamin B12 content in foods including microbiological assays, chemiluminescence assays, polarographic, spectrophotometric and high-performance liquid chromatography [49]. The microbiological assay has been the most commonly used assay technique for foods, utilizing certain vitamin B12–requiring microorganisms, such as *Lactobacillus delbrueckii* subsp.*lactis* ATCC7830 [19]. However, it is no longer the reference method due to the high measurement uncertainty of vitamin B12 [50]. Furthermore, this assay requires overnight incubation and may give false results if any inactive vitamin B12 analogues are present in the foods [38]. Currently, radioisotope dilution assay (RIDA) with labelled vitamin B12 and hog IF (pigs) have been used to determine vitamin B12 content in food [19]. Previous reports have suggested that the RIDA method is able to detect higher concentrations of vitamin B12 in foods compared to the microbiological assay method [19, 49]. New techniques employing more specific monoclonal antibodies and specific binding proteins are expected to advance the detection of vitamin B12 in food products [51].

### 1.4.4 Recommended dietary intake of vitamin B12

The recommended dietary intake (RDI) of vitamin B12 varies between countries. The European Union recommends 1 µg of vitamin B12, whilst the government of the United Kingdom and United States recommends a daily intake of 1.5 µg and 2.4 µg, respectively [23]. The requirements of vitamin B12 also varies according to age and whether a woman is pregnant or lactating, as shown in **Table 2**.

**Table 2: Recommended Dietary Allowance (RDA)/ Recommended Nutrient Intake (RNI) for vitamin B12**

| Age or condition | Vitamin B12 requirement (µg/day) in healthy U.S and Canadian populations [45] | Age or condition | Vitamin B12 requirement (µg/day) in a healthy UK population [44] |
|---|---|---|---|
| Pregnant | 2.6 | Pregnant | 1.5 |
| Breast-feeding | 2.8 | Breast-feeding | 2.0 |
| 0-6 mo | 0.4 | 0-6 mo | 0.3 |
| 7-12 mo | 0.5 | 7-12 mo | 0.4 |
| 1-3 yr | 0.9 | 1-3 yr | 0.5 |
| 4-8 yr | 1.2 | 4-6 yr | 0.8 |
| 9-13 yr | 1.8 | 7-10 yr | 1.0 |
| 14-18 yr | 2.4 | 11-14 yr | 1.2 |
| 19-50 yr | 2.4 | 15+ yr | 1.5 |
| 51+ yr | 2.4 | | |

The RNI of vitamin B12 for healthy British adult men and women is 1.5 µg/day and is based on the estimated average requirement (EAR) of vitamin B12 which is 1.25 µg/day for over 15 year olds (with no different recommendations for pregnant women)**[44]**. Although the daily requirement of vitamin B12 for people over the age of 50 is the same as younger adults (1.5 µg/day), this serves many problems. Individuals over the age of 51 years are at greater risk of vitamin B12 malabsorption, due to inadequate stomach acid and gastritis. As a result, the US institute of Medicine has recommended that individuals over 51 should take vitamin B12 supplements or consume a greater amount of fortified vitamin B12 products [45, 50]. The storage of vitamin B12 in the body is approximately 1000 - 5000 µg, which is relatively high [23]. Therefore, vitamin B12 deficiency may not appear for several years, until stores deplete.

However, an inadequate dietary consumption of vitamin B12 is recommended to prevent the onset of vitamin B12 deficiency.

For pregnant women in the UK, the RNI (1.5 µg/day) does not take into consideration the foetal deposition of vitamin B12 (0.10-0.2 µg/day). Furthermore, there is evidence that the maternal absorption of vitamin B12 is more efficient during pregnancy. During lactation, the RNI is further increased to 2.0 µg/day to take account of the approximate secretion of 0.33 µg vitamin B12/day in breast milk [38, 45].

## 1.5 Vitamin B12 deficiency

### 1.5.1 Symptoms of vitamin B12 deficiency

The clinical manifestations of vitamin B12 deficiency vary in severity and can affect multiple systems in the body. The following section summarizes the current knowledge of the adverse functional effects of vitamin B12 deficiency.

### 1.5.2 Prevalence of vitamin B12 deficiency from world-wide studies

According to the World Health Organization (WHO), vitamin B12 deficiency may be considered a global public health problem affecting millions of individuals [52]. However, the incidence and prevalence of vitamin B12 deficiency worldwide is unknown due to the limited population-based data available (**Table 3**).

Developed countries such as the United States, Germany and the United Kingdom have relatively constant mean vitamin B12 concentrations [3]. The data from the National Health and Nutrition Examination Survey (NHANES) reported the prevalence of serum vitamin B12 concentrations in the United States population between 1999 to 2002 [53, 54]. Serum vitamin B12 concentrations of <148 pmol/L was present in < 1% of children and adolescents. In adults aged 20-39 years, concentrations were below this cut-off in ≤3% of individuals. In the elderly (70 years and older), ≈ 6% of persons had a vitamin B12 concentration below the cut-off.

Furthermore, ≈ 14-16% of adults and >20% of elderly individuals showed evidence of marginal vitamin B12 depletion (serum vitamin B12: 148-221 pmol/L) [53, 54]. In the United Kingdom, a National Diet and Nutrition Survey (NDNS) was conducted in adults aged between 19 to 64 years in 2000–2001 [55] and in elderly individuals (≥ 65 years) in 1994–95 [56]. Six percent of men (n = 632) and 10% of women (n = 667) had low serum vitamin B12 concentrations, defined as <150 pmol/L. In a subgroup of women of reproductive age (19 to 49 years), 11% had low serum B12 concentrations <150 pmol/L (n=476). The prevalence of vitamin B12 deficiency increased substantially in the elderly, where 31% of the elderly had vitamin B12 levels below 130 pmol/L. In the most recent NDNS survey conducted between 2008-2011, serum vitamin B12 was measured in 549 adults [57]. The mean serum vitamin B12 concentration for men (19-64 years) was 308 pmol/L, of which 0.9% of men had low serum B12 concentrations <150 pmol/L. In women aged between 19-64 years, the mean serum vitamin B12 concentration was slightly lower than men (298 pmol/L), with 3.3% having low vitamin B12 concentrations <150 pmol/L [57]. In Germany, a national survey in 1998 was conducted in 1,266 women of childbearing age. Approximately, 14.7% of these women had mean serum vitamin B12 concentrations of <148 pmol/L [58].

Few studies have reported vitamin B12 status on a national level in non-Western countries [59]. Of these reported studies, vitamin B12 deficiency was prevalent among school-aged children in Venezuela (11.4% ) [60], children aged 1-6 years in Mexico (7.7%) [61], women of reproductive age in Vietnam (11.7%) [62], pregnant women in Venezuela (61.34%) [60] and in the elderly population (>65 years) in New Zealand (12%) [63]. Currently, there are no nationally representative surveys for any African or South Asian countries. However, the very few surveys which have investigated vitamin B12 deficiency in these countries have been based on local or district level data. These surveys have reported a high prevalence of vitamin B12 deficiency (<150 pmol/L), among 36% of breastfed and 9% of non-breastfed children

(n=2482) in New Delhi [64] and 47% of adults (n=204) [65] in Pune, Maharashtra, India. Furthermore, in Kenya a local district survey in Embu (n=512) revealed that 40% of school-aged children in Kenya had vitamin B12 deficiency [66].

**Table 3: Worldwide Prevalence of vitamin B12 deficiency (serum/plasma B12 < 148 or 150 pmol/L)**

| Group | Number of studies | Number of participants | Vitamin B12 deficiency (%) |
|---|---|---|---|
| Children (< 1y – 18 years) | 14 | 22,331 | 12.5 |
| Pregnant women | 11 | 11,381 | 27.5 |
| Non-pregnant women | 16 | 18,520 | 16 |
| All adults (Under 60 years) | 18 | 81.438 | 6 |
| Elderly (60+ years) | 25 | 30,449 | 19 |

*Data derived from Table 2 available on* *https://doi.org/10.1016/bs.afnr.2017.11.005* [1]

### 1.5.3 Vitamin B12 and metabolic risk in offspring

Vitamin B12 is a critical micronutrient essential for supporting the increasing metabolic demands of the foetus during pregnancy [67]. B12 deficiency in pregnant women is increasingly common [68] and has been shown to be associated with major maternal health implications, including increased obesity [68], higher body mass index (BMI) [69], insulin resistance [67], gestational diabetes, and type 2 diabetes (T2D) in later life [70]. A study in a pregnant white non-diabetic population in England, found that for every 1% increase in BMI, there was 0.6% decrease in circulating B12 [67]. Furthermore, an animal study in ewes demonstrated that a B12, folate and methionine restricted diet around conception, resulted in offspring with higher adiposity, blood pressure and insulin resistance which could be accounted for altered DNA methylation patterns [71].

Both vitamin B12 and folate are involved in the one-carbon metabolism cycle. In this cycle, vitamin B12 is a necessary cofactor for methionine synthase, an enzyme involved in the methylation of homocysteine to methionine [72]. DNA methylation is involved in the functioning of genes and is an essential epigenetic control mechanism in mammals. This methylation is dependent on methyl donors such as vitamin B12 from the diet [73]. Vitamin B12 deficiency has the potential to influence methylation patterns in DNA, besides other epigenetic modulators such as micro (RNAs), leading to the altered expression of genes [74, 75]. Consequently, an altered gene expression can possibly mediate impaired foetal growth and the programming of non-communicable diseases [13, 74].

Vitamin B12 and folate status during pregnancy is associated with the increasing risk of low birth weight [68, 76], preterm birth [76], insulin resistance and obesity [67, 69] in the offspring. In addition it has been associated with adverse foetal and neonatal outcomes including neural tube defects (NTDs) [77-80] and delayed myelination or demyelination [81, 82]. The mother's B12 status can be important in determining the later health of the child, as shown in the Pune maternal Nutrition Study, conducted in India. In this study mothers with high folate concentrations and low vitamin B12 concentrations, led to babies having a higher adiposity and insulin resistance at age 6. In the same study, over 60% of pregnant women were deficient in vitamin B12 and this was considered to increase the risk of gestational and later diabetes in the mothers [69]. Increased longitudinal cohort studies or randomised controlled trials are required to understand the mechanisms between vitamin B12 and metabolic outcomes, and to potentially offer interventions to improve maternal and offspring health [83].

### 1.5.4 Vitamin B12 and cardiometabolic disease outcomes

Multiple studies have explored the association between vitamin B12 and metabolic disease outcomes, such as obesity, insulin resistance and the development of cardiovascular

disease. Results from two recent studies have indicated that vitamin B12 deficiency may be associated with obesity during childhood. Pinhas-Hamiel et al., reported that obese children and adolescents (n=164) had significantly lower vitamin B12 concentrations in comparison to normal-weight children (n=228) [84]. The report from the Canadian Health Measurement Survey showed that obese children and adolescents aged 6 to 19 years were more likely to have an inadequate vitamin B12 status compared to those with normal weight [85]. In adults, Madan et al., (2006) reported that 13% of patients referred to pre-operative bariatric surgery had vitamin B12 deficiency [86]. On the other hand, Schweiger et al., (2010) only observed vitamin B12 deficiency in 3.4% of patients evaluated in bariatric surgery (n=114) [87]. In addition, in a study conducted in post-menopausal women, vitamin B12 concentrations decreased in relation to an increase in BMI [88]. A long-term study where vitamin B12 was supplemented across a period of 10 years, led to lower levels of weight gain in overweight or obese individuals ($p < 0.05$) [89].

There are several mechanisms which may explain the relationship between obesity and decreased vitamin B12 status. Vitamin B12 is a major dietary methyl donor, involved in the one-carbon cycle of metabolism and a recent genome-wide association (GWA) analysis showed that increased DNA methylation is associated with increased BMI in adults [90], consequently a deficiency of vitamin B12 may disrupt DNA methylation and increase non-communicable disease risk. Vitamin B12 is also a co-enzyme which converts methylmalonyl-CoA to succinyl-CoA in the one carbon cycle. If this reaction cannot occur, methylmalonyl-CoA levels elevate, inhibiting the rate-limiting enzyme of fatty acid oxidation (CPT1 – carnitine palmitoyl transferase), leading to lipogenesis and insulin resistance [9]. Further to this, reduced vitamin B12 concentrations in the obese population is thought to result from repetitive short-term restrictive diets and increased vitamin B12 requirements secondary to increased growth and body surface area [84, 91]. It has also been hypothesised that low vitamin

B12 concentrations in obese individuals are a result of wrong feeding habits, where individuals consume a diet low in micronutrient density [92]. Finally, vitamin B12 is involved in the production of red blood cells, and vitamin B12 deficiency can result in anaemia, which causes fatigue and the lack of motivation to exercise [89].

It is important to screen vitamin B12 deficiency in obese individuals, due to its importance in energy metabolism, and relationship with homocysteine and its potential to modulate weight gain [92]. More studies are needed to test for the causality of vitamin B12 and obesity using genetic markers [93]. Furthermore, many studies have tested for the association of vitamin B12 with BMI. However, BMI does not accurately measure adiposity, and a high BMI does not necessary indicate that an individual is obese. More studies implementing x-ray absorptiometry, magnetic-resonance imaging computed tomography scans and analysing body fat % may be important for testing the link between obesity-related traits and vitamin B12 concentrations [93].

A few studies have also reported no deficiency of vitamin B12 in obese individuals [88, 94-96]. Lower vitamin B12 concentrations were observed in overweight Brazilian adolescents compared to normal-weight adolescents, however there was no statistically significant difference between the two groups [97]. Likewise, among Thai adults no statistically significant difference between overweight and obese individuals compared to normal control subjects was detected [98]. In the study by Baltaci et al [7], approximately 37.7% of overweight and 40.1% of obese Turkish individuals were deficient in vitamin B12. Despite overweight and obese individuals having lower B12 levels in comparison to control non-obese subjects, the difference between the groups were not statistically significant. In a Mendelian randomization study conducted in a Danish cohort [16], no significant associations were detected between genetically determined decreased serum vitamin B12 concentrations and BMI levels, indicating that there may not be a causal role of low serum vitamin B12 levels in obesity.

Finally, a recent literature review conducted over 19 studies, found no evidence of an inverse association between BMI and circulating vitamin B12 [93].

Previous clinical and population-based studies have indicated that vitamin B12 deficiency is prevalent amongst adults with type 2 diabetes [99-101]. Kaya et al., conducted a study in women with polycystic ovary syndrome, and found that obese women with insulin resistance had lower vitamin B12 concentrations compared to those without insulin resistance [102]. Similarly, in a study conducted in European adolescents, there was an association between high adiposity and higher insulin sensitivity with vitamin B12 concentrations. Individuals with a higher fat mass index and higher insulin sensitivity (high Homeostatic Model Assessment [HOMA] index) had lower plasma vitamin B12 concentrations [103]. Furthermore, a recent study conducted in India reported that mean levels of vitamin B12 decreased with increasing levels of glucose tolerance e.g. individuals with type 2 diabetes had the lowest values of vitamin B12, followed by individuals with pre-diabetes and normal glucose tolerance, respectively [5]. However, B12 levels of middle aged-women with and without metabolic syndrome [104] showed no difference in vitamin B12 levels between those with insulin resistance (IR) and those without. It is believed that malabsorption of vitamin B12 in diabetic patients, is due to individuals taking metformin therapy (an insulin sensitizer used for treating type 2 diabetes) [105]. Furthermore, obese individuals with type 2 diabetes are likely to suffer from gastroesophageal reflux disease [106], and take proton pump inhibitors, which further increased the risk of vitamin B12 deficiency [93].

The investigation into the relationship between cardiovascular disease (CVD) and vitamin B12 has been limited, and there is still controversy as to whether primary intervention with vitamin B12 will lower CVD [107]. Deficiency of vitamin B12 can impair the remethylation of homocysteine in the methionine cycle, and result in raised homocysteine levels [108]. There is much evidence linking elevated homocysteine concentrations with an

increased risk of CVD [109], and homocysteine lowering treatments have led to improvements in cardiovascular reactivity and coagulation factors [110]. In adults with metabolic syndrome, individuals with low levels of vitamin B12 had higher levels of homocysteine compared to healthy subjects [111]. It is thus possible that vitamin B12 deficiency enhances the risk of developing cardiovascular disease in individuals who are obese [84]. Alternatively, low levels of vitamin B12 may increase the levels of proinflammatory proteins which may induce ischaemic stroke [112, 113].

A recent literature review conducted over seven studies, found that there was limited evidence to show that low vitamin B12 status increased the risk of CVD and diabetes [114]. Only one study by Weikert et al. reported that low vitamin B12 status increased the risk of cerebral ischaemia [115]. After controlling for homocysteine, the relative risk of cerebral ischaemia reduced by approximately 10%, suggesting that the effects of low vitamin B12 are partially mediated by homocysteine [115]. In two other studies, higher vitamin B12 concentrations were associated with an increased risk of mortality, fatal and non-fatal coronary events [116, 117]. It is important to note that these discrepancies, may be the result of the study population including individuals who were diseased [116] or old [117]. Further to this, both studies did not assess whether individuals were taking vitamin B12 supplements or they did not exclude individuals with liver disease or malignancy, which is important as raised vitamin B12 levels could have been due to a functional deficit [114, 118]. Finally, the review did not identify any associations between vitamin B12 and CVD in the remaining four studies [114].

Currently, no data supports vitamin B12 supplementation on reducing the risk of CVD. In a dose-response meta-analysis of five prospective cohort studies, it was reported that the risk of coronary heart disease (CHD) did not change substantially with increasing dietary vitamin B12 intake [119]. Of these five studies, three of the studies stated a non-significant positive

association and two of the studies demonstrated an inverse association between vitamin B12 supplementation and CHD (only one of the studies was significant) [119].

### 1.5.5 Vitamin B12 and Neural tube defects (NTDs)

Neural tube defects (NTDs), including spina bifida, encephalocele and anencephaly, are debilitating birth defects which result from the failure of neural fold closure during embryonic development. The causes of NTDs are multifactorial, including folate deficiency, genetic and environment factors [120]. The WHO Technical Consultation has concluded that there is moderate evidence for the association between low vitamin B12 status and the increased risk of developing NTDs [121]. Given that vitamin B12 is a co-factor for methionine synthase within the folate cycle. If vitamin B12 supplies are depleted, folate becomes trapped and DNA synthesis and methylation reactions are impaired. DNA synthesis is critical for embryonic development. Further to this, cell-signalling events which control gene-expression are controlled by methylation reactions. As a result, adequate folate and vitamin B12 is needed to help prevent NTDs [77]. Many studies have shown associations between maternal vitamin B12 status and NTD affected pregnancy [77-80]. Low vitamin B12 concentrations have also been found in the amniotic fluid of NTD affected pregnancy [122, 123]. Additionally, a population-based case-control study (89 women with an NTD and 422 unaffected pregnant controls) in Canada conducted after the fortification of folic acid in flour, found almost a tripling in the risk of NTD, in the presence of low maternal vitamin B12 status (indicated by holoTC)[78]. Future studies, using interventions with vitamin B12 supplements or fortification with vitamin B12 is needed to confirm the relationship between vitamin B12 and NTDs.

### 1.5.6 Vitamin B12 and anaemia

In countries where vitamin B12 deficiency is common, it is generally assumed that there is a greater risk of developing anaemia. However, the overall contribution of vitamin B12

deficiency to the global incidence of anaemia may not be significant, except in elderly individuals and vegetarians [124]. There are relatively few studies which have assessed the impact of haematological measures in response to vitamin B12 supplementation. One study in 184 premature infants, reported that individuals given monthly vitamin B12 injections (100 μg) or taking supplements of vitamin B12 and folic acid (100 μg/day), had higher haemoglobin concentrations after 10-12 weeks, compared to those only taking folic acid or those taking no vitamin B12 injections [125]. In deficient Mexican adult women and pre-schoolers, it was found that vitamin B12 supplementation did not affect any haematologic parameters [126, 127]. Vitamin B12 deficiency is also a major factor leading to megoblastic anaemia, especially in those infants breastfed by strict vegetarian mothers [121, 128].

### 1.5.7 Vitamin B12 and Ageing

Vitamin B12 has been associated with disability in the elderly including the development of age-related macular degeneration (AMD) and the risk of frailty [50].

AMD is the leading cause of severe, irreversible vision loss in older adults [129]. During the advanced stages of AMD, individuals are impaired of carrying out basic activities such as driving, recognising faces and reading [130]. Several risk factors have been linked to AMD, including increasing age, family history, genetics, hypercholesterolemia, hypertension, sunlight exposure and lifestyle (smoking and diet) [131, 132]. A few cross-sectional studies have found associations between low vitamin B12 status and AMD cases [132, 133]. It has been shown that daily supplementation of vitamin B12, B6 and folate over a period of seven years can reduce the risk of AMD by 34% in women with increased risk of vascular disease (n=5,204) [134]. However, another study failed to find an association between AMD and vitamin B12 status in a sample of 3,828 individuals representative of the non-institutionalized US population [135].

Frailty is a geriatric condition which is characterized by diminished endurance, strength, and reduced physiological function that increases an individual's risk of mortality and impairs an individual from fulfilling an independent lifestyle [136]. Frailty is associated with an increased vulnerability to fractures, falls from heights, reduced cognitive function and more frequent hospitalisation [137]. The worldwide prevalence of frailty within the geriatric population is 13.9% [138], therefore there is an urgent need to eliminate any risk factors associated with frailty. Poor vitamin B status has been shown to be associated with an increased risk of frailty. Two cross sectional studies have reported that deficiencies of vitamin B12 were associated with the length of hospital stay, as observed by serum vitamin B12 concentrations and methylmalonic acid (MMA) concentrations [139, 140]. Furthermore, another study looking at elderly women (n=326), found that certain genetic variants associated with vitamin B12 status (Transcobalamin 2) may contribute to reduced energy metabolism, consequently contributing to frailty [141]. In contrast, a recent study by Dokuzlar et al., found that there was no association between vitamin B12 levels and frailty in the geriatric population (n=335) [142]. Given that there are limited studies, which have assessed the relationship between vitamin B12 and frailty status, more longitudinal studies are needed to clarify the relationship.

### 1.5.8 Vitamin B12 and neurological decline

Severe vitamin B12 deficiency is associated with subacute combined degeneration of the spinal cord, which involves demyelination of the posterior and lateral columns of the spinal cord [23]. Symptoms include memory and cognitive impairment, sensory loss, motor disturbances, loss of posterior column functions and disturbances in proprioception [143, 144]. In advanced stages of vitamin B12 deficiency, cases of psychosis, paranoia and severe depression have been observed, which may lead to permanent disability if left untreated [23, 143, 144]. Studies have shown the rapid reversal of the neurological symptoms of vitamin B12

deficiency, after treatment with high-dose of vitamin B12 supplementation; suggesting the importance of prompt treatment in reversing neurological manifestations [145].

### 1.5.9 Vitamin B12 and cognitive decline

Elderly individuals are currently assessed on vitamin B12 status during the screening process for dementia. Studies investigating the association between vitamin B12 concentrations and cognitive status have produced inconclusive results [50, 146, 147]. It has been shown that elevated MMA concentrations are associated with decreased cognitive decline and Alzheimer's Disease [148]. In addition, low vitamin B12 and folate intakes have shown associations with hyperhomocysteinemia, which is associated with cerebrovascular disease, cognitive decline and an increased risk of dementia in prospective studies [149].

There are limited intervention studies which have investigated the effect of supplementation of vitamin B12 and cognitive function. A Cochrane review, analysing two studies, found no effect of vitamin B12 supplementation on the cognitive scores of older adults [150]. A recent longitudinal study in elderly individuals, found that individuals had a higher risk of brain volume loss over a 5-year period, if they had lower vitamin B12 and holoTC levels and higher plasma tHcy and MMA levels [151]. More intervention studies are needed to determine the modifiable effects of vitamin B12 supplementation on cognition [50].

### 1.5.10 Vitamin B12 and Osteoporosis

There has been growing interest on the effect of low serum vitamin B12 concentrations on bone health [152, 153]. Recent studies have found a connection between elevated plasma tHcy and an increased risk of bone fractures, but is unknown whether this is related to the increased levels of tHcy or to vitamin B12 levels (which are involved in homocysteine metabolism) [154]. Results from the third NHANES conducted in the United States, found that individuals had significantly lower bone mass density (BMD) and higher osteoporosis rates

with each higher quartile of serum MMA (n= 737 men and 813 women) [155]. Given that poor bone mineralization has been found in individuals with pernicious anaemia [156], and that the content of vitamin B12 within bone cells in culture has shown to affect the functioning of bone forming cells (osteoblasts) [157]; it is possible that vitamin B12 deficiency is causally related to poor bone health.

Randomized intervention trials investigating the association of vitamin B12 supplementation and bone health have yielded mixed results. Two studies conducted in osteoporotic risk patients with hyperhomocysteinemia and individuals who had undergone a stroke, found positive effects between supplementation of B vitamins on BMD [158, 159]. However, no improvement in BMD was observed in a group of healthy older people [160]. Further, controlled trials are needed to confirm the impact and mechanisms vitamin B12 deficiency has on bone mineralization [121].

### 1.5.11 Causes of B12 deficiency

The most common reason for vitamin B12 deficiency in spite of eating a diet rich in animal products is poor absorption. It has been long known that vegans, lacto-ovo vegetarians and elderly individuals are at risk of vitamin B12 deficiency. Causes can also relate to having inadequate amounts of IF, gastric atrophy, intestinal disease, gastric surgery, bacterial overgrowth in the small intestine, alcohol consumption, a tapeworm infection, drug-nutrient interactions, as well as some genetic defects [45, 48, 161].

It is well known that strict vegans are at high risk of vitamin B12 deficiency. At present, there are very few studies analysing the association of vitamin B12 deficiency with veganism in large populations. In a group of 131 vegan adults from Germany (aged 20-82 years), individuals who followed a vegan diet for 7.1 years, had a 1.8 increased rate of deficiency compared to those who adhered to a vegan diet for less than 5 years. The study showed that

26% of strict vegans who did not take vitamin B12 supplements, had vitamin B12 deficiency with a cut-off point of 110 pmol/L [162]. Furthermore, in another study looking at 25 vegan adults from California aged 20 to 60 years, showed that 40% of individuals were vitamin B12 deficit; based on either low plasma cobalamin (< 150 pmol/L), macrocytosis, or elevated serum MMA (>376 nmol/L) [163]. Vegans are therefore recommended to take vitamin B12 fortified foods or supplements to meet their recommended daily intake.

Traditionally, vegans were suggested to be the only group at risk of vitamin B12 deficiency, but it is now acknowledged that individuals who consume low animal source foods are also at risk. Lacto-ovo vegetarians and individuals from less-industrialized (where the consumption of meat is rare) have a greater risk of vitamin B12 deficiency compared to individuals who consume an omnivorous diet [48]. Evidence shows that meat contains comparatively more vitamin B12 (1.3 µg/100 kcal cooked meat) than milk (0.6 µg/100 kcal) [48]. In a recent literature review addressing the vitamin B12 deficiency rates amongst vegetarians, it was reported that 32% of young adult vegetarians/ lacto-ovo vegetarians had vitamin B12 deficiency (MMA >271 nmol/L) [164]. As a result, lacto-ovo vegetarians are required to take supplemental vitamin B12 to meet their nutritional needs [50].

Vitamin B12 deficiency is also a common condition among the elderly. Elderly individuals are frequently malnourished, which enhances the risk of vitamin B12 deficiency. Whilst some of these reasons might be the result of underlying ill health, other influences include problems with dentition, depression or anxiety, mobility difficulties (e.g. difficulties with food preparation) and the use of medications which may interfere with appetite or absorption of vitamin B12 [165]. Atrophic gastritis is also a common condition observed in the elderly, which results in the inflammation of the stomach mucous membrane. In atrophic gastritis, there is a reduction or absence in gastric acid secretion which is needed to release vitamin B12 from proteins in food. However, elderly individuals still retain the ability to absorb

vitamin B12 in synthetic form (as it is not protein bound), due to sufficient intrinsic factor being secreted [48].

Pernicious anaemia is the final stage of an auto-immune gastritis (Type A atrophic gastritis). In autoimmune gastritis, parietal cells of the corpus and fundus of the stomach are destroyed. These parietal cells are responsible for producing hydrochloric acid and intrinsic factor, which is required for the uptake of vitamin B12 [166]. As there is no therapy at present for auto-immune gastritis, patients are required to take vitamin B12 injections, or large doses of vitamin B12 to prevent the development of megaloblastic anaemia and future neurological complications [50].

Vitamin B12 uptake in the ileum can be reduced by the overgrowth of bacteria or parasites. Intestinal bacteria may have the potential to compete for vitamin B12, convert the vitamin B12 into inactive analogs or impair the absorption of vitamin B12 [48]. At present, *Helicobacter pylori* infection is one of the most common gastric infections worldwide. *H. pylori* infection is characterized by gastritis, gastric and duodenal ulcers, achlorhydria and gastric atrophy. Numerous studies have suggested that there may be a causal relationship between *H. pylori* and food-bound vitamin B12 malabsorption [167]. Furthermore, diseases of the ileum such as Crohn's Disease, chronic bowel inflammatory disease and gastrointestinal surgery may induce vitamin B12 malabsorption [45].

Vitamin B12 malabsorption is also linked to genetic disorders which regulate the uptake and metabolism of vitamin B12. Vitamin B12 is a cofactor for methionine synthase (MS) and methylmalonyl CoA mutase (MCM). In order to function as a co-factor, the structure of vitamin B12 must be modified [168]. Obstructions in the intracellular processing of vitamin B12 into its co-factor forms; methylcobalamin (MeCbl) for (MS) and adenosylcobalamin (AdoCbl) for MCM or changes in the functional activity of MS or MCM can result in inborn errors of vitamin

B12 utilisation. The genetically inherited blocks can be detrimental for new-borns and children [168]. A number of inborn errors of intracellular vitamin B12 metabolism, designated cblA-cblG, have been determined by biochemical analysis of radioactive metabolites and B12 (complementation analysis). Methylmalonic acidemia (cblA, cblB, cblD variant 2), hyperhomocysteinemia (cblD variant 1, cblE, cblG) or combined methylmalonic acidemia and hyperhomocysteinemia (cblC, classic cblD, cblF) have so far been acknowledged as inborn errors [169]. These disorders and the genes involved in intracellular B12 metabolism are listed in **Table 4**. Further to this, vitamin B12 levels in the general population are underpinned by molecular mechanisms which are responsible for the absorption, distribution, metabolism and elimination of vitamin B12 [170]. The genetics of vitamin B12 status and genetic variation in different ethnicities within individuals without inborn errors of metabolism will be discussed in detail in **chapter 2.**

**Table 4: Inborn Errors of Cobalamin Transport and Metabolism**

| Disorder | Gene | Location | Phenotype (Inborn errors) | Function |
|---|---|---|---|---|
| Intrinsic factor deficiency | *GIF* | 11q13 | Intrinsic factor deficiency | Encodes a glycoprotein secreted by parietal cells of the gastric mucosa. The gene encodes the protein that is required for adequate absorption of vitamin B12. |
| Imerslund–Gräsbeck syndrome (Megaloblastic anaemia 1) | *AMN* | 14q32 | Intestinal absorption of dietary cobalamin is impaired (Partial loss of IF binding affinity to cobalamin or the cubam receptor complex) | Involved in the transfer of the cubilin-vitamin B12 complex into the intestinal cell |
| | *CUBN* | 10p12.1 | | It encodes the intestinal receptor Cubilin, which is expressed in the renal proximal tubule and intestinal mucosa. Cubilin recognizes the vitaminB12-intrinsic factor complex, and binds to another protein called Amnionless to facilitate the entry of vitamin B12 into the intestinal cells |
| Transcobalamin deficiency (Transcobalamin II deficiency) | *TCN2* | 22q11.2 | Decreased intestinal absorption of B12, uncorrected by intrinsic factor. | It encodes a transport protein called transcobalamin 2 (TC), which binds to vitamin B12 within the enterocyte. The TC-B12 complex enters the portal circulation and makes vitamin B12 available for cellular uptake in target tissues |
| Haptocorrin deficiency (Transcobalamin I deficiency) | *TCN1* | 11q11–q12 | Affects multiple specific granule proteins, and results in low serum B12 levels | It encodes a glycoprotein called Transcobalamin 1, also known as haptocorrin (HC), which binds to vitamin B12. It shields dietary vitamin B12 from the acidic environment of the stomach. |
| Transcobalamin receptor deficiency | *CD320* | 19p13.2 | Loss of a glutamate residue in the extracellular | It encodes the membrane receptor transcobalamin receptor (TCblR), which binds |

| | | | domain of the receptor. Decreased receptor-mediated uptake of TC-B12 in vitro. | to the transcobalamin-vitamin B12 complex, and mediates the uptake of vitamin B12 into cells |
|---|---|---|---|---|
| cblA | *MMAA* | 4q31.1–q31.2 | Adenosyl-vitaminB12 deficiency in cells | *MMAA* encodes a protein that may be involved in the translocation of vitamin B12 into the mitochondria. In addition, MMAA could play an important role in the protection and reactivation of Methylmalonyl-coA mutase (MCM) in vitro. |
| cblB | *MMAB* | 12q24 | Adenosyl-vitaminB12 deficiency in cells | Adenosylates cobalamin in an ATP-dependent manner |
| cblC | *MMACHC* | 1p23.2 | The inability to convert cynano-vitaminB12 into biological forms | The *MMACHC* gene encodes a chaperone protein MMAACHC (cblC protein) which binds to vitamin B12 in the cytoplasm and appears to catalyse the reductive decyanation of cyanocobalamin into cob(II)alamin |
| cblD | *MMADHC* | 2q23.2 | Improper targeting of vitamin B12 to cognate enzymes | This gene leads to the Branching of vitamin B12 within the cell to either the cytosol or the mitochondrion |
| CblE | *MTRR* | 5p15.3-p15.2 | Inactive methionine synthase | This gene is responsible for the reductive methylation of vitamin B12 to generate methylcobalamin from cob(II)alamin |
| CblF | *LMBRD1* | 6q13 | Accumulation of vitamin B12 within lysosome | Potentially helps in the transport of vitamin B12 out of the lysosome |
| CblG | *MTR* | 1q43 | Homocysteine accumulation | Transfers a methyl group from methyltetrahydrofolate to homocysteine to produce methionine |

| CblJ | *ABCD4* | 14q24.3 | Accumulation of cobalamin within lysosome | Transports vitamin B12 from lysosomes to the cytosol |
|---|---|---|---|---|
| Methylmalonyl CoA mutase deficiency | *MUT* | 6p21 | Methylmalonic acid accumulation | The enzyme converts methylmalonylCoA and succinylCoA, reversibly. |

*Data derived from Table 1 available on https://doi.org/10.1016/bs.afnr.2017.11.005* ***[1]*** *and from Table 3 available on https://doi.org/10.1186/s12263-018-0591-9*

Currently there is concern that the mandatory fortification of folic acid to cereals and grains, may in fact conceal the macrocytic anaemia associated with vitamin B12 deficiency, consequently eliminating an important diagnostic tool [171]. The combination of high folate and low serum vitamin B12 is associated with higher concentrations of methylmalonic acid and homocysteine, contributing to hematologic and neurologic disturbances. The National Health and Nutrition Examination Survey (NHANES) collected on older adults during 1999-2002 showed that high folate intakes were related to impaired mental functioning and cognitive decline among individuals with a low vitamin B12 status [172]. Considering these findings, there has been interest as to whether vitamin B12 fortification in flour should be implemented. However, as of yet there is not enough data evaluating the bioavailability of the vitamin from fortified flour in specific population groups (such as the elderly with food-bound vitamin B12 malabsorption and others with gastric atrophy) to make a firm decision [121].

### 1.5.12 Drug-nutrient interactions

There are some drugs which are thought to interfere with the absorption or metabolism of vitamin B12 [50]. These include $H_2$-receptor antagonists, proton pump inhibitors and metformin. Cimetidine is a $H_2$-receptor antagonist which is used to treat peptic ulcers and alleviate heartburn. Cimetidine inhibits the secretion of gastric acid and pepsin and has been reported to inhibit IF secretion [173, 174]. A >1000 mg/day dose may in fact lead to

malabsorption of protein-bound vitamin B12 by peptic ulcer patients (n=9 male) and normal subjects (n=4 male) [175], however this malabsorption was shown to be reversible upon discontinuation of cimetidine in another study [174] .

Proton pump inhibitors (PPIs) such as omeprazole and lansoprazole are widely prescribed to treat gastroesophageal reflux disease. It has been suggested that prolonged use of PPIs may influence vitamin B12 status, by inhibiting gastric acid secretion. The effect of omeprazole on vitamin B12 absorption is dose-related, with intakes of 20 mg/day reducing food-bound vitamin B12 absorption by 70%, whilst 40 mg/day reducing absorption by 90% [176]. At present the current literature on the association between PPI usage and vitamin B12 status is mainly based on case-reports or retrospective observational studies, which have produced relatively inconsistent findings [177].

Metformin therapy is used as the first line of therapy for individuals with type 2 diabetes mellitus. Studies have shown that Metformin induces vitamin B12 malabsorption and impaired intrinsic factor secretion in the ileum [178-181]. The mechanism of metformin-related vitamin B12 deficiency is still under debate. Metformin delays glucose absorption in the upper small intestine affecting the motility of the small bowel, which stimulates bacterial overgrowth and consequential vitamin B12 deficiency [178, 179]. Metformin has also been shown to enhance competitive inhibition or inactivation of vitamin B12 absorption, leading to alterations in intrinsic factor (IF) levels and interactions with the cubulin endocytic receptor. Additionally, Metformin inhibits the calcium dependent absorption of the vitamin B12-IF complex at the terminal ileum [181], as a result increasing calcium intake may improve the uptake of vitamin B12 in metformin users [180].

## 1.6 Assessment of Vitamin B12 status

Traditionally, measuring serum cobalamin remains the preferred choice for determining vitamin B12 deficiency. However, using serum vitamin B12 concentrations alone does not

confirm the uncertainties of underlying functional and biochemical deficiencies. Nowadays other methods such as measuring plasma methylmalonic acid, serum holotranscobalamin and plasma homocysteine are also used [23].

### 1.6.1 Vitamin B12 and Holotranscobalamin

The measurement of serum vitamin B12 levels is the most widely used assay to screen vitamin B12 deficiency. However this method is rarely used alone, as it is known to have a poor sensitivity and specificity in detecting vitamin B12 deficiency [182]. Serum B12 assays measures both serum holohaptocorrin (HoloHC) and serum holotranscobalamin (holoTC). HoloHC, represent 70-90% of vitamin B12, but is biologically inert as no cellular receptors exist, except on the liver. On the other hand, HoloTC contains biologically active vitamin B12, which can be taken up by cells, and represents 10-30% of circulating vitamin B12 [183]. Given that the majority of vitamin B12 is bound to HC, results would mask the true deficiency or would falsely infer vitamin deficiency [23]. Vitamin B12 is usually measured using an automated method and a competitive-binding immune chemiluminescence, a low cost test [23]. Depending on the technique used to measure vitamin B12, vitamin B12 deficiency is usually considered when the plasma vitamin B12 concentration is less than 200 pg/mL. However, there is no gold standard value to represent subclinical deficiency of vitamin B12 (**Table 5**) [184].

Currently, the TC bound to plasma vitamin B12, is more relevant for assessing the functional vitamin B12 status. HoloTC reflects the absorptive capacity of vitamin B12, and any deficiency of TC has been previously associated with neurological and haematological complications [182]. HoloTC is usually measured by immunoassay, and cut-off values for low HoloTC depend on the specific laboratory guidelines [23].

**Table 5: Biomarkers of vitamin B12 status**

| Biomarker (Unit) | Assay Type | Tentative reference interval[a*] | Tentative cut-off value for vitamin B12 deficiency[b*] | Tentative cut-off value for repletion of vitamin B12[*] |
|---|---|---|---|---|
| Plasma B12 (pmol/L) | Competitive-binding immune chemiluminescence method/ protein binding assay | 200-600 | <148 | >221 |
| Holotranscobalamin (pmol/L) | Immunological | 40-100 | <35 | >40 |
| Homocysteine (µmol/l) | Immunological, Liquid chromatography– mass spectrometry or gas chromatography mass spectrometry | 8-15 | >15 | <8 |
| Methylmalonic acid (µmol/l) | Liquid chromatography– mass spectrometry or gas chromatography mass spectrometry | 0.04-0.37 | >0.37 | <0.27 |

*[a]The Tentative reference intervals cover approximately 95% of B12 replete individuals. [b]The tentative cut-off value for vitamin B12 deficiency includes both clinical and subclinical deficiency.*The values indicated in this table are based on previously cited literature. Data derived from Table 1 available on https://doi.org/10.1038/nrdp.2017.40 [2]*

### 1.6.2 Homocysteine and Methylmalonic acid

Homocysteine (Hcy) and methylmalonic acid (MMA) can be used as sensitive biomarkers to detect an underlying vitamin B12 deficiency, even when no apparent sign of clinical vitamin B12 deficiency or low serum vitamin B12 levels are present [182]. There are two B12-dependent enzymatic reactions which use MMA and Hcy as substrates. Vitamin B12 in combination with folic acid is required to convert Hcy to methionine, and vitamin B12 is used to convert MMA to succinyl-CoA [185]. As a result, MMA is a more sensitive indicator of vitamin B12 deficiency compared to Hcy. These two biomarkers can be confounded by both

environmental and physiological conditions [161]. Renal failure, heart transplantation, thyroid dysfunction, certain medications, genetic variation in the methylenetetrahydrofolate reductase (*MTHFR*) gene and high folate and vitamin B6 deficiency can contribute to elevated Hcy concentrations. Furthermore, MMA is elevated in renal impairment and rare inborn errors affecting methylmalonate-CoA mutase activity [50, 186].

Elevated Hcy and MMA concentrations, have been found to be 99.8% sensitive for diagnosing vitamin B12 deficiency [187]. Both Hcy and MMA are usually measured using Liquid chromatography– mass spectrometry or gas chromatography mass spectrometry [188]. According to Carmel (2006), an inadequate vitamin B12 status is described as serum vitamin B12 $< 148$ pmol/L, or 148–258 pmol/L and MMA $> 0.30$μmol/L, or tHcy $> 13$ nmol/L (females) and $>15$ nmol/L (males) [189]. However, it should be noted that the reference range depends on the individual techniques used to measure Hcy and MMA; as the published estimates for the specificity and sensitivity for diagnosing vitamin B12 deficiency varies extensively (**Table 5**)[184].

## 1.7 Treatment of vitamin B12 deficiency

### 1.7.1 Parenteral treatment

In the United Kingdom and other western countries worldwide, most patients with vitamin B12 deficiency are given intramuscular injections of vitamin B12. Intramuscular vitamin B12 exists in two forms: cyanocobalamin or hydroxocobalamin. Hydroxocobalamin is generally used as the first line of treatment, as it is retained in the body for a longer period of time and it can be administered at intervals of up to three months [190]. Approximately 10% (100 μg) of injected hydroxocobalamin is retained in the body after administration of 1000 μg [22].

The standard treatment for patients without neurological symptoms is three injections of intramuscular hydroxocobalamin (1000 μg) three times a week, for a duration of two weeks.

On the other hand, for patients with neurological involvement, injections are given intramuscularly (1000 µg) on alternative days for three weeks or until clear improvement is shown. Individuals with pernicious anaemia are given lifelong treatment. Individuals with severe anaemia and cardiac symptoms are usually treated with transfusion and diuretic agents [22, 23, 191].

Hydroxocobalamin is usually well-tolerated, with serious adverse reactions being rare. However, injections can cause significant amount of pain in thin patients and can be dangerous in anticoagulated patients [190]. Side effects which are rarely observed for hydroxocobalamin include: chills, fever, hot flushes, itching, nausea, dizziness, skin rash and anaphylaxis [23].

### 1.7.2 Oral treatment

When vitamin B12 deficiency is related to an individual's diet, a dose of 50-150 µg cyanocobalamin is given between meals [23]. Oral therapy is considered during mild or subclinical vitamin B12 deficiency and when there are no concerns of compliance or abnormalities associated with absorption [192].

Previous case control studies have suggested that the oral administration of vitamin B12, is equally safe and effective at eliminating vitamin B12 deficiency [193, 194]. Vitamin B12 taken in the oral route, can be absorbed both actively and passively. In passive absorption, the vitamin B12 is absorbed without binding to IF. Approximately 0.5-4% of radioactively labelled oral vitamin B12 can be absorbed by passive diffusion in both healthy and patients with pernacious anemia [190, 195]. On the other hand, in active absorption, vitamin B12 binds to IF in the terminal ileum [190].

It has been noted that patients with IF deficiency can still adequately absorb vitamin B12, provided that they are given high doses of vitamin B12 (1000 µg daily) [190]. A cochrane review of two randomised controlled trials comparing oral with intramuscular administration

in 108 participants, found that high oral doses (1000 µg and 2000 µg daily) are as effective as intramuscular injections in responding to neurological and haemotological symptoms [190]. However, due to the limited data available to present the support of oral therapy in individuals with neurological dysfunction, parenteral vitamin B12 is still the prefered method of treatment.

At present, oral vitamin B12 is widely prescribed in Canada and Sweden [190]. However, high doses of oral vitamin B12 are unavailable for prescription under the NHS in the United Kingdom. Given that intramuscular injections require patients to visit a health facility or have a health care visitor to administer an injection, using oral vitamin B12 instead, could potentially save NHS resources and the time of medical staff [190].

## 1.8 Vitamin B12 Toxicity

High serum vitamin B12 is defined as a value above 950 pg/ml; this refers to the upper limit of biological normality [196]. At present, few studies have looked at the toxic effects associated with a high serum vitamin B12 concentration. One study observed that when vitamin B12 was administered at 2 mg (2,000 µg) daily by mouth or 1 mg monthly by intramuscular (IM) injection to treat pernicious anaemia, no toxic effects were identified [197]. It is known that only a certain percentage of vitamin B12 can be absorbed by the body, and any ingested amounts which exceed the absorption capacity of vitamin B12 intrinsic factor receptors are excreted through the urine or faeces. This could partly explain the low toxicity [38]. On the other hand, other studies have noted that high doses of vitamin B12 supplements were associated with a greater risk of CVD in individuals with diabetic nephropathy [198] and a greater risk of autism spectrum disorder in the offspring of pregnant women [199].

It is possible that an increase in plasma vitamin B12 could be a result of a functional deficit. The destruction of hepatocytes in chronic hepatitis, can stimulate the binding of vitamin B12 to haptocorrin (HC) in the plasma, to form holohatocorrin (holoHC- inactive form of vitamin B12) leading to a decline of vitamin B12 attaching to holotranscobalamin (holoTC) II

(active form of vitamin $B_{12}$). As a result, there is an increase in vitamin B12 in the plasma, as vitamin B12 cannot be delivered to the cells [200]. Furthermore, elevated vitamin B12 concentrations could be due to the leakage of vitamin B12 from damaged liver tissue into the plasma [196]. As a result, high vitamin B12 levels are not always beneficial and could underlie a number of underlying pathologies [196].

## 1.9 Nutrigenetics approach

Nutrigenetics is a branch of science that investigates the effect of genetic variants in response to dietary manipulation. The ultimate goal of nutrigenetics is to investigate the molecular and physiological basis of genetic variants associated with health and disease, and how these genotype-phenotype associations can be modified by dietary intake [201]. The field of nutrigenetics is rapidly evolving, with the hope that one day in the future nutritionists will be able to provide personalised dietary recommendations to patients to delay or prevent the onset of disease [202].

### 1.9.1 Genetic factors and ethnic variation

Vitamin B12 absorption and metabolism involves complex biological pathways containing multiple steps. Genetic variants may alter vitamin B12 tissue status by affecting the proteins involved in vitamin B12 absorption, cellular uptake and intracellular metabolism [143]. In a study using monozygotic and dizygotic twins, the heritability of B12 levels was estimated to be 59%, indicating that the magnitude of genetic influence on vitamin B12 levels are considerable [203]. At present, genetic studies of vitamin B12 status suggest that it is a multifactorial trait (also called complex trait), where several single nucleotide polymorphisms (SNPs) in multiple genes interact with the environment to cause the altered B12 status [204]. The genetics of vitamin B12 status and the genetic variation in different ethnicities are discussed in detail in **chapter 2**.

A review article from Surendran et al., (2018) which is found in chapter 2 of this thesis, identified 59 vitamin B12-related gene polymorphisms associated with vitamin B12 status, from the following populations: African American, Brazilian, Canadian, Chinese, Danish, English, European ancestry, Icelandic, Indian, Italian, Latino, Northern Irish, Portuguese and residents of the USA [14]. The most compelling evidence has been accumulated for the fucosyltransferase 2 (*FUT2*) SNP (rs602662), for which homozygosity of the minor G allele has been associated with lower vitamin B12 status. Variants in other B12 metabolic genes, including methylmalonyl CoA mutase (*MUT)*, cubulin (*CUBN)* and transcobalamin-I (*TCN1)* have been reported in European populations [205]. Furthermore, an additional four loci, membrane-spanning 4-domains (*MS4A3)*, citrate lyase beta like (*CLYBL)*, fucosyltransferase 6 (*FUT6)* and 5q32 were constricted to the Chinese population [206]. It has been suggested that ethnic-specific associations are involved in the genetic determination of vitamin B12 concentrations. However, despite recent success in genetic studies, most of the identified genes that could explain variation in vitamin B12 concentrations were from Caucasian populations. As a result, further research utilizing larger sample sizes of non-Caucasian populations is necessary in order to better understand these ethnic-specific associations [14].

Genes alone are not responsible for explaining the variation in vitamin B12 concentrations, as lifestyle factors e.g. dietary factors, also influence vitamin B12 concentrations. Therefore, this is investigated by identifying gene-diet interactions (Nutrigenetics). In my thesis, I aimed to investigate the interaction between dietary factors (modifiable factor) and genetic markers (non-modifiable factors) on vitamin B12 concentrations and metabolic disease trait outcomes.

### 1.9.2 Rational for studying gene-diet interactions

Many SNPs have been shown to be associated with vitamin B12 status and these SNPs only represent a fraction of the heritability of vitamin B12 status [14]. It is well known that environmental factors, such as diet, can modulate the effects of genes on metabolic traits [12]. However, it is unknown whether dietary factors can interact with genes to impact vitamin B12 status; hence the interaction between genetic and dietary factors must be considered. Findings from gene-diet interactions will contribute to identifying the interactions of genes and diet in the development of vitamin B12 deficiency. Therefore, this knowledge is essential for the primary prevention of vitamin B12 deficiency, and for developing effective dietary strategies for the prevention of vitamin B12 deficiency and its related metabolic outcomes.

### 1.9.3 Importance of studying gene-diet interactions in different genetic groups

It has been established that genetic studies looking at vitamin B12 status in healthy adults, especially large-scale ones, have been unable to capture the level of diversity which exists worldwide, as they are mainly based on individuals of European ancestry [14]. The under-representation of diverse ethnic groups hampers our full understanding of the genetic architecture of vitamin B12 levels [207]. Furthermore, the limited genetic data on non-Caucasian populations in relation to genetic susceptibility to vitamin B12 deficiency, can also impede our ability to translate genetic research into clinical care, and will exacerbate health inequalities across the current public health policy [207]. Given that vitamin B12 status can also be determined by environmental factors, it is also important to explore gene-diet interactions in different ethnic groups, so that it will be eventually possible to personalise diet according to each ethnic sub-group. It is important to note that, different ethnic groups respond differently to specific dietary interventions [12]. Therefore, using estimates of genetic risk for vitamin B12 deficiency from European-based studies in non-Europeans may result in an

inaccurate assessment of risk of vitamin B12 deficiency and could result in an inappropriate environmental intervention (dietary or physical activity) in under-studied populations.

### 1.9.4 Study designs and their role in identifying gene-diet interactions

Multiple lines of evidence suggest that SNPs may modify gene expression and consequently influence metabolic disease outcomes. Besides, it is well known that interactions may exist between genes and dietary factors to influence metabolic outcomes [12]. Several genes are involved in vitamin B12 metabolism [14] and variants in these genes may modify cardio-metabolic disease outcomes [16, 17]. Beyond the independent gene effects, no studies have evaluated interactions between vitamin B12 gene polymorphisms and macronutrient intake on cardiometabolic disease outcomes. A more detailed understanding of gene- diet interactions is needed to generate information required to develop strategies for diet modification to reduce the incidence of cardiometabolic disease related traits in individuals with specific variants related to vitamin B12 absorption and metabolism. The following section describes the potential study designs which can be employed for gene-diet interactions.

The most commonly used study design is the cross-sectional design. A cross-sectional design is a study design, where disease related-outcomes and exposures in study participants are measured at a single point in time [208]. One of the limitations of a cross-sectional study design is that it is a one-time measurement of exposure and outcome, thus it is difficult to derive causal relationships between risk factors and a disease. Another limitation is that these studies are prone to confounding. Thus, it is important, that confounding factors are adjusted during statistical analysis (within the regression model) [208].

A case-control study determines whether an exposure is associated with an outcome of interest (e.g. disease). In simple terms, a case-control design, is a study which compares a group of individuals who have a disease or an outcome of interest (cases), with patients who are free

from a disease/outcome at a given point of time [209]. The study design is very similar to a cross-sectional study design, and both studies share many strengths. The main strengths of observational studies are that they can be used to generate a hypothesis, or they may be the only study design which is feasible or ethically viable to be carried out. Furthermore, observational studies are quick, easy and relatively inexpensive. They have the potential for large numbers of samples to be collected [210]. Under a nutrigenetics perspective, observational studies (cross sectional and case control) can impose substantial limitations. Firstly, phenotypes can vary across different time periods. For example, when testing gene-diet interactions, TAG concentrations vary upon the time of collection [211], thus only collecting fasting blood samples may be a limitation. Further to this, observational studies lack replication, making it difficult to conclude whether the findings are due to chance. Observational studies rely on FFQs, which are self-reported by participants and this can introduce bias. Ultimately, cross-sectional studies are beneficial as they are able to identify genetic variants which may be associated with diseases, and these variants are less likely to be affected by confounding variables [212].

The next study design is a randomized clinical trial (RCT). RCTs are part of an experimental study design, where volunteers are randomly assigned to receive an experimental treatment (intervention) or a control treatment (where they receive the current standard treatment: this could be no treatment, a placebo or the best existing treatment currently available) [213]. As a result, any observed changes in the outcome e.g. vitamin B12 levels, is a result of the intervention treatment. The main advantages of an experimental trial are that both participants and trialists are unaware of whether the participant is receiving the treatment or control diet, until the study is completed. Although randomised control trials are powerful tools, studies are often limited by the sample size. It is difficult to have large sample sizes as it

is not cost effective, furthermore participants may drop out or have poor compliance with the treatment [214].

Alternatively, another type of experimental study is the ‘cross over’ study design. In a cross over study design, half of the study samples are randomly assigned to a control diet for a certain period of time, they then undergo a wash out period, and they then switch to the experimental dietary intervention. The other half of the study sample, start off the experimental dietary intervention, undergo a wash out period, and then switch to the control diet [215]. In this type of study, the groups exchange their respective arms at a specific point of the experiment. The advantages of following cross-over studies are that they verify the findings of the first phase of the study, by reproducing it in the second phase, consequently reinforcing the conclusion of the study. Furthermore, intervention studies minimize the effect of confounding factors. However, one of the main concerns of dietary intervention studies is the need for a washout period between studies and that the trials often have a small sample size, which may reduce the power of detecting gene-diet interaction effect sizes [212, 216].

The postprandial study design (sequential meal design) is a type of experimental design. In this protocol two test meals are given to participants at different time intervals. The purpose of the following test design is to determine how chronic dietary fat or cereal/non-digestible carbohydrate supplements manipulate the lipaemic response. Secondly, this test design is used to determine the acute impact of specific fatty acids on the first meal on the postprandial lipaemic response of the second meal. As a result, looking at the changes in biochemical variables during the postprandial state highlights the importance of using the postprandial design in investigating gene-diet interactions [217]. An overview of the different study designs employed in gene-diet interactions is shown in **Table 6**.

**Table 6: Types of studies used to perform gene-diet interactions**

| Type of Study design | Overview | Strengths | Disadvantages |
|---|---|---|---|
| Cross-sectional | Disease related-outcomes and exposures in study participants are measured at a single point in time. | • Can be used to generate a hypothesis.<br>• Quick and Easy<br>• Relatively inexpensive.<br>• Potential for a large sample to be collected<br>• Provides estimates of prevalence of all factors measured | • It is not possible to say whether the exposure or the outcome is the cause, and which is the effect.<br>• Results are prone to confounding, so it important that confounding factors are adjusted during statistical analysis (within the regression model)<br>• Phenotypes can vary across different time periods, thus only collecting fasting blood samples may be a limitation.<br>• Using an FFQ, measures the current diet in a group of individuals. The current diet may be altered by the presence of a disease.<br>• The reliance of FFQs, which are self-reported by participants, can introduce Bias. |
| Case-control | A study which compares a group of individuals who have a disease or an outcome of interest (cases), with patients who are free from a disease/outcome at a given point of time | • Can be used to generate a hypothesis.<br>• Can study several exposure factors simultaneously<br>• Quick<br>• Easy | • Phenotypes can vary across different time periods, thus only collecting fasting blood samples may be a limitation.<br>• Selection bias<br>• Not useful for rare exposures |

| | | | |
|---|---|---|---|
| | | • Relatively inexpensive.<br>• Potential for a large sample to be collected | • The reliance of food frequency questionnaires, which are self-reported by participants, can introduce Bias.<br>• Incidence rate cannot be computed |
| Randomized clinical trials | Volunteers are randomly assigned to receive an experimental treatment (intervention) or a control treatment | • In blinded study designs, both participants and trialists are unaware of whether the participant is receiving the treatment or control diet, until the study is completed. However, often in nutrition studies it is difficult to blind interventions.<br>• Ability to detect causal relationships | • Studies are usually limited by sample size.<br>• It is not cost effective to have a large sample size.<br>• Participants may have poor compliance with the treatment. |
| Cross-over | Half of the study samples are randomly assigned to a control diet for a certain period of time, they then undergo a wash out period, and they then switch to the experimental dietary intervention. The other half of the study sample, start off the experimental dietary intervention, undergo a wash out period, and then switch to the control die | • Verification of the findings of the first phase of the study can be conducted, by reproducing it in the second phase, consequently reinforcing the conclusion of the study.<br>• This study has minimal effect from confounding factors | • The small sample size, which may reduce the power for detecting gene-diet interaction effect sizes. |
| Postprandial | In this protocol two test meals are given to participants at different time intervals. The purpose of the following test design is to determine how chronic dietary fat or cereal/non- | • The frequency of blood sampling, with on average 10–13 blood samples taken during each postprandial assessment | • Determination of the postprandial response is complex<br>• Lack of standardisation of methodologies, test meal size and composition, between different studies and research groups |

| | | | |
|---|---|---|---|
| | digestible carbohydrate supplements manipulate the lipaemic response. Secondly, this test design is used to determine the acute impact of specific fatty acids on the first meal on the postprandial lipaemic response of the second meal. | | • Small subject numbers |

### 1.9.5 From Nutrigenetics to Personalised nutrition

It is becoming increasingly evident that genes and nutrients interact and influence an individual's risk of developing metabolic disease related traits [11]. Approximately over 1000 genes have been shown to be associated with human diseases, [218]; however, many of these genes will not increase the risk of developing a disease without exposure to certain dietary compounds [219]. Given that 80% of chronic diseases can be prevented by lifestyle and dietary modifications [220], it is important that dietary prevention strategies and dietary guidelines are revised. It is now possible to individualise diets using dietary, phenotype and genotypic data [221]. Greater attention is now being placed in switching dietary interventions from being population-based to being 'personalised' according to an individual's genotype. The concept of personalised nutrition is continually changing as research is developing in the field. Grimaldi et al, describes it as an approach that 'uses information on individual characteristics to develop targeted nutritional advice, products, or services' [222]. The importance of personalised nutrition was shown in a retrospective study, which found that participants who were truly matched to a diet based on their genotype, had a twofold to threefold greater reduction in body weight during a 12-month period, compared to individuals falsely matched to a diet [223].

At present, personalised nutrition is in its infancy. The success of personalised dietary advice relies on its ability to drive dietary change and attract consumer interest [221]. Although nutrient-gene interactions are a promising field of research, the molecular and pathophysiological mechanisms underlying these interactions is unclear. It is important that functional studies are carried out to clarify the biological significance and potential clinical applications of gene-diet interactions [12]. Furthermore, it has been shown that gut microbiota could interact with gene-diet interactions, to modify the risk of developing metabolic diseases [224]. As a result, future studies should profile individuals for metabolites, so that personalised dietary advice can be based on an individual's metabotype [12]. It is essential that before

personalised nutrition is introduced; larger, well-powered studies should be conducted in a range of ethnic groups. Furthermore, other modifiable factors (e.g. physical activity), which could interact with genetic factors should be taken into account.

## 1.10 Conclusions

The findings from these studies indicate that diet modifications, which attempts to optimize vitamin B12 concentrations and other lipid traits, must consider genetic factors. Gene-diet interaction studies are important for clarifying the relationship between nutrients, genetic variants and vitamin B12 status. Although nutrigenetics research is developing and garnering public health interest, consistent challenges have emerged surrounding the nature of nutrigenetics research. Several unregulated websites offering tests and dietary advice are available, with limited scientific evidence [225]. It is believed that there are no defined standards of how to conduct nutrigenetics studies. Additionally, the majority of nutrigenetics studies, have been published as secondary analyses to studies, the purpose of which was not to study gene-diet interactions [226]. Future studies will require an appropriate study design and a well-powered sample size. Furthermore, certain genetic variants may contribute to interindividual variability during postprandial states [211]; therefore, gene-diet interactions studies must examine both fasting and postprandial states.

In summary, there is a need to increase the number of nutrigenetics studies to establish the link between SNPs, dietary factors and health outcomes. It is also important to identify how gene-diet interactions influence vitamin B12 metabolic and lipid metabolism pathways at the molecular level, in order to determine the mechanism of action. Once this has been determined and validated in various ethnic groups, personalised dietary advice can be enforced to prevent diet-related diseases.

## 1.11 Thesis aims and outlines of the thesis

Based on the hypothesis that SNPs would influence serum vitamin B12 concentrations that may be modulated by lifestyle factors across different ethnic groups, the aims of this thesis are outlined below and is visually represented in **Figure 3** (This diagram is a generic diagram that is modified throughout the thesis, depending on the study nature):

1. To examine the association of selected common SNPs associated with vitamin B12 concentrations and SNPs associated with metabolic traits with vitamin B12 concentrations and metabolic outcomes in different ethnic groups.
2. To examine the interaction between these SNPs and lifestyle factors [dietary (fat, carbohydrate, and protein as total energy %) and physical activity levels] on vitamin B12 concentrations and metabolic outcomes using various study designs.

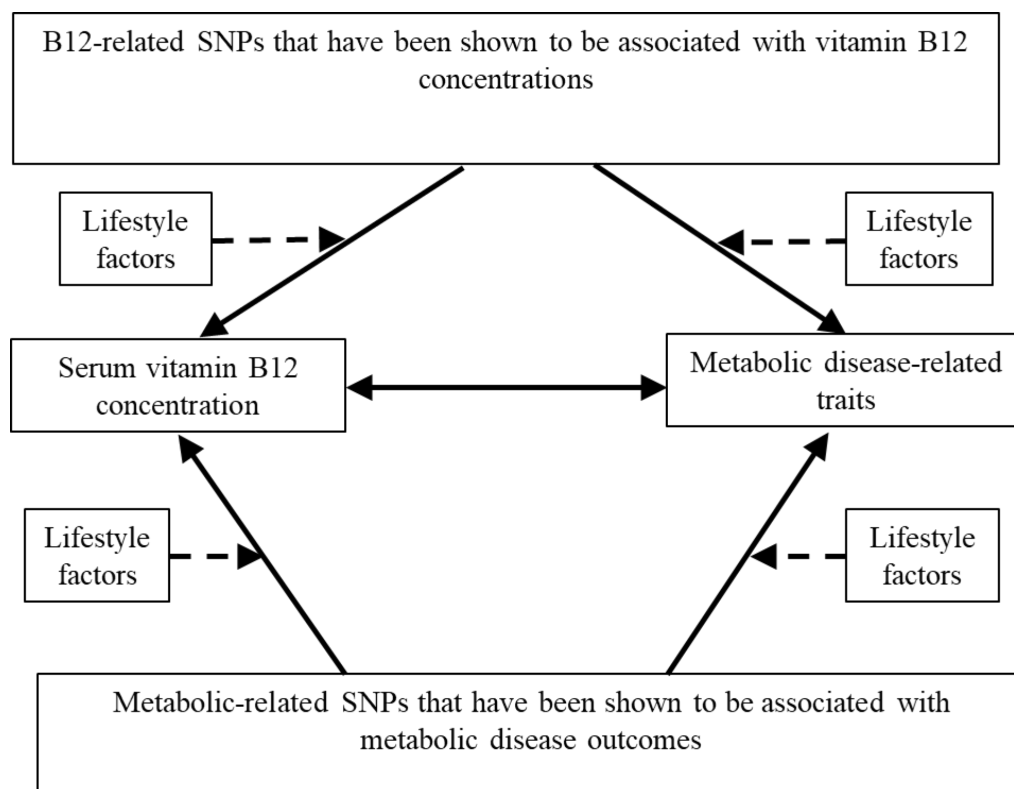


**Figure 3: The aims of this thesis**

*The diagram shows four possible associations, and four possible interactions. One-sided arrows with unbroken lines represent genetic associations and one-sided arrows with broken lines*

*represent interactions between a lifestyle factor and SNPs on serum vitamin B12/ metabolic traits. The first aim was to test for the associations between the B12-related SNPs and vitamin B12 status and metabolic disease related traits. Next, the association between the metabolic-related SNPs and vitamin B12 concentrations and metabolic disease-related traits was tested. The second aim was to test whether these genetic associations were modified by lifestyle factors (macronutrient intake and physical activity levels).*

The hypothesis and aims of each chapter are outlined below and is summarized in **Table 7**:

**Chapter 2**: Based on the candidate gene and GWA studies, associations between genetic loci in several genes involved in vitamin B12 metabolism have been identified. The aim of this literature review was to establish a reliable list of genetic variations that will allow us to predict the vitamin B12 status of an individual by knowing their genotype in these genetic variations. This review identified the genetic determinants of circulating vitamin B12 levels by focusing on new findings from GWA and candidate gene association studies, as well as results from Mendelian randomization analyses conducted so far for vitamin B12 pathway genes. The review also discussed the role of the genes involved in B12 status and reported the genetic variants specific to particular ethnic groups.

**Chapter 3**: Cardiovascular disease (CVD) has remained the leading cause of mortality in Brazil since the late 1960s and may be influenced by abnormal concentrations of vitamin B12, homocysteine, folic acid and lipids in our body. To date, common variants in genes of the one-carbon metabolism pathway have been reported to influence the concentrations of vitamin B12, folic acid, homocysteine and lipids. However, the interaction between SNPs involved in the one-carbon metabolism pathway and macronutrient intake on cardiovascular risk factors in the Brazilian population has not yet been investigated. Hence, the present study investigated whether the association of ten SNPs involved in the one-carbon metabolism pathway with vitamin B12,

folic acid, homocysteine and lipid levels, and examined the interaction of these SNPs with lifestyle factors (dietary factors and physical activity) in adolescents (n=119) with cardiovascular risk.

**Chapter 4:** Observational studies in South Asian populations have suggested an association between vitamin B12 status and metabolic traits; however, the findings have been inconclusive. Given that there are no gene-diet interaction studies, to date, in the Sri Lankan population, I used a genetic approach to explore the relationship between metabolic traits and vitamin B12 status in a South Asian Sri Lankan population and investigated whether these relationships were modified by lifestyle factors (dietary factors and physical activity) in 109 Sinhalese adults (61 men and 48 women aged 25-50 years).

**Chapter 5:** Low vitamin B12 concentrations have been associated with major clinical outcomes, including adiposity, in Indian populations. The Fat mass and obesity associated gene (*FTO*) is an established obesity-susceptibility locus; however, it remains unknown whether it influences vitamin B12 status. Hence, I investigated the association of two previously studied *FTO* polymorphisms (rs2388405 and rs8050136) with vitamin B12 concentrations and metabolic disease-related outcomes and examined whether these associations were modified by dietary factors and physical activity in an Asian Indian population (300 Type 2 Diabetic cases, 300 pre-diabetics and 300 normal glucose-tolerant (NGT)).

**Chapter 6:** Adverse effects of maternal vitamin B12 deficiency have been linked to major clinical outcomes, including increased body mass index and gestational diabetes, however, less is known about vitamin B12 nutrition in non-pregnant women. The aim of this study was to use a gene-based approach to explore the relationship between metabolic traits and vitamin B12 status in a cohort of 117 Minangkabau women (25-60 years) in Padang, West Sumatra, Indonesia, and investigated whether these relationships were modified by lifestyle factors (dietary factors and physical activity).

**Chapter 7:** Low vitamin B12 status has been shown to be a risk factor for several cardiometabolic traits such as obesity, diabetes and cardiovascular disease (CVD). Animal models have shown that the modification of dietary fat intake can affect vitamin B12 status. Hence, we investigated whether vitamin B12- and metabolic disease-related genetic variants modify vitamin B12 concentrations and cardiometabolic traits in response to replacement of saturated fatty acids (SFA) with monounsaturated (MUFA) or n-6 polyunsaturated (PUFA) fatty acids. A retrospective analysis was conducted on 119 participants in the Dietary Intervention and VAScular function (DIVAS) study.

**Chapter 8**: This chapter focuses on the discussion, which is based on the findings from all the studies, and the future prospects of this PhD work.

**Table 7: Summary of the SNPs that were examined in each chapter**

| Chapters | Population | Study design | B12-related SNPs analysed | Metabolic disease SNPs analysed | Journal name and status of publication |
|---|---|---|---|---|---|
| **Chapter 2:** An update on vitamin B12-related gene polymorphisms and B12 status. | Multi-ethnic | Literature review | • 59 B12-related SNPs from 19 genes were analysed. | • Not applicable | Genes & Nutrition (**Published,** DOI number: 10.1186/s12263-018-0591-9) |
| **Chapter 3:** The Influence of One-carbon Metabolism Gene Polymorphisms and Gene–environment Interactions on Homocysteine, Vitamin B12, Folate and Lipids in a Brazilian Adolescent Population | Brazilian | Cross-sectional study | • Fucosyltransferase [*FUT2*]- rs602662<br>• Transcobalamin 2 [*TCN2*]- rs1801198<br>• 5-methyltetrahydrofolate-homocysteine methyltransferase or methionine synthase [*MTR*]- rs1805087[a]<br>• 5-methyltetrahydrofolate-homocysteine methyltransferase reductase or methionine synthase reductase [*MTRR*]- rs1801394[a]<br>• Betaine-homocysteine S-methyltransferase | • Catechol-o-methyl transferase [*COMT*]- rs4680 and rs4633 | Journal of Diabetology (**Published,** DOI number: 10.4103/jod.jod_37_18) |

| | | | | | |
|---|---|---|---|---|---|
| | | | [*BHMT*]-rs3797546 and rs492842[b]<br>• methylenetetrahydrofolate reductase [*MTHFR*]-rs1801131[c]<br>• methylenetetrahydrofolate reductase [*MTHFR*]-rs1801133[d] | | |
| **Chapter 4:** A genetic approach to examine the relationship between vitamin B12 status and metabolic traits in a South Asian population | Sri Lankan | Cross-sectional study | • *MTHFR*- rs1801133<br>• Carbamoyl-phosphate synthase 1 [*CPS1*]-rs1047891<br>• Cubulin [*CUBN*]-rs1801222<br>• CD320 molecule [*CD320*]- rs2336573<br>• *TCN2*- rs1131603<br>• Citrate lyase beta like [*CLYBL*]- rs41281112<br>• *FUT2*- rs602662<br>• Transcobalamin 1 [*TCN1*]- rs34324219<br>• Fucosyltransferase 6 [*FUT6*]- rs778805<br>• Methylmalonyl-CoA mutase [*MUT*]-rs1141321) | • Fat mass and obesity-associated [*FTO*]-rs9939609 and rs8050136<br>• Melanocortin 4 Receptor [*MC4R*]-rs17782313 and rs2229616<br>• Transcription factor 7-like 2 [*TCF7L2*]-rs12255372 and rs7903146<br>• Potassium voltage-gated channel subfamily J member 11 [*KCNJ11*]-rs5219 | International Journal of Diabetes in Developing Countries (**Published**, DOI number: https://doi.org/10.1007/s13410-019-00749-8) |

| | | | | • Calpain 10 [*CAPN10*]- rs3792267, rs2975760 and rs5030952 | |
|---|---|---|---|---|---|
| **Chapter 5:** Evidence for the association between *FTO* gene variants and vitamin B12 concentrations in an Asian Indian population | Indian | Case-Control | • Not applicable | • *FTO*- rs9939609 and rs2388405 | Genes & Nutrition (**Published,** DOI number: https://doi.org/10.1186/s12263-019-0649-3) |
| **Chapter 6:** A nutrigenetic approach for investigating the relationship between vitamin B12 status and metabolic traits in Indonesian women | Indonesian | Cross-sectional study | • *MTHFR*- rs1801133<br>• *CPS1*- rs1047891<br>• *CUBN*- rs1801222<br>• *CD320*- rs2336573<br>• *TCN2*- rs1131603<br>• *FUT2*- rs602662<br>• *TCN1*- rs34324219<br>• *FUT6*- rs778805<br>• *MUT*- rs1141321 | • *FTO*- rs9939609 and rs8050136<br>• *MC4R*- rs17782313 and rs2229616<br>• *TCF7L2*- rs12255372 and rs7903146<br>• *KCNJ11*- rs5219<br>• *CAPN10*- rs3792267 and rs5030952 | Journal of Diabetes & Metabolic Disorders (**Published,** DOI number: https://doi.org/10.1007/s40200-019-00424-z) |
| **Chapter 7:** DIVAS A genetic | British | Dietary intervention study | • *FUT2*- rs602662, rs492602 and rs16982241 | • *TCF7L2*- rs12255372 and rs7903146 | Lipids in Health and Disease **(Under review)** |

| | | | | | |
|---|---|---|---|---|---|
| approach to investigate the relationship between vitamin B12 status and cardiometabolic traits in response to changes in dietary fat composition in adults with moderate cardiovascular disease risk | | | | • *MC4R*- rs17782313 and rs2229616<br>• *FTO*- rs9939609 and rs8050136. | |

[a]SNPs which have shown associations with folate

[b] SNPs which have shown associations with homocysteine

[c]SNPs which have shown associations with folate and homocysteine

[d] SNPs which have shown associations with vitamin B12, folate and homocysteine

# Chapter 2

## An update on vitamin B12-related gene polymorphisms and B12 status

For this literature review, I extracted and interpreted genetic variants related to vitamin B12 status. I conducted a literature search and identified 10,534 articles from the PubMed database. Following this, 10,482 articles were excluded according to the established exclusion criteria. In addition, the reference lists of identified publications were hand searched to identify any further studies. Further exclusions were applied and as a result, only 23 articles were selected for analysis. I was also responsible for contacting corresponding authors to provide any additional information where needed. I wrote the manuscript and revised the manuscript based on comments from the co-authors. I was also involved in drafting the responses to the comments from the reviewers.

**Published (The published version of the paper is attached as an appendix at the end of the thesis)**

Surendran, S., Adaikalakoteswari, A., Saravanan, P., Shatwaan, I. A., Lovegrove, J. A. and Vimaleswaran, K. S. (2018) An update on vitamin B12-related gene polymorphisms and B12 status. Genes & Nutrition, 13 (1). pp. 1555-8932. ISSN 1865-3499 doi: https://doi.org/10.1186/s12263-018-0591-9

### 2.1 Abstract

**Background:** Vitamin B12 is an essential micronutrient in humans needed for health maintenance. Deficiency of vitamin B12 has been linked to dietary, environmental and genetic

factors. Evidence for the genetic basis of vitamin B12 status is poorly understood. However, advancements in genomic techniques have increased the knowledge-base of the genetics of vitamin B12 status. Based on the candidate gene and genome wide association (GWA) studies, associations between genetic loci in several genes involved in vitamin B12 metabolism have been identified.

**Objective:** The objective of this literature review was to identify and discuss reports of associations between single nucleotide polymorphisms (SNPs) in vitamin B12 pathway genes, and their influence on the circulating levels of vitamin B12.

**Methods:** Relevant articles were obtained through a literature search on PubMed through to May 2017. An article was included if it examined an association of a SNP with serum or plasma vitamin B12 concentration. Beta coefficients and odds ratios were used to describe the strength of an association, and a $P < 0.05$ was considered as statistically significant. Two reviewers independently evaluated the eligibility for the inclusion criteria and extracted the data.

**Results:** From twenty-three studies which fulfilled the selection criteria, sixteen studies identified SNPs that showed statistically significant associations with vitamin B12 concentrations. Fifty-nine vitamin B12-related gene polymorphisms associated with vitamin B12 status were identified in total, from the following populations: African American, Brazilian, Canadian, Chinese, Danish, English, European ancestry, Icelandic, Indian, Italian, Latino, Northern Irish, Portuguese and residents of the United States.

**Conclusion:** Overall, the data analysed suggests that ethnic-specific associations are involved in the genetic determination of vitamin B12 concentrations. However, despite recent success in genetic studies, the majority of identified genes that could explain variation in vitamin B12 concentrations were from Caucasian populations. Further research utilizing larger sample sizes

of non-Caucasian populations is necessary in order to better understand these ethnic-specific associations.

## 2.2 Introduction

Vitamin B12, also known as cobalamin (Cbl), is an essential water-soluble micronutrient required to be ingested by humans to maintain health. The nutritional deficiency of vitamin B12 has been linked to many complications including an increased risk of macrocytic anaemia, neuropsychiatric symptoms [227], cardiovascular diseases [228], and the onset of different forms of cancer [229, 230]. To maintain adequate vitamin B12 status, individuals must ingest sufficient dietary vitamin B12 and retain the ability to absorb vitamin B12. The absorption, transport and cellular uptake of vitamin B12 is dependent upon the co-ordinated action of the binding proteins: haptocorrin (HC), intrinsic factor (IF), transcobalamin II (TC) and other specific cell receptors. After vitamin B12 binds to HC in the stomach and IF in the duodenum, it binds to TC within the enterocyte and is then released into the blood stream. The vitamin B12-TC complex then binds to the transcobalamin receptor (TC-R) and is taken up by cells via endocytosis [231].

Genetic variants may alter vitamin B12 tissue status by affecting the proteins involved in vitamin B12 absorption, cellular uptake and intracellular metabolism [143]. In a study using monozygotic and dizygotic twins, the heritability of B12 levels was estimated to be 59%, indicating that the magnitude of genetic influence on vitamin B12 levels are considerable [203]. At present, genetic studies of vitamin B12 status suggest that it is a multifactorial trait, where several single nucleotide polymorphisms (SNPs) in multiple genes interact with the environment to cause the altered B12 status [204]. Most of the SNPs related to vitamin B12 status have been examined using a candidate gene approach [204]. However, it is now possible to use an unbiased genome-wide association (GWA) study to associate DNA sequence

variations across the human genome with the risk factors of developing a disease [232]. Despite a number of informative genome-wide association studies and candidate gene analyses, the complex relationship between an individual's genotype and their vitamin B12 status remains poorly understood. This article is the first literature review to discuss the results of genetic studies associated with vitamin B12 status in healthy individuals. Understanding the possible underlying genetic factors of vitamin B12 metabolism will lead to an increased understanding of the biological mechanisms underlying vitamin B12 status.

## 2.3 Materials and Methods

### 2.3.1 Study identification

In order to identify published articles, literature searches were completed using the PubMed database (https://www.ncbi.nlm.nih.gov/pubmed/), from the earliest date of indexing until May 2017. The following keywords were used to identify articles from PubMed: 'vitamin B12 and genetics' (n=2,792), 'vitamin B12 and gene polymorphisms' (n=447), 'genetic variants of vitamin B12' (n=115), 'genetic variants of cobalamin' (n=95), 'genetics of cobalamin' (n=2,574), 'genetics of vitamin B12'(n= 2,721)','vitamin B12 and genes (n=932) and 'cobalamin and genes' (n=858). In addition, reference lists of identified publications were hand searched to identify other studies potentially eligible for inclusion.

No limits on geographical location were placed in the literature search, and only articles written in English were selected. After inclusion and exclusion criteria were applied, a comprehensive list of relevant articles was included in this review.

### 2.3.2 Study selection

The abstracts of all articles with relevant titles were reviewed first and were further assessed if they reported original data on testing for an association of a SNP with plasma or serum vitamin B12 concentrations. Articles were excluded if: 1) they included non-human

subjects 2) they were limited to a subset of the population (e.g. pregnant women / carrying a disease) and 3) the sample size of the population was less than 10.

Based on the search criteria and keywords used, 10,534 articles were identified from the PubMed database. Following this, 10,482 articles were excluded according to the established exclusion criteria, and 52 articles were then considered as potentially relevant for the review. The full text of the 52 articles was read, which resulted in the exclusion of a further 29 articles. As a result, only 23 articles were selected for analysis (**Figure 4**). A $P< 0.05$ was considered as statistically significant.

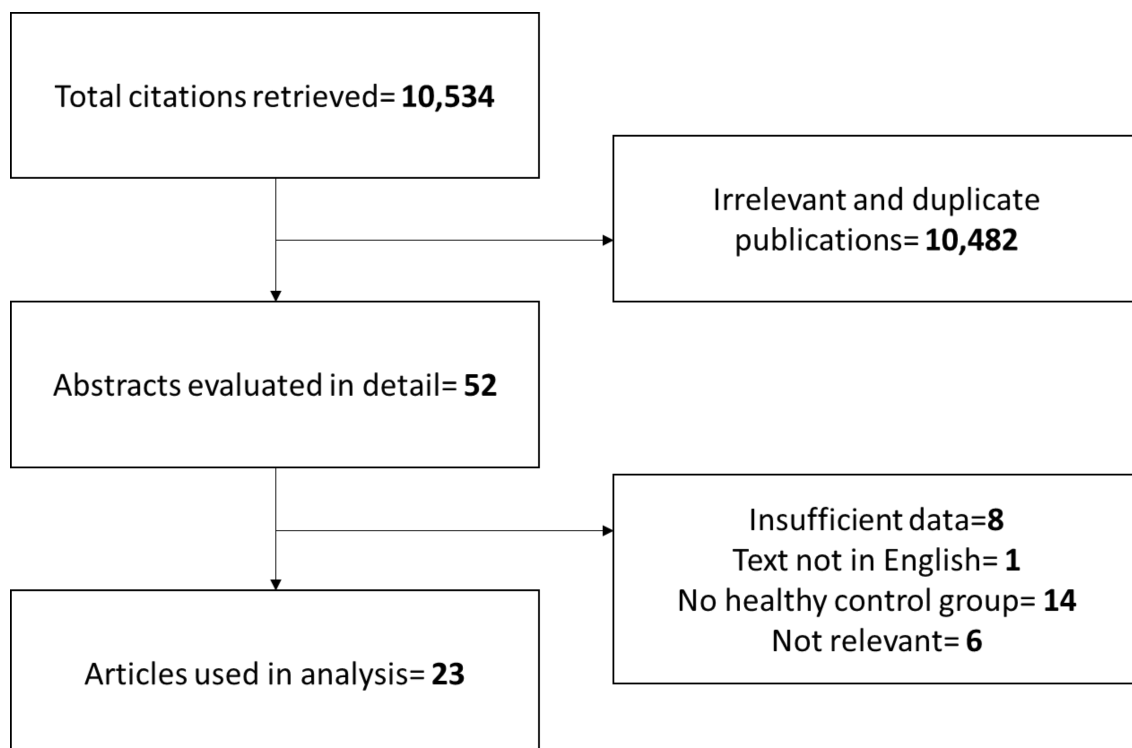


**Figure 4: Flow diagram of studies identified in the literature search for the identification of genetic variants associated with vitamin B12 concentrations**

### 2.3.3 Data extraction:

The studies were identified by a single investigator (SS) and the following data were double-extracted independently by two reviewers (VKS and IAS): first author, publication

year, location or ethnicity of participants, sample size, mean age, study design, SNP position, name and rs ID, genotype and allele distribution by vitamin B12 status. For the outcome data, the beta coefficients of vitamin B12 concentrations per risk allele, odds ratios (ORs) with their corresponding 95% confidence intervals (95% CIs) were extracted. Any discrepancies over extracted data were settled through discussion between the two independent reviewers (VKS and IAS). Finally, corresponding authors were contacted to provide any additional information where needed.

## 2.4 Results of Database search: Genes associated with vitamin B12 status

The following section reviews studies of genetic variants which have been associated with vitamin B12 status. These variants have been grouped as: a) co-factors or regulators essential for the transport of vitamin B12 (b) membrane transporters actively facilitating membrane crossing (c) involved in the catalysis of enzymatic reactions in the one carbon cycle (d) involved in cell cycle regulation, (e) mitochondrial proteins and (f) other genes (**Figure 5 and 6**. A summary of GWA and candidate gene association studies that have been reported to be associated with circulating plasma or serum B12 concentrations are presented in **Table 8** and **Table 9.** The location and function of the most frequently studied genes associated with vitamin B12 concentrations are summarized in **Table 10**.

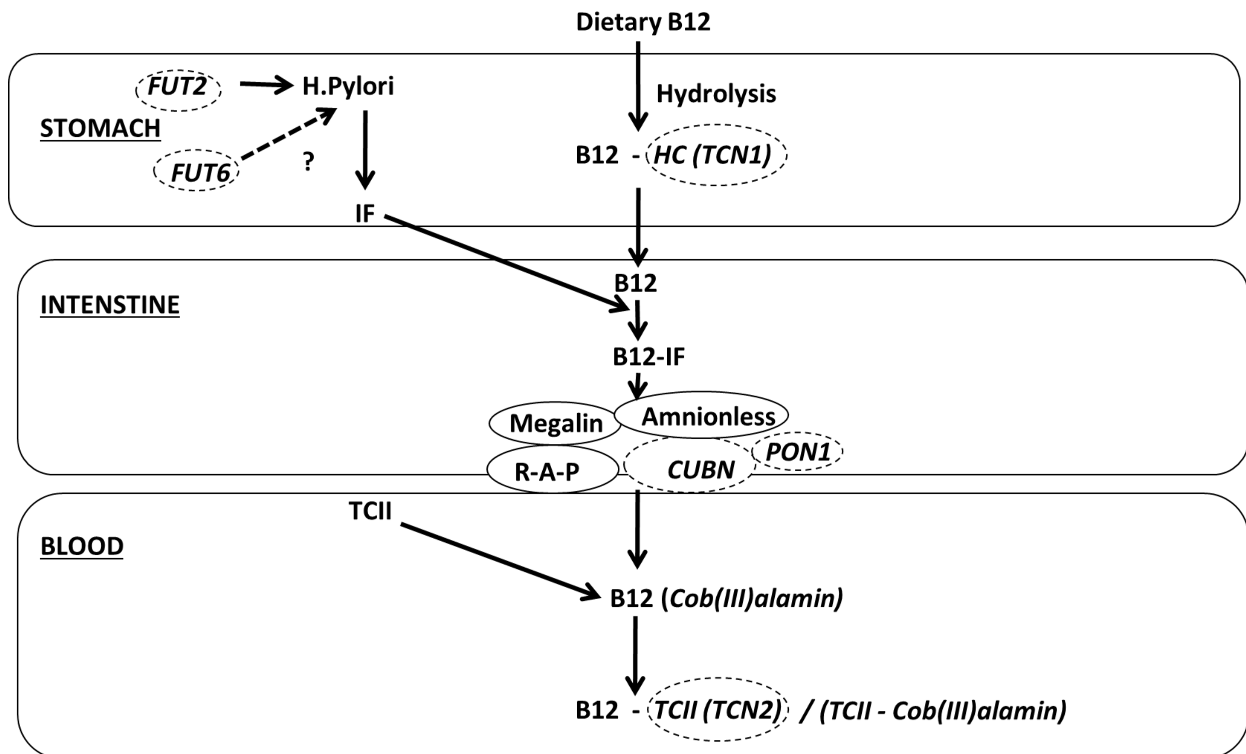


**Figure 5: Diagram representing the genes associated with vitamin B12 status**

*The diagram shows the proteins involved in the metabolism of vitamin B12 from dietary intake to reaching the circulatory system. Genes identified to harbour variants regulating serum levels of B12 are surrounded by dashed lines. B12: vitamin B12; CUBN: cubilin (intrinsic factor-cobalamin receptor); FUT2: fucosyl-transferase 2; FUT6: fucosyl-transferase 6; HC: Haptocorrin (TCN1); H. pylori: Helicobacter pylori; IF: Intrinsic factor; PON1: serum paraoxonase/arylesterase 1; R-A-P: Receptor-Associated-Protein; TCII: Transcobalamin II (TCN2); TCII-R: Transcobalamin II receptor (CD320).*

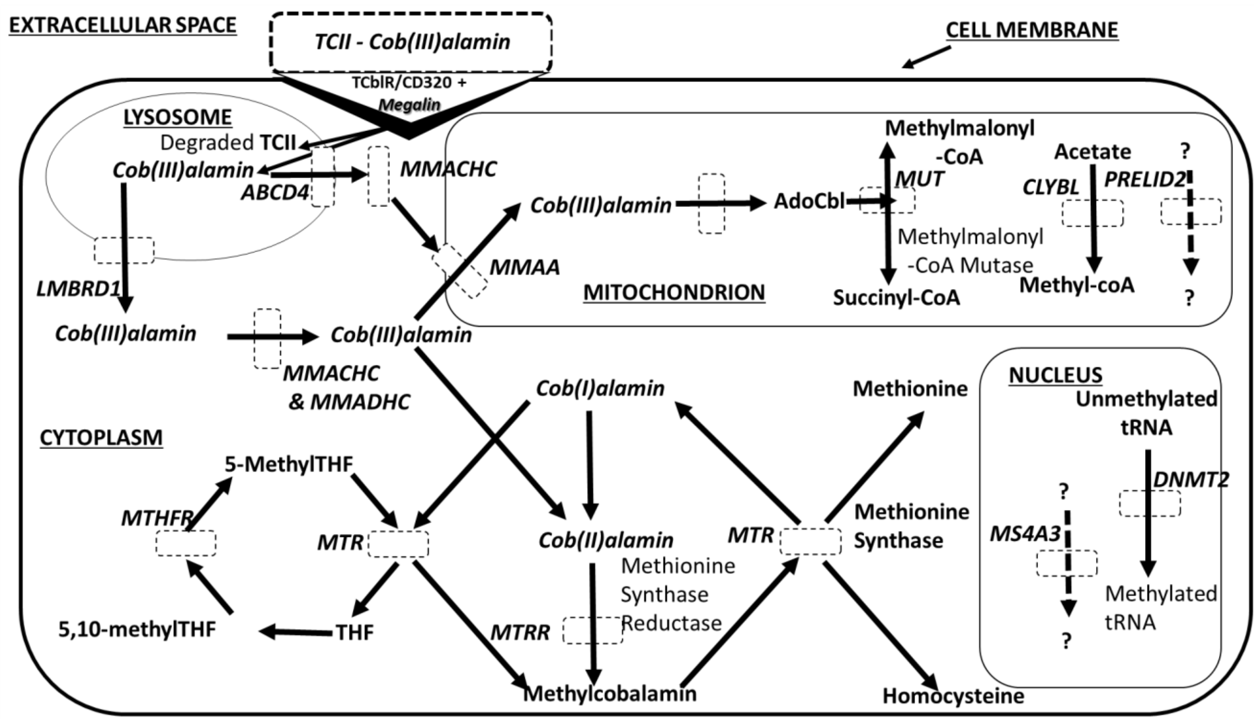


**Figure 6: Diagram representing the genes associated with vitamin B12 status**

*The diagram shows the proteins involved in the metabolism of vitamin B12 from the extracellular space to being internalised within the cell. Genes identified to harbour variants regulating serum levels of B12 are surrounded by dashed lines. Ado-B12: Adenosyl-cobalamin; ABDC4: ATP-binding cassette, sub-family D (ALD), member 4; CD320: CD320 Molecule; CLYBL: Citrate Lyase Beta Like; DNMT2: DNA methyltransferase 2 gene; LMBD1: LMBR1 domain containing 1; LMBRD1: LMBR1 Domain Containing 1; MMAA: Methylmalonic Aciduria (Cobalamin Deficiency) CblA Type; MMAB: Methylmalonic Aciduria (Cobalamin Deficiency) CblB Type; MMACHC: Methylmalonic aciduria and homocystinuria, cblC type; MMADHC:Methylmalonic Aciduria (Cobalamin Deficiency) CblD Type, With Homocystinuria; MS4A3: Membrane-Spanning 4-Domains, Subfamily A, Member 3 (Hematopoietic Cell-Specific); MTHFR: 5-methyl-tetrahydrafolate reductase; MTR:5-Methyltetrahydrofolate-Homocysteine Methyltransferase; MTRR: 5-Methyltetrahydrofolate-Homocysteine Methyltransferase Reductase; MUT: Methylmalonyl CoA Mutase; PRELID2: PRELI Domain Containing 2; THF: Tetrahydrofolate; 5,10-Methyl THF: 5,10-Methyl-tetrahydrofolate*

**Table 8: Genome-wide association studies showing the association of SNPs with vitamin B12 concentrations**

| Chromosome location | Gene name (Gene symbol) | Reference SNP Cluster ID | Sample size & Ethnicity | Age (years) | Minor allele + Minor allele frequency | Effect size | *P*-value | References |
|---|---|---|---|---|---|---|---|---|
| 1p34.1 | Methylmalonic aciduria and homocystinuria type C protein (*MMACHC*) | rs12272669 | Icelandic sample: n = 37283 | 63 ± 24 | A = 0.002 | Effect: A allele<br>Other: G allele<br><br>β = 0.51 pmol/L | $3.00 \times 10^{-9}$ | Garup et al., 2013 [12] [205] [205] |
| 1q42.2 | Intergenic | rs583228 | Initial sample: n = 1999 Chinese Han men | 38 ± 11 | T = 0.220 | Effect: T allele<br>Other: C allele<br><br>β = Not available | $7.68 \times 10^{-6}$ | Lin et al., 2012 [19] |
| | | | Replication sample: n = 1496 Chinese men | 37± 11 | | Effect: T allele<br>Other: C allele<br><br>β = Not available | >0.05 | |
| | | | Combined total: n = 3495 | | | Effect: T allele<br>Other: C allele<br><br>β = 25.50 pg/ml<br>SE = 7.19 | $3.92 \times 10^{-4}$ | |

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|---|---|---|---|---|---|---|---|---|
| 2q34 | Carbamoyl-Phosphate Synthase 1 (*CPS1*) | rs1047891 | Icelandic sample: n = 37283 | 63 ± 24 | A = 0.372 | Effect: C allele<br>Other: A allele<br><br>β = 0.04 pmol/L | 7.60 x $10^{-6}$ | Grarup et al., 2013 [12] |
| | | | Danish Inter99 population: n = 5481 | 46 ± 8 | | Effect: C allele<br>Other: A allele<br><br>β = 0.10 pmol/L | 5.50 x $10^{-4}$ | |
| | | | Danish - Health 2006: n = 2812 | 49 ± 13 | | Effect: C allele<br>Other: A allele<br><br>β = 0.03 pmol/L | >0.05 | |
| | | | Combined total: n = 45574 | | | Effect: C allele<br>Other: A allele<br><br>β = Not available | 3.00 x $10^{-8}$ | |
| 4q31.21 | Methylmalonic aciduria (cobalamin deficiency) cblA type (*MMAA*) | rs2270655 | Parents of PMNS cohort*: n = 1001 Indian | 36 ± 5 | C = 0.157# | Effect allele: C<br><br>β = -0.07 pmol/L | >0.05 | Nongmaithem et al., 2017 [22] [233] [233] |
| | | | Adults: n = 724 Indian | 38 ± 11 | | Effect allele: C<br><br>β = 0.00 pmol/L | >0.05 | |

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|---|---|---|---|---|---|---|---|---|
| | | | PMNS children*:<br>n = 690<br>Indian | 11 ± 1 | | Effect allele: C<br><br>β = -0.09 pmol/L | >0.05 | |
| | | | PS children†:<br>n = 534<br>Indian | 5 ± 0 | | Effect allele: C<br><br>β = -0.20 pmol/L | 2.00 x $10^{-2}$ | |
| 4q31.21 | Methylmalonic aciduria (cobalamin deficiency) cblA type (*MMAA*) | rs2270655 | Icelandic sample:<br>n = 37283 | 63 ± 24 | C = 0.059 | Effect: G allele<br>Other: C allele<br><br>β = 0.07 pmol/L | 3.50 x $10^{-5}$ | Grarup et al., 2013 [12] [205] [205] |
| | | | Danish Inter99 population:<br>n = 5481 | 46 ± 8 | | Effect: G allele<br>Other: C allele<br><br>β = 0.30 pmol/L | 2.80 x $10^{-7}$ | |
| | | | Danish - Health 2006:<br>n = 2812 | 49 ± 13 | | Effect: G allele<br>Other: C allele<br><br>β = 0.25 pmol/L | 5.80 x $10^{-8}$ | |
| | | | Combined total:<br>n = 45576 | | | Effect: G allele<br>Other: C allele<br><br>β = Not available | 2.20 x $10^{-13}$ | |
| 4q31.21 | Methylmalonic aciduria (cobalamin deficiency) cblA type (*MMAA*) | rs114699496 | Icelandic sample:<br>n = 25960 | 63 ± 24 | T = 0.046** | Effect: T<br>Other: C<br><br>β = -0.07 pmol/L | 7.60 x $10^{-6}$ | Grarup et al., 2013 [12] |

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|---|---|---|---|---|---|---|---|---|
| 5q32 | Intergenic | rs10515552 | Initial sample:<br>n = 1999<br>Chinese Han men | 38 ± 11 | C = 0.162 | Effect: C allele<br>Other: T allele<br><br>β = Not available | 8.52 x $10^{-7}$ | Lin et al., 2012 [19] |
|  |  |  | Replication sample:<br>n = 1496<br>Chinese men | 37 ± 11 |  | Effect: C allele<br>Other: T allele<br><br>β = Not available | 5.15 x $10^{-3}$ |  |
|  |  |  | Combined total:<br>n = 3495 |  |  | Effect: C allele<br>Other: T allele<br><br>β = 43.93 pg/ml<br>SE = 7.98 | 3.94 x $10^{-8}$ |  |
| 6p12.3 | Methylmalonyl-CoA Mutase (*MUT*) | chr6:49508102 | Icelandic sample:<br>n = 25960 | 63 ± 24 | Not available | Effect: C allele<br>Other: G allele<br><br>β = 0.07 pmol/L | 1.60 x $10^{-18}$ | Grarup et al., 2013 [12] |
| 6p12.3 | Methylmalonyl-CoA Mutase (*MUT*) | rs1141321 (rs9473558) | Icelandic sample:<br>n = 37283 | 63 ± 24 | T = 0.373 | Effect: C allele<br>Other: T allele<br><br>β = 0.06 pmol/L | 1.40 x $10^{-16}$ | Grarup et al., 2013 [12] |
|  |  |  | Danish Inter99 population:<br>n = 5481 | 46 ± 8 |  | Effect: C allele<br>Other: T allele<br><br>β = 0.12 pmol/L | 1.40 x $10^{-5}$ |  |
|  |  |  | Danish- Health 2006:<br>n = 2812 | 49 ± 13 |  | Effect: C allele<br>Other: T allele<br><br>β = 0.11 pmol/L | 1.40 x $10^{-7}$ |  |

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|---|---|---|---|---|---|---|---|---|
|  |  |  | Combined total:<br>n = 45574 |  |  | Effect: C allele<br>Other: T allele<br><br>β = Not available | $3.60 \times 10^{-26}$ |  |
| 6p12.3 | Methylmalonyl-CoA Mutase (*MUT*) | rs1141321 (rs9473558) | Initial sample:<br>n = 1999<br>Chinese Han men | 38 ± 11 | T = 0.237 | Effect: T allele<br>Other: C allele<br><br>β= -30.34 pg/ml<br>SE = 8.91 | $5.51 \times 10^{-4}$ | Lin et al., 2012 [19] [206] [206] |
| 6p12.3 | Methylmalonyl-CoA Mutase (*MUT*) | rs1141321 (rs9473558) | NHS-CGEMS[‡]:<br>n = 1658<br>Caucasian women | 59 ± 6 | T = 0.350 | Effect: T allele<br>Other: C allele<br><br>β = −0.03 pg/ml<br>SE = 0.01 | $4.27 \times 10^{-2}$ | Hazra et al., 2009 [20] [234][234] |
|  |  |  | SHARe[§]:<br>n = 1647<br>Caucasian women | 59 ± 10 |  | Effect: T allele<br>Other: C allele<br><br>β = −0.03 pg/ml<br>SE = 0.01 | $1.87 \times 10^{-2}$ |  |
|  |  |  | SHARe[§]:<br>n = 1458<br>Caucasian men | 59 ± 10 |  | Effect: T allele<br>Other: C allele<br><br>β = −0.07 pg/ml<br>SE = 0.01 | $3.96 \times 10^{-7}$ |  |
|  |  |  | Combined total:<br>n = 4763 |  |  | Effect: T allele<br>Other: C allele<br><br>β = −0.04 pg/ml<br>SE = 0.01 | $4.05 \times 10^{-8}$ |  |

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|---|---|---|---|---|---|---|---|---|
| 6p12.3 | Methylmalonyl-CoA Mutase (*MUT*) | rs9473555 | Icelandic sample: n = 25960 | 63 ± 24 | C = 0.402 | Effect: C allele Other: G allele β = -0.06 pmol/L | $5.40 \times 10^{-17}$ | Grarup et al., 2013 [12] |
| 6p12.3 | Methylmalonyl-CoA Mutase (*MUT*) | rs9473555 | Initial sample: n = 1999 Chinese Han men | 38 ± 11 | C = 0.238 | Effect: C allele Other: G allele β = -31.00 pg/ml SE = 8.860 | $4.06 \times 10^{-4}$ | Lin et al., 2012 [19] |
| 6p12.3 | Methylmalonyl-CoA Mutase (*MUT*) | rs9473555 | NHS-CGEMS[‡]: n = 1658 Caucasian women | 59 ± 6 | C = 0.350 | Effect: C allele Other: G allele β= −0.03 pg/ml SE = 0.01 | $4.27 \times 10^{-2}$ | Hazra et al., 2009 [20] |
| | | | SHARe[§]: n = 1647 Caucasian women | 59 ± 10 | | Effect: C allele Other: G allele β= −0.03 pg/ml SE = 0.01 | $2.26 \times 10^{-2}$ | |
| | | | SHARe[§]: n = 1458 Caucasian men | 59 ± 10 | | Effect: C allele Other: G allele β= −0.07 pg/ml SE = 0.01 | $3.71 \times 10^{-7}$ | |
| | | | Combined total: n = 4763 | | | Effect: C allele Other: G allele β= −0.04 pg/ml | $4.91 \times 10^{-8}$ | |

| | | | | | | SE = 0.01 | | |
|---|---|---|---|---|---|---|---|---|
| 6q15 | Nearest gene:<br>Sperm Acrosome Associated 1<br>(*SPACA1*) | Chr6_88792234 | Icelandic sample:<br>n = 37283 | 63 ± 24 | G = 0.006 | Effect: G allele<br>Other: A allele<br><br>β = 0.26 pmol/L | 2.80 x $10^{-7}$ | Grarup et al., 2013 [12] [205] [205] |
| 7q21.3 | Paraoxonase 1<br>(*PON1*) | rs3917577 | n = 3114<br>Canadian (85% Caucasian, 15% non-Caucasian) | 20 – 79<br>(range) | G = 0.020 | Effect: A allele<br>Other: G allele<br><br>Vitamin B-12 below adequate (< 220 pmol/L):<br>OR: 0.67 (95% CI: 0.54, 0.81) pmol/L | 7.20 x $10^{-5}$ | Zinck et al., 2015 [18] |
| 8q21.13 | Nearest gene:<br>Zinc Finger and BTB Domain Containing 10<br>(*ZBTB10*) | rs62515066 | Icelandic sample:<br>n = 37283 | 63 ± 24 | G = 0.025 | Effect: G allele<br>Other: A allele<br><br>β = 0.12 pmol/L | 5.40 x $10^{-7}$ | Grarup et al., 2013 [12] |
| 9p21.1 | NONE<br>(Intergenic) | rs12377462 | Initial sample:<br>n = 1999<br>Chinese Han men | 38 ± 11 | T = 0.366 | Effect: T allele<br>Other: C allele<br><br>β = Not available | 3.34 x $10^{-7}$ | Lin et al., 2012 [19] |
| | | | Replication sample:<br>n = 1496<br>Chinese men | 37 ± 11 | | Effect: T allele<br>Other: C allele<br><br>β = Not available | >0.05 | |

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|---|---|---|---|---|---|---|---|---|
| | | | Combined total: n = 3495 | | | Effect: T allele<br>Other: C allele<br><br>β = 28.53 pg/ml<br>SE = 5.99 | $2.02 \times 10^{-6}$ | |
| 10p12.31 | Cubulin (*CUBN*) | rs1801222 | n = 3114 Canadian (85% Caucasian, 15% non-Caucasian) | 20 – 79 (range) | A = 0.100 | Effect: G allele<br>Other: A allele<br><br>Vitamin B12 deficiency (< 148 pmol/L):<br>OR: 1.61 (95% CI: 1.24, 2.09) pmol/L | $3.00 \times 10^{-4}$ | Zinck et al., 2015 [18] |
| 10p12.31 | Cubulin (*CUBN*) | rs1801222 | n = 3114 Canadian (85% Caucasian, 15% non-Caucasian) | 20 – 79 (range) | A = 0.100 | Effect: G allele<br>Other: A allele<br><br>Vitamin B-12 below adequate (< 220 pmol/L):<br>OR: 1.39 (95% CI: 1.23, 1.58) pmol/L | $2.00 \times 10^{-7}$ | Zinck et al., 2015 [18] |
| 10p12.31 | Cubulin ( *CUBN*) | rs1801222 | Icelandic sample: n = 37283 | 63 ± 24 | A = 0.407 | Effect: G allele<br>Other: A allele<br><br>β = 0.10 pmol/L | $1.10 \times 10^{-52}$ | Garup et al., 2013 [12] |
| | | | Danish Inter99 population: n = 5481 | 46 ± 8 | | Effect: G allele<br>Other: A allele<br><br>β = 0.14 pmol/L | $7.60 \times 10^{-8}$ | |

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|---|---|---|---|---|---|---|---|---|
| | | | Danish - Health 2006:<br>n = 2812 | 49 ± 13 | | Effect: G allele<br>Other: A allele<br><br>β = 0.17 pmol/L | $2.90 \times 10^{-18}$ | |
| | | | Combined total:<br>n = 45576 | | | Effect: G allele<br>Other: A allele<br><br>β = Not available | $3.30 \times 10^{-75}$ | |
| 10p12.31 | Cubulin<br>( *CUBN*) | rs1801222 | NHS-CGEMS[‡]:<br>n = 1658<br>Caucasian women | 59 ± 6 | A = 0.280 | Effect: A allele<br>Other: G allele<br><br>β = −0.05 pg/ml<br>SE = 0.01 | $9.04 \times 10^{-5}$ | Hazra et al., 2009 [20] |
| | | | SHARe[§]:<br>n = 1647<br>Caucasian women | 59 ± 10 | | Effect: A allele<br>Other: G allele<br><br>β = −0.04 pg/ml<br>SE = 0.02 | $6.32 \times 10^{-3}$ | |
| | | | SHARe[§]:<br>n = 1458<br>Caucasian men | 59 ± 10 | | Effect: A allele<br>Other: G allele<br><br>β = −0.05 pg/ml<br>SE = 0.02 | $3.56 \times 10^{-4}$ | |
| | | | Combined total:<br>n = 4,763 | | | Effect: A allele<br>Other: G allele<br><br>β = −0.05 pg/ml<br>SE = 0.01 | $2.87 \times 10^{-9}$ | |

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|---|---|---|---|---|---|---|---|---|
| 10p12.31 | Cubulin (*CUBN*) | rs4748353 | n = 3114 Canadian (85% Caucasian, 15% non-Caucasian) | 20 – 79 (range) | C = 0.000 | Effect: C allele<br>Other: T allele<br><br>Vitamin B12 deficiency (< 148 pmol/L):<br>OR: 2.14 (95% CI: | $8.00 \times 10^{-4}$ | Zinck et al., 2015 [18] |
| 10p12.31 | Cubulin (*CUBN*) | rs11254363 | n = 3114 Canadian (85% Caucasian, 15% non-Caucasian) | 20 – 79 (range) | G = 0.010 | Effect: A allele<br>Other: G allele<br><br>Vitamin B-12 below adequate (< 220 pmol/L):<br>OR: 0.81 (95% CI: 0.70, 0.93) pmol/L | $3.00 \times 10^{-3}$ | Zinck et al., 2015 [18] |
| 10p12.31 | Cubulin (*CUBN*) | rs11254363 | GWAS Meta-analysis:<br><br>InCHIANTI study:<br>n = 1175 Italian<br><br>SardiNIA study:<br>n = 1115 Italian<br><br>BLSA study[¶]:<br>n = 640 | InCHIANTI:<br>68 ± 16<br><br>SardiNIA:<br>45 ± 18<br><br>BLSA[g]:<br>68 ± 16 | G = 0.300 | Effect: A allele<br>Other: G allele<br><br>$\beta$ = -39.16 pg/ml<br>SE = 9.18 | $7.24 \times 10^{-8}$ | Tanaka et al., 2009 [21] |
| | | | Replication study: | 47 ± 13 | | Effect: A allele | >0.05 | |

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|---|---|---|---|---|---|---|---|---|
| | | | Progetto Nutrizione study:<br>n = 687<br>Italian | | | Other: G allele<br><br>β = 3.62 pg/ml<br>SE = 10.94 | | |
| | | | Combined meta-analysis (GWAS Meta-analysis + Replication study):<br>n = 3613 | | | Effect: A allele<br>Other: G allele<br><br>β = -21.49 pg/ml<br>SE = 7.03 | $1.11 \times 10^{-6}$ | |
| 10p12.31 | Cubulin (*CUBN)* | rs12243895 | Initial sample:<br>n = 1999<br>Chinese Han men | 38 ± 11 | A = 0.243 | Effect: A allele<br>Other: G allele<br><br>β = 23.49 pg/ml<br>SE = 9.06 | $7.11 \times 10^{-3}$ | Lin et al., 2012 [19] |
| 10p12.31 | Cubulin (*CUBN)* | rs12780845 | Parents of PMNS cohort*:<br>n = 1001<br>Indian | 36 ± 5 | G = 0.415# | Effect allele: G<br><br>β = 0.09 pmol/L | >0.05 | Nongmaithem et al., 2017 [22] |
| | | | Adults:<br>n = 724<br>Indian | 38 ± 11 | | Effect allele: G<br><br>β = 0.09 pmol/L | >0.05 | |

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| | | | PMNS children*: n = 690 Indian | 11 ± 1 | | Effect allele: G<br><br>β = 0.08 pmol/L | >0.05 | |
| | | | PS children†: n = 534 Indian | 5 ± 0 | | Effect allele: G<br><br>β = 0.03 pmol/L | >0.05 | |
| 10p13 | DNA methyltransferase gene (*DNMT2*) / TRNA Aspartic Acid Methyltransferase 1 (*TRDMT1*) | rs2295809 | n = 3114 Canadian (85% Caucasian, 15% non-Caucasian) | 20 – 79 (range) | T = 0.240 | Effect: A allele Other: T allele<br><br>Vitamin B-12 below adequate (< 220 pmol/L): OR: 0.82 (95% CI: 0.73, 0.92) pmol/L | $1.00 \times 10^{-3}$ | Zinck et al., 2015 [18] |
| 10p13 | DNA methyltransferase gene (*DNMT2*) / TRNA Aspartic Acid Methyltransferase 1 (*TRDMT1*) | rs56077122 | Icelandic sample: n = 25960 | 63 ± 24 | A = 0.335 | Effect: A allele Other: C allele<br><br>β = 0.09 pmol/L | $4.80 \times 10^{-21}$ | Grarup et al., 2013 [12] |

| 11q12.1 | Intergenic<br><br>Nearest gene:<br>Transcobalamin 1 (*TCN1*) | rs1174560<br>53 | Icelandic sample:<br>n = 25960 | 63 ± 24 | A = 0.024 | Effect: G allele<br>Other: A allele<br><br>β = 0.16 pmol/L | 1.90 x<br>$10^{-9}$ | Grarup et al., 2013 [12] |
|---|---|---|---|---|---|---|---|---|
| 11q12.1 | Membrane Spanning 4-Domains A3<br>*(MS4A3)* | rs2298585 | Icelandic sample:<br>n = 25960 | 63 ± 24 | T = 0.001 | Effect: T allele<br>Other: C allele<br><br>β = 0.21 pmol/L | >0.05 | Grarup et al., 2013 [12] [205] [205] |
| 11q12.1 | Membrane Spanning 4-Domains A3<br>*(MS4A3)* | rs2298585 | Initial sample:<br>n = 1999<br>Chinese Han men | 38 ± 11 | T = 0.120 | Effect: T allele<br>Other: C allele<br><br>β = Not available | 1.71 x $10^{-10}$ | Lin et al., 2012 [19] |
| | | | Replication sample:<br>n = 1496<br>Chinese men | 37 ± 11 | | Effect: T allele<br>Other: C allele<br><br>β = Not available | 1.58 x $10^{-6}$ | |
| | | | Combined total:<br>n = 3495 | | | Effect: T allele<br>Other: C allele<br><br>β = 71.80 pg/ml<br>SE = 9.04 | 2.64 x<br>$10^{-15}$ | |
| 11q12.1 | Transcobalamin 1<br>(*TCN1*) | rs526934 | Adults:<br>n = 724<br>Indian | 38 ± 11 | G = $0.216^{\#}$ | Effect allele: G<br><br>β = -0.07 pmol/L | >0.05 | Nongmaithem et al., 2017 [22] |

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|---|---|---|---|---|---|---|---|---|
| | | | PMNS children*: n = 690 Indian | 11 ± 1 | | Effect allele: G<br><br>β = -0.10 pmol/L | >0.05 | |
| | | | PS children†: n = 534 Indian | 5 ± 0 | | Effect allele: G<br><br>β = -0.16 pmol/L | $2.00 \times 10^{-2}$ | |
| 11q12.1 | Transcobalamin 1 (*TCN1*) | rs526934 | n = 3114 Canadian (85% Caucasian, 15% non-Caucasian) | 20 – 79 (range) | G = 0.080 | Effect: A allele<br>Other: G allele<br><br>Vitamin B-12 below adequate (< 220 pmol/L):<br>OR: 1.38 (95% CI: 1.21, 1.57) pmol/L | $1.40 \times 10^{-6}$ | Zinck et al., 2015 [18] |
| 11q12.1 | Transcobalamin 1 (*TCN1*) | rs526934 | Icelandic sample: n = 25960 | 63 ± 24 | G = 0.296 | Effect: G allele<br>Other: A allele<br><br>β = -0.12 pmol/L | $2.30 \times 10^{-48}$ | Grarup et al., 2013 [12] |
| 11q12.1 | Transcobalamin 1 (*TCN1*) | rs526934 | Initial sample: n = 1999 Chinese Han men | 8 ± 11 | G = 0.189 | Effect: G allele<br>Other: A allele<br><br>β= -30.39 pg/ml<br>SE = 9.66 | $1.78 \times 10^{-3}$ | Lin et al., 2012 [19] |

| 11q12.1 | Transcobalamin 1 (*TCN1*) | rs526934 | NHS-CGEMS[‡]: n = 1658 Caucasian women | 59 ± 6 | G = 0.270 | Effect: G allele Other: A allele $\beta = -0.05$ pg/ml SE = 0.01 | $1.27 \times 10^{-3}$ | Hazra et al., 2009 [20] |
|---|---|---|---|---|---|---|---|---|
| | | | SHARe[§]: n = 1647 Caucasian women | 59 ± 10 | | Effect: G allele Other: A allele $\beta = -0.06$ pg/ml SE = 0.02 | $6.69 \times 10^{-5}$ | |
| | | | SHARe[§]: n = 1458 Caucasian men | 59 ± 10 | | Effect: G allele Other: A allele $\beta = -0.06$ pg/ml SE = 0.02 | $1.64 \times 10^{-4}$ | |
| | | | Combined total: n = 4763 | | | Effect: G allele Other: A allele $\beta = -0.05$ pg/ml SE = 0.01 | $2.25 \times 10^{-10}$ | |

<table>
<tr><td rowspan="3">11q12.1</td><td rowspan="3">Transcobalamin 1<br>(TCN1)</td><td rowspan="3">rs526934</td><td>GWAS Meta-analysis:<br><br>InCHIANTI study:<br>n = 1175<br>Italian<br><br>SardiNIA study:<br>n = 1115<br>Italian<br><br>BLSA study[¶]:<br>n = 640</td><td>InCHIANTI:<br>68 ± 16<br><br>SardiNIA:<br>45 ± 18<br><br>BLSA[g]:<br>68 ± 16</td><td rowspan="3">G = 0.330</td><td>Effect: A allele<br>Other: G allele<br><br>β = 36.76 pg/ml<br>SE = 10.35</td><td>$8.33 \times 10^{-7}$</td><td rowspan="3">Tanaka et al., 2009 [21]</td></tr>
<tr><td>Replication study: Progetto Nutrizione study:<br>n = 687<br>Italian</td><td>47 ± 14</td><td>Effect: A allele<br>Other: G allele<br><br>β = 12.83 pg/ml<br>SE = 13.24</td><td>>0.05</td></tr>
<tr><td>Combined meta-analysis (GWAS Meta-analysis + Replication study):<br>n = 3613</td><td></td><td>Effect: A allele<br>Other: G allele<br><br>β = 27.62 pg/ml<br>SE = 8.15</td><td>$1.51 \times 10^{-6}$</td></tr>
<tr><td rowspan="2">11q12.1</td><td rowspan="2">Transcobalamin 1<br>(TCN1)</td><td rowspan="2">rs34324219</td><td>Adults:<br>n = 724<br>Indian</td><td>38 ± 11</td><td rowspan="2">A = 0.041[††]</td><td>Effect allele: A<br><br>β = -0.30 pmol/L</td><td>$2.00 \times 10^{-2}$</td><td rowspan="2">Nongmaithem et al., 2017 [22]</td></tr>
<tr><td>PMNS children[*]:<br>n = 690</td><td>11 ± 1</td><td>Effect allele: A</td><td>>0.05</td></tr>
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|---|---|---|---|---|---|---|---|---|
| | | | Indian | | | β = -0.14 pmol/L | | |
| | | | PS children†:<br>n = 534<br>Indian | 5 ± 0 | | Effect allele: A<br><br>β = -0.65 pmol/L | 9.50 x $10^{-7}$ | |
| 11q12.1 | Transcobalamin 1<br>(*TCN1*) | rs3432421<br>9 | Icelandic sample:<br>n = 37283 | 63 ± 24 | A = 0.119 | Effect: C allele<br>Other: A allele<br><br>β = 0.21 pmol/L | 8.80 x<br>$10^{-71}$ | Grarup et al.,<br>2013 [12] |
| | | | Danish Inter99<br>population:<br>n = 5481 | 46 ± 8 | | Effect: C allele<br>Other: A allele<br><br>β = 0.40 pmol/L | 3.20 x<br>$10^{-23}$ | |
| | | | Danish - Health<br>2006:<br>n = 2812 | 49 ± 13 | | Effect: C allele<br>Other: A allele<br><br>β = 0.30 pmol/L | 3.50 x<br>$10^{-24}$ | |
| | | | Combined total:<br>n = 45576 | | | Effect: C allele<br>Other: A allele<br><br>β = Not available | 1.10 x $10^{-111}$ | |
| 11q12.1 | Transcobalamin 1<br>(*TCN1*) | rs3452891<br>2 | Adults:<br>n = 724<br>Indian | 38 ± 11 | T =<br>0.006†† | Effect allele: T<br><br>β = -0.79 pmol/L | 1.00 x $10^{-2}$ | Nongmaithem et<br>al., 2017 [22] |

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|---|---|---|---|---|---|---|---|---|
| | | | PMNS children*: n = 690 Indian | 11 ± 1 | | Effect allele: T<br><br>β = 0.38 pmol/L | >0.05 | |
| | | | PS children†: n = 534 Indian | 5 ± 0 | | Effect allele: T<br><br>β = -0.47 pmol/L | $3.00 \times 10^{-2}$ | |
| 11q12.1 | Transcobalamin 1 (*TCN1*) | rs34528912 | Icelandic sample: n = 25960 | 63 ± 24 | T = 0.036 | Effect: T allele<br>Other: C allele<br><br>β = 0.17 pmol/L | $2.10 \times 10^{-15}$ | Grarup et al., 2013 [12] |
| 13q32.3 | Citrate Lyase Beta Like (*CLYBL*) | rs41281112 | Initial sample: n = 1999 Chinese Han men | 38 ± 11 | T = 0.044 | Effect: T allele<br>Other: C allele<br><br>β = Not available | $1.09 \times 10^{-8}$ | Lin et al., 2012 [19] |
| | | | Replication sample: n = 1496 Chinese men | 37 ± 11 | | Effect: T allele<br>Other: C allele<br><br>β = Not available | $7.41 \times 10^{-3}$ | |
| | | | Combined total: n = 3495 | | | Effect: T allele<br>Other: C allele<br><br>β = −83.60 pg/ml<br>SE = 13.62 | $9.23 \times 10^{-10}$ | |
| 13q32.3 | Citrate Lyase Beta Like (*CLYBL*) | rs41281112 | Icelandic sample: n = 37283 | 63 ± 24 | T = 0.052 | Effect: C allele<br>Other: T allele<br><br>β = 0.17 pmol/L | $9.60 \times 10^{-27}$ | Grarup et al., 2013 [12] |

| | | | Danish Inter99 population: n = 5481 | 46 ± 8 | | Effect: C allele Other: T allele β = 0.24 pmol/L | 1.30 x $10^{-3}$ | |
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| | | | Danish - Health 2006: n = 2812 | 49 ± 13 | | Effect: C allele Other: T allele β = 0.29 pmol/L | 2.50 x $10^{-7}$ | |
| | | | Combined total: n = 45576 | | | Effect: C allele Other: T allele β = Not available | 8.90 x $10^{-35}$ | |
| 14q24.3 | ATP Binding Cassette Subfamily D Member 4 (*ABCD4*) | rs3742801 | Icelandic sample: n = 37283 | 63 ± 24 | T = 0.294 | Effect: T allele Other: C allele β = 0.05 pmol/L | 5.30 x $10^{-8}$ | Grarup et al., 2013 [12] |
| | | | Danish Inter99 population: n = 5481 | 46 ± 8 | | Effect: T allele Other: C allele β = 0.09 pmol/L | 7.60 x $10^{-4}$ | |
| | | | Danish - Health 2006: n = 2812 | 49 ± 13 | | Effect: T allele Other: C allele β = 0.08 pmol/L | 4.50 x $10^{-5}$ | |
| | | | Combined total: n = 45571 | | | Effect: T allele Other: C allele β = Not available | 1.70 x $10^{-13}$ | |

| 14q24.3 | ATP Binding Cassette Subfamily D Member 4 (*ABCD4*) | rs4619337 | Icelandic sample:<br>n = 25960 | 63 ± 24 | C = 0.292[‡‡] | Effect: C allele<br>Other: T allele<br><br>β = 0.05 pmol/L | $3.40 \times 10^{-8}$ | Grarup et al., 2013 [12] |
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| 19p13.2 | Actin Like 9 (*ACTL9*) | rs2340550 | Initial sample:<br>n = 1999<br>Chinese Han men | 38 ± 11 | A = 0.134 | Effect: A allele<br>Other: G allele<br><br>β = Not available | $9.34 \times 10^{-7}$ | Lin et al., 2012 [19] |
| | | | Replication sample:<br>n = 1496<br>Chinese men | 37 ± 11 | | Effect: A allele<br>Other: G allele<br><br>β = Not available | >0.05 | |
| | | | Combined total:<br>n = 3495 | | | Effect: A allele<br>Other: G allele<br><br>β = 23.39 pg/ml<br>SE = 8.56 | $6.32 \times 10^{-3}$ | |
| 19p13.2 | CD320 molecule (*CD320*) / Transcobalamin II Receptor (*TcblR*) | rs2336573 | n = 3114<br>Canadian (85% Caucasian, 15% non-Caucasian) | 20 – 79 (range) | T = 0.010 | Effect: C allele<br>Other: T allele<br><br>Vitamin B-12 below adequate (< 220 pmol/L):<br>OR: 0.62 (95% CI: 0.45, 0.86) pmol/L | $3.0 \times 10^{-3}$ | Zinck et al., 2015 [18] |

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| 19p13.2 | CD320 molecule (*CD320*) / Transcobalamin II Receptor (*TcblR*) | rs2336573 | Icelandic sample:<br>n = 37283 | 63 ± 24 | T = 0.031 | Effect: T allele<br>Other: C allele<br><br>β = 0.32 pmol/L | $1.10 \times 10^{-51}$ | Grarup et al., 2013 [12] |
|  |  |  | Danish Inter99 population:<br>n = 5481 | 46 ± 8 |  | Effect: T allele<br>Other: C allele<br><br>β = 0.22 pmol/L | $5.70 \times 10^{-3}$ |  |
|  |  |  | Danish - Health 2006:<br>n = 2812 | 49 ± 13 |  | Effect: T allele<br>Other: C allele<br><br>β = 0.31 pmol/L | $1.70 \times 10^{-8}$ |  |
|  |  |  | Combined total:<br>n = 45575 |  |  | Effect: T allele<br>Other: C allele<br><br>β = Not available | $8.40 \times 10^{-59}$ |  |
| 19p13.2 | CD320 molecule (*CD320*) / Transcobalamin II Receptor (*TcblR*) | rs8109720 | Icelandic sample:<br>n = 25960 | 63 ± 24 | Not available | Effect: G allele<br>Other: A allele<br><br>β = 0.32 pmol/L | $5.80 \times 10^{-52}$ | Grarup et al., 2013 [12] |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs281379 | Parents of PMNS cohort*:<br>n = 1001<br>Indian | 36 ± 5 | A = 0.222# | Effect allele: A<br><br>β = 0.20 pmol/L | $4.60 \times 10^{-4}$ | Nongmaithem et al., 2017 [22] |
|  |  |  | Adults:<br>n = 724<br>Indian | 38 ± 11 |  | Effect allele: A<br>β = 0.05 pmol/L | >0.05 |  |
|  |  |  | PMNS children*:<br>n = 690<br>Indian | 11 ± 1 |  | Effect allele: A<br><br>β = 0.24 pmol/L | $4.50 \times 10^{-4}$ |  |

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| | | | PS children[†]:<br>n = 534<br>Indian | 5 ± 0 | | Effect allele: A<br>β = 0.13 pmol/L | >0.05 | |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs492602 | n = 3114<br>Canadian (85% Caucasian, 15% non-Caucasian) | 20 – 79 (range) | A = 0.210 | Effect: G allele<br>Other: A allele<br><br>Vitamin B12 deficiency (< 148 pmol/L):<br>OR: 0.60 (95% CI: 0.54, 0.70) pmol/L | $2.00 x 10^{-4}$ | Zinck et al., 2015 [18] |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs492602 | n = 3114<br>Canadian (85% Caucasian, 15% non-Caucasian) | 20 – 79 (range) | A = 0.210 | Effect: G allele<br>Other: A allele<br><br>Vitamin B-12 below adequate (< 220 pmol/L):<br>OR: 0.71 (95% CI: 0.65, 0.81) pmol/L | $9.00 x 10^{-8}$ | Zinck et al., 2015 [18] |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs492602 | NHS-CGEMS[‡]:<br>n = 1658<br>Caucasian women | 59 ± 6 | G = 0.440 | Effect: G allele<br>Other: A allele<br><br>β = 0.09 pg/ml<br>SE = 0.01 | $5.39 x 10^{-11}$ | Hazra et al., 2009 [20] |
| | | | SHARe[§]:<br>n = 1647<br>Caucasian women | 59 ± 10 | | Effect: G allele<br>Other: A allele<br><br>β = 0.04 pg/ml<br>SE = 0.02 | $5.89 x 10^{-3}$ | |

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| | | | SHARe§:<br>n = 1458<br>Caucasian men | 59 ± 10 | | Effect: G allele<br>Other: A allele<br><br>β = 0.05 pg/ml<br>SE = 0.01 | $2.36 \times 10^{-4}$ | |
| | | | Combined total:<br>n = 4763 | | | Effect: G allele<br>Other: A allele<br><br>β = 0.06 pg/ml<br>SE = 0.01 | $1.30 \times 10^{-14}$ | |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs492602 | NHS-CGEMS‡:<br>n = 1637<br>Caucasian women | 59 (Mean) | G = 0.490 | Effect: A allele<br>Other: G allele<br><br>β = -0.08 pg/ml<br>SE = 0.01 | $2.68 \times 10^{-10}$ | Hazra et al., 2008 [29] |
| | | | Replication:<br>n = 1059<br>Caucasian women | 63 (Mean) | | Effect: A allele<br>Other: G allele<br><br>β = -0.10 pg/ml<br>SE = 0.02 | $5.60 \times 10^{-9}$ | |
| | | | Combined meta-analysis:<br>n = 2696 | | | Effect: A allele<br>Other: G allele<br><br>β = -0.09 pg/ml<br>SE = 0.01 | $5.36 \times 10^{-17}$ | |

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| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs516316 | Icelandic sample: n = 25960 | 63 ± 24 | C = 0.469‡‡ | Effect: C allele<br>Other: G allele<br>β = 0.17 pmol/L | 3.60 x $10^{-103}$ | Grarup et al., 2013 [12] |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs601338 | Adults:<br>n = 724<br>Indian | 38 ± 11 | A = 0.230# | Effect: A<br>Other: G<br><br>β = 0.05 pmol/L | >0.05 | Nongmaithem et al., 2017 [22] |
| | | | PMNS children*:<br>n = 690<br>Indian | 11 ± 1 | | Effect: A<br>Other: G<br><br>β = 0.25 pmol/L | 3.8 x $10^{-5}$ | |
| | | | PS children†:<br>n = 534<br>Indian | 5 ± 0 | | Effect: A<br>Other: G<br><br>β = 0.18 pmol/L | 4.30 x $10^{-3}$ | |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs601338 | n = 25960<br>Icelandic | 63 ± 24 | G = 0.384 | Effect: G allele<br>Other: A allele<br><br>β = -0.16 pmol/L | 2.40 x $10^{-95}$ | Grarup et al., 2013 [12] |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs601338 | NHS-CGEMS‡:<br>n = 1658<br>Caucasian women | 59 ± 6 | A = 0.450 | Effect: A allele<br>Other: G allele<br><br>β = 0.09 pg/ml<br>SE = 0.01 | 4.25 x $10^{-11}$ | Hazra et al., 2009 [20] |
| | | | SHARe§:<br>n = 1647<br>Caucasian women | 59 ± 10 | | Effect: A allele<br>Other: G allele | 2.63 x $10^{-3}$ | |

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| | | | | | | $\beta$ = 0.05 pg/ml<br>SE = 0.01 | | |
| | | | SHARe§:<br>n = 1458<br>Caucasian men | 59 ± 10 | | Effect: A allele<br>Other: G allele<br><br>$\beta$ = 0.05 pg/ml<br>SE = 0.01 | $4.02 \times 10^{-4}$ | |
| | | | Combined total:<br>n = 4763 | | | Effect: A allele<br>Other: G allele<br><br>$\beta$ = 0.06 pg/ml<br>SE = 0.01 | $6.92 \times 10^{-15}$ | |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs601338 | NHS-CGEMS‡:<br>n = 1658<br>Caucasian women | 59<br>(Mean) | G = 0.490 | Effect: G allele<br>Other: A allele<br><br>$\beta$ = -0.08 pg/ml<br>SE = 0.01 | $4.11 \times 10^{-10}$ | Hazra et al., 2008 [29] |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs602662 | Adults:<br>n = 724<br>Indian | 38 ± 11 | A = 0.233# | Effect allele: A<br><br>$\beta$ = 0.10 pmol/L | >0.05 | Nongmaithem et al., 2017 [22] |
| | | | PMNS children*:<br>n = 690<br>Indian | 11 ± 1 | | Effect allele: A<br><br>$\beta$ = 0.25 pmol/L | $1.90 \times 10^{-5}$ | |
| | | | PS children†:<br>n = 534 | 5 ± 0 | | Effect allele: A | $1.40 \times 10^{-3}$ | |

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| | | | Indian | | | β = 0.20 pmol/L | | |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs602662 | n = 3114<br>Canadian (85% Caucasian, 15% non-Caucasian) | 20 – 79 (range) | G = 0.230 | Effect: A allele<br>Other: G allele<br><br>Vitamin B12 deficiency (< 148 pmol/L):<br>OR: 0.61 (95% CI: 0.47, 0.80) pmol/L | 3.00 x $10^{-4}$ | Zinck et al., 2015 [18] |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs602662 | n = 3114<br>Canadian (85% Caucasian, 15% non-Caucasian) | 20 – 79 (range) | G = 0.230 | Effect: A allele<br>Other: G allele<br><br>Vitamin B-12 below adequate (< 220 pmol/L):<br>OR: 0.74 (95% CI: 0.66, 0.84) pmol/L | 1.20 x $10^{-6}$ | Zinck et al., 2015 [18] |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs602662 | Icelandic sample:<br>n = 37283 | 63 ± 24 | G = 0.404 | Effect: A allele<br>Other: G allele<br><br>β = 0.16 pmol/L | 4.10 x $10^{-96}$ | Grarup et al., 2013 [12] |
| | | | Danish Inter99 population:<br>n = 5481 | 46 ± 8 | | Effect: A allele<br>Other: G allele<br><br>β = 0.19 pmol/L | 3.50 x $10^{-13}$ | |

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| | | | Danish - Health 2006: n = 2812 | 49 ± 13 | | Effect: A allele<br>Other: G allele<br><br>β = 0.23 pmol/L | 1.90 x $10^{-34}$ | |
| | | | Combined total n = 45568 | | | Effect: A allele<br>Other: G allele<br><br>β = Not available | 2.40 x $10^{-139}$ | |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs602662 | NHS-CGEMS[‡]: n = 1658 Caucasian women | 59 ± 6 | G = 0.440 | Effect: G allele<br>Other: A allele<br><br>β = - 0.08 pg/ml<br>SE = 0.01 | 3.09 x $10^{-10}$ | Hazra et al., 2009 [20] |
| | | | SHARe[§]: n = 1647 Caucasian women | 59 ± 10 | | Effect: G allele<br>Other: A allele<br><br>β = -0.05 pg/ml<br>SE = 0.02 | 3.80 x $10^{-4}$ | |
| | | | SHARe[§]: n = 1458 Caucasian men | 59 ± 10 | | Effect: G allele<br>Other: A allele<br><br>β = - 0.05 pg/ml<br>SE = 0.01 | 2.80 x $10^{-4}$ | |
| | | | Combined total: n = 4763 | | | Effect: G allele<br>Other: A allele<br><br>β = - 0.07 pg/ml<br>SE = 0.01 | 1.83 x $10^{-15}$ | |

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| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs602662 | GWAS Meta-analysis:<br><br>InCHIANTI study:<br>n = 1175<br>Italian<br><br>SardiNIA study:<br>n = 1115<br>Italian<br><br>BLSA study[¶]:<br>n = 640<br>Residents from the USA | InCHIANTI:<br>68 ± 16<br><br>SardiNIA:<br>45 ± 18<br><br>BLSA[g]:<br>68 ± 16 | G = 0.470 | Effect: A allele<br>Other: G allele<br><br>β = 44.20 pg/ml<br>SE = 8.26 | $2.43 \times 10^{-12}$ | Tanaka et al., 2009 [21] |
| | | | Replication study:<br>Progetto Nutrizione study:<br>N = 687<br>Italian | 47 ± 13 | | Effect: A allele<br>Other: G allele<br><br>β = 58.65 pg/ml<br>SE = 10.43 | $2.19 \times 10^{-10}$ | |
| | | | Combined meta-analysis (GWAS Meta-analysis + Replication study):<br>n = 3613 | | | Effect: A allele<br>Other: G allele<br><br>β = 49.77 pg/ml<br>SE = 6.47 | $2.83 \times 10^{-20}$ | |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs602662 | NHS-CGEMS[‡]:<br>n = 1658<br>Caucasian women | 59<br>(Mean) | G = 0.490 | Effect: G allele<br>Other: A allele<br><br>β = -0.08 pg/ml<br>SE = 0.01 | $6.54 \times 10^{-10}$ | Hazra et al., 2008 [29] |

| | | | Replication:<br>n = 1056<br>Caucasian women | 63<br>(Mean) | | Effect: G allele<br>Other: A allele<br><br>β = -0.08 pg/ml<br>SE = 0.02 | $1.13 \times 10^{-6}$ | |
|---|---|---|---|---|---|---|---|---|
| | | | Combined meta-analysis:<br>n = 2714 | | | Effect: G allele<br>Other: A allele<br><br>β = -0.08 pg/ml<br>SE = 0.01 | $3.52 \times 10^{-15}$ | |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs838133 | Adults:<br>n = 724<br>Indian | 38 ± 11 | T = 0.205[#] | Effect allele: A<br><br>β = 0.05 pmol/L | >0.05 | Nongmaithem et al., 2017 [22] |
| | | | PMNS children[*]:<br>n = 690<br>Indian | 11 ± 1 | | Effect allele: A<br><br>β = 0.27 pmol/L | $2.00 \times 10^{-4}$ | |
| | | | PS children[†]:<br>n = 534<br>Indian | 5 ± 0 | | Effect allele: A<br><br>β = 0.06 pmol/L | >0.05 | |
| 19q13.33 | Fucosyl transferase 2 gene (*FUT2*) | rs1047781 | Initial sample:<br>n = 1999<br>Chinese Han men | 38 ± 11 | T = 0.459 | Effect: T allele<br>Other: A allele<br><br>β = Not available | $4.63 \times 10^{-17}$ | Lin et al., 2012 [19] |

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| | | | Replication sample:<br>n = 1496<br>Chinese men | 37 ± 11 | | Effect: T allele<br>Other: A allele<br><br>β = Not available | $6.79 \times 10^{-22}$ | |
| | | | Combined total:<br>n = 3495 | | | Effect: T allele<br>Other: A allele<br><br>β = 70.21 pg/ml<br>SE = 5.53 | $3.62 \times 10^{-36}$ | |
| 19p13.3 | Fucosyltransferase 6 (*FUT6*) | rs708686 | Adults:<br>n = 724<br>Indian | 38 ± 11 | T = 0.335[#] | Effect: T allele<br><br>β = 0.13 pmol/L | $1.0 \times 10^{-2}$ | Nongmaithem et al., 2017 [22] |
| | | | PMNS children[*]:<br>n = 690<br>Indian | 11 ± 1 | | Effect: T allele<br><br>β = 0.22 pmol/L | $2.20 \times 10^{-4}$ | |
| | | | PS children[†]:<br>n = 534<br>Indian | 5 ± 0 | | Effect: T allele<br><br>β = 0.23 pmol/L | $2.70 \times 10^{-4}$ | |
| 19p13.3 | Fucosyltransferase 6 (*FUT6*) | rs708686 | N = 25960<br>Icelandic | 63 ± 24 | T = 0.301[‡‡] | Effect: T allele<br>Other: C allele<br><br>β = 0.05 pmol/L | $2.90 \times 10^{-9}$ | Grarup et al., 2013 [12] |

| 19p13.3 | Fucosyltransferase 6 / Fucosyltransferase 3 (*FUT6*/*FUT3*) | rs3760775 | Parents of PMNS cohort*: n = 1001 Indian | 36 ± 5 | A = 0.188# | Effect allele: A β = 0.24 pmol/L | 6.00 x $10^{-6}$ | Nongmaithem et al., 2017 [22] |
|---|---|---|---|---|---|---|---|---|
| | | | Adults: n = 724 Indian | 38 ± 11 | | Effect allele: A β = 0.24 pmol/L | 9.90 x $10^{-5}$ | |
| | | | PMNS children*: n = 690 Indian | 11 ± 1 | | Effect allele: A β = 0.31 pmol/L | 2.90 x $10^{-6}$ | |
| | | | PS children†: n = 534 Indian | 5 ± 0 | | Effect allele: A β = 0.24 pmol/L | 2.10 x $10^{-4}$ | |
| 19p13.3 | Fucosyltransferase 6 (*FUT6*) | rs3760776 | Parents of PMNS cohort*: n = 1001 Indian | 36 ± 5 | T = 0.161# | Effect allele: T β = 0.10 pmol/L | >0.05 | Nongmaithem et al., 2017 [22] |
| | | | Adults: n = 724 Indian | 38 ± 11 | | Effect allele: T β = 0.23 pmol/L | 4.40 x $10^{-4}$ | |
| | | | PMNS children*: n = 690 Indian | 11 ± 1 | | Effect allele: T β = 0.30 pmol/L | 3.30 x $10^{-6}$ | |

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| | | | PS children[†]:<br>n = 534<br>Indian | 5 ± 0 | | Effect allele: T<br><br>$\beta$ = 0.18 pmol/L | 6.50 x $10^{-3}$ | |
| 19p13.3 | Fucosyltransferase 6 (*FUT6*) | rs3760776 | n = 25960<br>Icelandic | 63 ± 24 | A = 0.071 | Effect: A allele<br>Other: G allele<br><br>$\beta$ = 0.07 pmol/L | 4.40 x $10^{-6}$ | Grarup et al., 2013 [12] |
| 19p13.3 | Fucosyltransferase 6 (*FUT6*) | rs3760776 | Initial sample:<br>n = 1999<br>Chinese Han men | 38 ± 11 | A = 0.212 | Effect: A allele<br>Other: G allele<br><br>$\beta$ = Not available | 4.23 x $10^{-10}$ | Lin et al., 2012 [19] |
| | | | Replication sample:<br>n = 1496<br>Chinese men | 37 ± 11 | | Effect: A allele<br>Other: G allele<br><br>$\beta$ = Not available | 1.98 x $10^{-4}$ | |
| | | | Combined total:<br>n = 3495 | | | Effect: A allele<br>Other: G allele<br><br>$\beta$ = 49.78 pg/ml<br>SE = 6.82 | 3.68 x $10^{-13}$ | |
| 19p13.3 | Fucosyltransferase 6 (*FUT6*) | rs7788053 | Icelandic sample:<br>n = 37283 | 63 ± 24 | A = 0.254 | Effect: A allele<br>Other: G allele<br><br>$\beta$ = 0.05 pmol/L | 2.10 x $10^{-7}$ | Grarup et al., 2013 [12] |
| | | | Danish Inter99 population:<br>n = 5481 | 46 ± 8 | | Effect: A allele<br>Other: G allele<br><br>$\beta$ = 0.05 pmol/L | >0.05 | |

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| | | | Danish - Health 2006:<br>n = 2812 | 49 ± 13 | | Effect: A allele<br>Other: G allele<br><br>β = 0.07 pmol/L | 7.20 x $10^{-4}$ | |
| | | | Combined total:<br>n = 45575 | | | Effect: A allele<br>Other: G allele<br><br>β = Not available | 1.70 x $10^{-10}$ | |
| 19p13.3 | Fucosyltransferase 6<br>(*FUT6*) | rs7806069<br>8 | Parents of PMNS cohort*:<br>n = 1001<br>Indian | 36 ± 5 | A =<br>0.130†† | Effect allele: A<br><br>β = 0.21 pmol/L | 2.90 x $10^{-4}$ | Nongmaithem et al., 2017 [22] |
| | | | Adults:<br>n = 724<br>Indian | 38 ± 11 | | Effect allele: A<br><br>β = 0.20 pmol/L | 3.70 x $10^{-3}$ | |
| | | | PMNS children*:<br>n = 690<br>Indian | 11 ± 1 | | Effect allele: A<br><br>β = 0.27 pmol/L | 1.20 x $10^{-4}$ | |
| | | | PS children†:<br>n = 534<br>Indian | 5 ± 0 | | Effect allele: A<br><br>β = 0.19 pmol/L | 8.20 x $10^{-3}$ | |
| 21q22.3 | Cystathionine beta synthase<br>(*CBS*) | rs2124459 | n = 3114<br>Canadian (85% Caucasian, 15% non-Caucasian) | 20 – 79<br>(range) | C = 0.180 | Effect: T allele<br>Other: C allele<br><br>Vitamin B-12 below adequate (< 220 pmol/L):<br>OR: 0.82 (95% CI: 0.73, 0.93) pmol/L | 2.00 x $10^{-3}$ | Zinck et al., 2015 [18] |

| 22q12.2 | Transcobalamin 2 (*TCN2*) | rs757874 | n = 3114 Canadian (85% Caucasian, 15% non-Caucasian) | 20 – 79 (range) | T = 0.080 | Effect: G allele Other: T allele<br><br>Vitamin B-12 below adequate (< 220 pmol/L):<br>OR: 1.42 (95% CI: 1.11, 1.72) pmol/L | 3.30 x $10^{-4}$ | Zinck et al., 2015 [18] |
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| 22q12.2 | Transcobalamin 2 (*TCN2*) | rs1131603 | Adults:<br>n = 724<br>Indian | 38 ± 11 | C = 0.023[#] | Effect: C allele<br><br>β = 0.43 pmol/L | 4.00 x $10^{-2}$ | Nongmaithem et al., 2017 [22] |
| | | | PMNS children[*]:<br>n = 690<br>Indian | 11 ± 1 | | Effect: C allele<br><br>β = 0.05 pmol/L | >0.05 | |
| | | | PS children[†]:<br>n = 534<br>Indian | 5 ± 0 | | Effect: C allele<br><br>β = 0.44 pmol/L | 5.00 x $10^{-2}$ | |
| 22q12.2 | Transcobalamin 2 (*TCN2*) | rs1131603 | Icelandic sample:<br>n = 37283 | 63 ± 24 | C = 0.055 | Effect: C allele<br>Other: T allele<br><br>β = 0.19 pmol/L | 4.30 x $10^{-28}$ | Grarup et al., 2013 [12] |
| | | | Danish Inter99 population:<br>n = 5481 | 46 ± 8 | | Effect: C allele<br>Other: T allele<br><br>β = 0.33 pmol/L | 1.80 x $10^{-9}$ | |

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|---|---|---|---|---|---|---|---|---|
| | | | Danish - Health 2006: n = 2812 | 49 ± 13 | | Effect: C allele Other: T allele $\beta$ = 0.33 pmol/L | 5.30 x $10^{-17}$ | |
| | | | Combined total: n = 45575 | | | Effect: C allele Other: T allele $\beta$ = Not available | 4.90 x $10^{-49}$ | |
| 22q12.2 | Transcobalamin 2 (*TCN2*) | rs5753231 | Icelandic sample: n = 25960 | 63 ± 24 | T = 0.210 | Effect: C allele Other: T allele $\beta$ = 0.06 pmol/L | 7.50 x $10^{-10}$ | Grarup et al., 2013 [12] |

*All studies have a cross-sectional study design.*

*SNP, Single Nucleotide Polymorphism*

** Pune Maternal Nutrition Study (PMNS)*

*† Parthenon Study (PS)*

*‡ Nurses' Health Study (NHS) NCI-Cancer Genetic Markers of Susceptibility (CGEMS) project*

*§ Framingham-SNP-Health Association Resource (SHARe)*

*¶ Baltimore Longitudinal Study of Aging (BLSA)*

*# Data refers to the HapMap-GIH population, with data collected from Gujarati Indians from Houston, Texas*

*** Data refers to European populations collected from: Utah Residents (CEPH) with Northern and Western European Ancestry, Toscani in Italia, Finnish in Finland, British in England and Scotland and Iberian Population in Spain*

*†† Data refers to South Asian populations collected from: Gujarati Indian from Houston, Texas, Punjabi from Lahore, Pakistan, Bengali from Bangladesh, Sri Lankan Tamil from the UK and Indian Telugu from the UK*

*‡ Data refers to the HapMap-CEU population, with data collected from Utah Residents (CEPH) with Northern and Western European Ancestry*

**Table 9: Candidate gene association studies examining the association of SNPs with vitamin B12 concentrations**

| Chromosome location | Gene name (Gene symbol) | Reference SNP Cluster ID | Sample size & Ethnicity | Study design | Age (years) | Minor allele frequency | Effect size | *P*-value | References |
|---|---|---|---|---|---|---|---|---|---|
| 1p34.1 | Methylmalonic aciduria and homocystinuria type C protein (*MMACHC*) | rs10789465 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | C = 0.469† | Not available | $1.00 \times 10^{-3}$ | Andrew et al., 2013 [13] |
| 1p36.3 | Methylenetetrahydrofolate Reductase (*MTHFR*) | rs1801131 | n = 988 French women | Cross-sectional | 40 – 65 (range) | C = 0.290 | Not available | >0.05 | De Batlle et al., 2016 [79] |
| 1p36.3 | Methylenetetrahydrofolate Reductase (*MTHFR*) | Rs1801131 | n = 6784 Danish | Cross-sectional | 30 – 60 (range) | C = 0.340 | Not available | >0.05 | Thuesen et al., 2010 [57] |
| 1p36.3 | Methylenetetrahydrofolate Reductase (*MTHFR*) | Rs1801131 | n = 220 Brazilian | Cross-sectional | 1 – 8 (range) | C = 0.240 | Not available | >0.05 | Aléssio et al., 2004 [78] |
| 1p36.3 | Methylenetetrahydrofolate Reductase (*MTHFR*) | rs1801133 | n = 988 French women | Cross-sectional | 40 – 65 (range) | T = 0.360 | Not available | >0.05 | De Batlle et al., 2016 [79] |
| 1p36.3 | Methylenetetrahydrofolate Reductase (*MTHFR*) | rs1801133 | n = 731 English (White Caucasian) | Cross-sectional | 85 | T = 0.330 | $\beta = 5.00 \times 10^{-5}$ pmol/L‡ | >0.05 | Mendonca et al., 2016 [28] |
| 1p36.3 | Methylenetetrahydrofolate Reductase (*MTHFR*) | rs1801133 | Elderly individuals: n = 262 Brazilian | Cross-sectional | 60 – 91 (range) | T = 0.370 | Not available | >0.05 | Barnabe et al., 2015 [77] |

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|---|---|---|---|---|---|---|---|---|---|
| | | | Children:<br>n = 106<br>Brazilian | | 0.5 – 6<br>(range) | T = 0.290 | Not available | >0.05 | |
| 1p36.3 | Methylenetetrahydro folate Reductase (*MTHFR*) | rs1801133 | n = 6784<br>Danish | Cross-sectional | 30 – 60<br>(range) | T = 0.290 | Effect allele: Not available<br>Other allele: Not available<br><br>Low serum vitamin B12:<br>OR: 1·78 (95% CI:<br>1.25, 2.54) pmol/L | $3.00 \times 10^{-3}$ | Thuesen et al., 2010 [57] |
| 1p36.3 | Methylenetetrahydro folate Reductase (*MTHFR*) | rs1801133 | n = 153<br>Spanish | Cross-sectional | 13 – 19<br>(range) | T = 0.380 | Not available | >0.05 | Al-Tahan et al., 2008 [81] |
| 1p36.3 | Methylenetetrahydro folate Reductase (*MTHFR*) | rs1801133 | n = 10601<br>Norwegian | Cross-sectional | 56 | T = 0.280 | Not available | >0.05 | Hustad et al., 2007 [80] |
| 1p36.3 | Methylenetetrahydro folate Reductase (*MTHFR*) | rs1801133 | n = 220<br>Brazilian | Cross-sectional | 1 – 8<br>(range) | T = 0.320 | Not available | >0.05 | Aléssio et al., 2004 [78] |
| 1q43 | 5-Methyltetrahydrofolate-Homocysteine methyltransferase (MTR) | rs1805087 | n = 731<br>English (White Caucasian) | Cross-sectional | 85 | G = 0.180 | $\beta = 4.00 \times 10^{-3}$ pmol/L[‡] | >0.05 | Mendonca et al.,2016 [28] |

| 1q43 | 5-Methyltetrahydrofolate-Homocysteine methyltransferase (MTR) | rs1805087 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | G = 0.161† | Not available | >0.05 | Andrew et al., 2013 [13] |
|---|---|---|---|---|---|---|---|---|---|
| 1q43 | 5-Methyltetrahydrofolate-Homocysteine methyltransferase (MTR) | rs1805087 | n = 6784 Danish | Cross-sectional | 30 – 60 (range) | G = 0.200 | Not available | >0.05 | Thuesen et al., 2010 [57] |
| 1q43 | 5-Methyltetrahydrofolate-Homocysteine methyltransferase (MTR) | rs2275568 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | A = 0.460† | Not available | >0.05 | Andrew et al., 2013 [13] |
| 1q43 | 5-Methyltetrahydrofolate-Homocysteine methyltransferase (MTR) | rs2789352 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | T = 0.381† | Not available | >0.05 | Andrew et al., 2013 [13] |
| 1q43 | 5-Methyltetrahydrofolate-Homocysteine methyltransferase (MTR) | rs3768142 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | G = 0.384† | Not available | >0.05 | Andrew et al., 2013 [13] |
| 1q43 | 5-Methyltetrahydrofolate-Homocysteine methyltransferase (MTR) | rs10733118 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | T = 0.381† | Not available | >0.05 | Andrew et al., 2013 [13] |
| 1q43 | 5-Methyltetrahydrofolate-Homocysteine methyltransferase (MTR) | rs10925257 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | G = 0.155† | Not available | >0.05 | Andrew et al., 2013 [13] |

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|---|---|---|---|---|---|---|---|---|---|
| 1q43 | 5-Methyltetrahydrofolate-Homocysteine methyltransferase (MTR) | rs11800413 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | G = 0.431$^{\dagger}$ | Not available | >0.05 | Andrew et al., 2013 [13] |
| 1q43 | 5-Methyltetrahydrofolate-Homocysteine methyltransferase (MTR) | rs12060264 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | A = 0.438$^{\dagger}$ | Not available | >0.05 | Andrew et al., 2013 [13] |
| 2q23.2 | Methylmalonic Aciduria and Homocystinuria,Cbl D Type (*MMADHC*) | rs7580915 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | G = 0.228$^{\dagger}$ | Not available | >0.05 | Andrew et al., 2013 [13] |
| 4p14 | Replication Factor C Subunit 1 (*RFC1*) | rs1051266 | Elderly individuals: n = 262 Brazilian | Cross-sectional | 60 – 91 (range) | A = 0.430 | Not available | >0.05 | Barnabe et al., 2015 [77] |
| | | | Children: n = 106 Brazilian | | 1– 6 (range) | A/G = 0.500 | Not available | >0.05 | |
| 4q31.21 | Methylmalonic aciduria (cobalamin deficiency) cblA type (*MMAA*) | rs4835011 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | G = 0.080$^{\dagger}$ | Not available | >0.05 | Andrew et al., 2013 [13] |
| 4q31.21 | Methylmalonic aciduria (cobalamin deficiency) cblA type (*MMAA*) | rs4835012 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | G = 0.178$^{\dagger}$ | Not available | $3.00 \times 10^{-2}$ | Andrew et al., 2013 [13] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 4q31.21 | Methylmalonic aciduria (cobalamin deficiency) cblA type (*MMAA*) | rs4835014 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | T = 0.031† | Not available | >0.05 | Andrew et al., 2013 [13] |
| 4q31.21 | Methylmalonic aciduria (cobalamin deficiency) cblA type (*MMAA*) | rs11728906 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | G = 0.235† | Not available | >0.05 | Andrew et al., 2013 [13] |
| 5q14.1 | Betaine-homocysteine S-methyltransferase (*BHMT*) | rs3733890 | n = 6784 Danish | Cross-sectional | 30 – 60 (range) | A = 0.290 | Not available | >0.05 | Thuesen et al., 2010 [57] |
| 5p15.31 | Methionine synthase reductase (*MTRR*) | rs10380 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | T = 0.156† | Not available | >0.05 | Andrew et al., 2013 [13] |
| 5p15.31 | Methionine synthase reductase (*MTRR*) | rs162031 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | T = 0.205† | Not available | >0.05 | Andrew et al., 2013 [13] |
| 5p15.31 | Methionine synthase reductase (*MTRR*) | rs162036 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | G = 0.186† | Not available | $4.00 \times 10^{-2}$ | Andrew et al., 2013 [13] |
| 5p15.31 | Methionine synthase reductase (*MTRR*) | rs162040 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | C = 0.124† | Not available | >0.05 | Andrew et al., 2013 [13] |

| 5p15.31 | Methionine synthase reductase (*MTRR*) | rs162048 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | G = 0.164[†] | Not available | 5.00 x $10^{-2}$ | Andrew et al., 2013 [13] |
|---|---|---|---|---|---|---|---|---|---|
| 5p15.31 | Methionine synthase reductase (*MTRR*) | rs326120 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | G = 0.155[†] | Not available | >0.05 | Andrew et al., 2013 [13] |
| 5p15.31 | Methionine synthase reductase (*MTRR*) | rs1532268 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | A = 0.308[†] | Not available | 1.00 x $10^{-2}$ | Andrew et al., 2013 [13] |
| 5p15.31 | Methionine synthase reductase (*MTRR*) | rs1801394 | n = 6784 Danish | Cross-sectional | 30 – 60 (range) | A = 0.430 | Not available | >0.05 | Thuesen et al., 2010 [57] |
| 5p15.31 | Methionine synthase reductase (*MTRR*) | rs1801394 | n = 220 Brazilian | Cross-sectional | 1 – 8 (range) | A = 0.490 | Not available | >0.05 | Aléssio et al., 2004 [78] |
| 5p15.31 | Methionine synthase reductase (*MTRR*) | rs2966952 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | T = 0.167[†] | Not available | >0.05 | Andrew et al., 2013 [13] |
| 5p15.31 | Methionine synthase reductase (*MTRR*) | rs3776455 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | G = 0.389[†] | Not available | 2.00 x $10^{-3}$ | Andrew et al., 2013 [13] |
| 5p15.31 | Methionine synthase reductase (*MTRR*) | rs6555501 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | C = 0.473[†] | Not available | >0.05 | Andrew et al., 2013 [13] |

| 6p12.3 | Methylmalonyl-CoA Mutase (*MUT*) | rs6458687 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | T = 0.372[†] | Not available | >0.05 | Andrew et al., 2013 [13] |
|---|---|---|---|---|---|---|---|---|---|
| 6p12.3 | Methylmalonyl-CoA Mutase (*MUT*) | rs6458690 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | G = 0.363[†] | Not available | 2.00 x $10^{-4}$ | Andrew et al., 2013 [13] |
| 6p12.3 | Methylmalonyl-CoA Mutase (*MUT*) | rs9381784 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | T = 0.363[†] | Not available | 3.00 x $10^{-2}$ | Andrew et al., 2013 [13] |
| 6q13 | LMBR1 Domain Containing 1 (*LMBRD1*) | rs991974 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | T = 0.044[†] | Not available | >0.05 | Andrew et al., 2013 [13] |
| 6q13 | LMBR1 Domain Containing 1 (*LMBRD1*) | rs1457498 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | T = 0.084[†] | Not available | >0.05 | Andrew et al., 2013 [13] |
| 6q13 | LMBR1 Domain Containing 1 (*LMBRD1*) | rs3778241 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | T = 0.398[†] | Not available | >0.05 | Andrew et al., 2013 [13] |
| 6q13 | LMBR1 Domain Containing 1 (*LMBRD1*) | rs3799105 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | C = 0.384[†] | Not available | >0.05 | Andrew et al., 2013 [13] |
| 6q13 | LMBR1 Domain Containing 1 (*LMBRD1*) | rs6455338 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | C = 0.387[†] | Not available | >0.05 | Andrew et al., 2013 [13] |

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|---|---|---|---|---|---|---|---|---|---|
| 6q13 | LMBR1 Domain Containing 1 (*LMBRD1*) | rs9294851 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | T = 0.384† | Not available | >0.05 | Andrew et al., 2013 [13] |
| 11q12.1 | Transcobalamin 1 (*TCN1*) | rs526934 | n = 731 English (White Caucasian) | Cross-sectional | 85 | G = 0.270 | $\beta = 4.00 \times 10^{-3}$ pmol/L‡ | >0.05 | Mendonca et al., 2016 [28] |
| 12q24.11 | Methylmalonic aciduria (Cobalamin deficiency) cblB type (*MMAB*) | rs2287182 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | A = 0.128† | Not available | >0.05 | Andrew et al., 2013 [13] |
| 12q24.11 | Methylmalonic aciduria (Cobalamin deficiency) cblB type (*MMAB*) | rs3759387 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | A = 0.235† | Not available | >0.05 | Andrew et al., 2013 [13] |
| 12q24.11 | Methylmalonic aciduria (Cobalamin deficiency) cblB type (*MMAB*) | rs7134594 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | C = 0.487† | Not available | >0.05 | Andrew et al., 2013 [13] |
| 12q24.11 | Methylmalonic aciduria (Cobalamin deficiency) cblB type (*MMAB*) | rs7957619 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | A = 0.110 † | Not available | >0.05 | Andrew et al., 2013 [13] |
| 12q24.11 | Methylmalonic aciduria (Cobalamin deficiency) cblB type (*MMAB*) | rs12314392 | n = 262 Caucasian women of North European descent | Cross-sectional (Twin Study) | 48 ± 13 | G = 0.433 † | Not available | >0.05 | Andrew et al., 2013 [13] |

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|---|---|---|---|---|---|---|---|---|---|
| 19p13.2 | CD320 molecule<br>(*CD320*) /<br>Transcobalamin II<br>Receptor (*TcblR*) | rs2336573 | n = 591<br>Caucasian women | Cross-<br>sectional | 77 ± 7 | A = 0.050 | Not available | >0.05 | Kurnat-Thoma et<br>al., 2015 [59] |
| | | | n = 198<br>African American<br>women | | 75 ± 6 | A = 0.330 | Not available | 4.0 x $10^{-2}$ | |
| | | | n = 797<br>Combined total | | | | Not available | 2.0 x $10^{-2}$ | |
| 19q13.33 | Fucosyl transferase<br>2 gene<br>(*FUT2*) | rs492602 | n = 731<br>English (White<br>Caucasian) | Cross-<br>sectional | 85 | A = 0.450 | β = 0.05 pmol/L[‡] | <0.001 | Mendonca et al.,<br>2016 [28] |
| 19q13.33 | Fucosyl transferase<br>2 gene<br>(*FUT2*) | rs602662 | Vegetarian:<br>n = 553<br>North Indian | Cross-<br>sectional | 50 (41 –<br>59)<br><br>Median<br>(interqua<br>rtile<br>range) | A = 0.310 | Effect: A allele<br>Other: G allele<br><br>β = 0.12 pmol/L | 5.0 x $10^{-3}$ | Tanwar et al.,<br>2013 [27] |
| | | | Non-Vegetarian:<br>n = 593<br>North Indian | Cross-<br>sectional | 47 (37 –<br>55)<br><br>Median<br>(interqua | | Effect: A allele<br>Other: G allele<br><br>β = 0.12 pmol/L | 4.0 x $10^{-3}$ | |

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|---|---|---|---|---|---|---|---|---|---|
| | | | | | rtile range) | | | | |
| | | | Combined total:<br>n = 1146<br>North Indian | Cross-sectional | 49 (40 – 57)<br><br>Median (interquartile range) | | Effect: A allele<br>Other: G allele<br><br>β = 0.12 pmol/L | 4.0 x $10^{-5}$ | |
| 22q12.2 | Transcobalamin 2 (*TCN2*) | rs1801198 | NORCAP cohort[*]:<br>n = 2411<br>Norwegian<br><br>Serum holoTC could be analysed in only 2379 individuals | Cross-sectional | 50 – 64<br>(range) | G = 0.440 | Effect: C allele<br>Other: G allele<br><br>Total holo-TC:<br>β = 0.02 pmol/L[‡] | >0.05[‡] | Riedel et al.,2011 [55] |
| | | | | | | | Effect: C allele<br>Other: G allele<br><br>Plasma Cbl:<br>β = 0.03 pmol/L[‡] | >0.05[‡] | |
| 22q12.2 | Transcobalamin 2 (*TCN2*) | rs1801198 | n = 122<br>Portuguese | Cross-sectional | 46 ± 13 | G = 0.480 | Not available | Vitamin B12: >0.05<br>Holo-TC:<br><0.05 | Castro et al., 2010 [52] |
| 22q12.2 | Transcobalamin 2 (*TCN2*) | rs1801198 | n = 554<br>Participants of Latino ancestry residing in USA | Cross-sectional | 69 ± 6 | G = 0.350 | Not available | Vitamin B12: >0.05 | Garrod et al., 2010 [56] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Total holo TC: >0.05 | |
| 22q12.2 | Transcobalamin 2 (*TCN2*) | rs1801198 | n = 613 Northern Irish Men (Caucasian) | Cross-sectional | 30 – 49 (range) | G = 0.450 | Not available | 1.00 x $10^{-2}$ | Stanislawska-Sachadyn et al.,2010 [54] |
| 22q12.2 | Transcobalamin 2 (*TCN2*) | rs1801198 | n = 6,784 Danish | Cross-sectional | 30 – 60 (range) | G = 0.440 | Not available | >0.05 | Thuesen et al., 2010 [57] |
| 22q12.2 | Transcobalamin 2 (*TCN2*) | rs1801198 | n = 207 Brazilian | Cross-sectional | 1 – 8 (range) | G = 0.360 | Not available | >0.05 | Alessio et al., 2007 [58] |
| 22q12.2 | Transcobalamin 2 (*TCN2*) | rs4820888 | n = 591 Caucasian women | Cross-sectional | 77 ± 7 | G = 0.430 | Not available | >0.05 | Kurnat-Thoma et al., 2015 [59] |
| | | | n = 198 African American women | | 75 ± 6 | G = 0.450 | Not available | >0.05 | |
| | | | n = 797 Combined total | | | | Not available | 2.0 x $10^{-2}$ | |
| 22q12.2 | Transcobalamin 2 (*TCN2*) | rs9606756 | NORCAP cohort[*]: n = 2411 Norwegian<br><br>Serum holoTC could be analysed in only 2379 individuals | Cross-sectional | 50 – 64 (range) | G = 0.120 | Effect: A allele Other: G allele<br><br>Total holo-TC: $\beta$ = -0.21 pmol/L[‡] | <0.001[‡] | Riedel et al.,2011 [55] |
| | | | | | | | Effect: A allele | >0.05[‡] | |

| | | | | | | | Other: G allelePlasma Cbl:<br>β = -0.02 pmol/L[‡] | | |
|---|---|---|---|---|---|---|---|---|---|
| 22q12.2 | Transcobalamin 2<br>(*TCN2*) | rs9606756 | n = 6784<br>Danish | Cross-<br>sectional | 30 – 60<br>(range) | G = 0.120 | Not available | >0.05 | Thuesen et al., 2010 [57] |
| 1p36.3<br><br>19q13.33 | Methylenetetrahydro folate Reductase<br>(*MTHFR*)<br>+<br>Fucosyl transferase 2 gene<br>(*FUT2*) | rs180133<br>rs180131<br>rs492602 | n = 988<br>Brazilian | Cross-<br>sectional | 5 ± 3 | rs180133<br>T = 0.320<br>rs180131<br>C = 0.220<br>rs492602<br>G = 0.390 | β for GRS= −0·11 pmol/L | <0·001 | Cobayashi et al., 2015 [105] |

*All studies have a cross sectional design*

*SNP, Single Nucleotide Polymorphism*

[*]*NORwegian Colorectal CAncer Prevention (NORCCAP) cohort*

[†]*Data refers to HapMap European population, with data collected from Utah Residents (CEPH) with Northern and Western European Ancestry*

[‡]*The specific data available is not published elsewhere and was obtained by contacting the corresponding author*

**Table 10: A summary of the most frequently studied genes associated with vitamin B12 concentrations**

| Vitamin B12-related proteins | Gene name | Location | Function |
|---|---|---|---|
| **Co-factors or regulators of co-factors essential for the transport of vitamin B12** | Methylmalonic aciduria and homocystinuria, cblC type (*MMACHC)* | 1p34.1 | The *MMACHC* gene encodes a chaperone protein MMAACHC (cblC protein) which binds to vitamin B12 in the cytoplasm and appears to catalyse the reductive decyanation of cyanocobalamin into cob(II)alamin [235]. |
| | Transcobalamin 1 (*TCN1*) | 11q12.1 | It encodes a glycoprotein called Transcobalamin 1, also known as haptocorrin (HC), which binds to vitamin B12. It shields dietary vitamin B12 from the acidic environment of the stomach [236]. |
| | Fucosyltransferase 2 (*FUT2*) | 19q13.33 | It encodes the enzyme fucosyltransferase 2 (FUT2), which is involved in the synthesis of antigens of the Lewis blood group [231]. These antigens mediate the attachment of gastric pathogens to the gastric mucosa, which can affect the absorption of vitamin B12 [109]. |
| | Fucosyltransferase 6 (*FUT6*) | 19p13.3 | It encodes the enzyme fucosyltransferase 6 (FUT6), which is involved in forming Lewis associated antigens. These antigens attach gastric pathogens to the gastric mucosa. It has been shown that these gastric pathogens can reduce the absorption of vitamin B12 in the gut [43,44]. |

| | | | |
|---|---|---|---|
| | Transcobalamin 2 (*TCN2*) | 22q12.2 | It encodes a transport protein called transcobalamin 2 (TC), which binds to vitamin B12 within the enterocyte. The TC-B12 complex enters the portal circulation [59] [237][237][237][237][237][237][237][237][237][237][237][237][237][237][237][237][237][237][237][237][237][237][237][237][236][236][236][236][236][236][236][236][236][236][236][236][236][236]and makes vitamin B12 available for cellular uptake in target tissues [238]. |
| **Membrane transporters that actively facilitates membrane crossing** | Cubilin (*CUBN*) | 10p13 | It encodes the intestinal receptor Cubilin, which is expressed in the renal proximal tubule and intestinal mucosa [239]. Cubilin recognizes the vitaminB12-intrinsic factor complex, and binds to another protein called Amnionless to facilitate the entry of vitamin B12 into the intestinal cells [240]. |
| | ATP Binding Cassette Subfamily D Member 4 (*ABCD4*) | 14q24.3 | *ABCD4* codes for an ABC transporter. It has been postulated that ABCD4 is involved in intracellular cobalamin processing [241], and is involved in transporting vitamin B12 from lysosomes to the cytosol. In the cytosol, vitamin B12 can be converted into methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl) [241]. |
| | CD320 Molecule (*CD320*) | 19p13.2 | It encodes the membrane receptor transcobalamin receptor (TCblR), which binds to the transcobalamin-vitamin B12 complex, and mediates the uptake of vitamin B12 into cells [[242]. |
| **Proteins involved in the catalysis of enzymatic** | Methylenetetrahydrofolate reductase (*MTHFR*) | 1p36 | *MTHFR* codes for a critical enzyme involved in homocysteine remethylation. MTHFR catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate in an irreversible reaction [[243]. |

| **reactions in the one carbon cycle** | | | |
|---|---|---|---|
| | Methionine synthase reductase (*MTRR*) | 5p15.31 | This gene is responsible for maintaining adequate levels of activated vitamin B12 (methylcob(III)alamin), which maintains the enzyme methionine synthase in its active state [83]. |
| **Proteins involved in cell cycle regulation** | Membrane Spanning 4-Domains A3 (*MS4A3*) | 11q12.1 | *MS4A3* encodes a protein involved as a hematopoietic cell cycle regulator [244]. *MS4A3* gene may have a role in the cell-cycle regulation in the GI tract, thus affecting the renewal of intestinal and gastric epithelial cells, and absorption of vitamin B12 [206, 244]. |
| **Mitochondrial protein** | Methylmalonic aciduria (cobalamin deficiency) cb1A type (*MMAA*) | 4q31 | *MMAA* encodes a protein that may be involved in the translocation of vitamin B12 into the mitochondria [245]. In addition, MMAA could play an important role in the protection and reactivation of Methylmalonyl-coA mutase (MCM) in vitro [246]. |
| | Methylmalonyl-CoA Mutase (*MUT*) | 6p12.3 | It encodes a Mitochondrial enzyme methylmalonyl-CoA mutase (MUT), which catalyses the isomerization of methylmalonyl-CoA to succinyl-CoA. This isomerization requires vitamin B12 as a cofactor in the form of 5-prime-deoxyadenosylcobalamin (AdoCbl) [168]. |
| | Citrate Lyase Beta Like (*CLYBL*) | 13q32.3 | It encodes a human mitochondrial enzyme, which is co-expressed with other co-enzymes in the mitochondrial B12 pathway [247]. |

### 2.4.1 Co-factors or regulators of co-factors essential for the transport of vitamin B12

#### 2.4.1.1 Methylmalonic aciduria and homocystinuria, cblC type (*MMACHC)*

The Methylmalonic aciduria and homocystinuria, cblC type (*MMACHC)* gene is located in the chromosome region 1p34.1 [248]. The *MMACHC* gene encodes a chaperone protein MMACHC (cblC protein) which binds to vitamin B12 in the cytoplasm and appears to catalyse the reductive decyanation of cyanocobalamin into cob(II)alamin [235].

Among the common variations, SNP rs12272669 has been associated with vitamin B12 status, where 'A' allele carriers had higher vitamin B12 concentrations compared with 'G' allele carriers (P=3.00 x $10^{-9}$, β=0.51 pmol/L) in 37,283 Icelandic individuals [205]. Furthermore, SNP rs10789465 was associated with vitamin B12 concentrations (P=1.00 x $10^{-3}$) in a candidate gene association study comprising 262 Caucasian women of North European descent [170]. Currently, it is unknown how these variants affect the regulation of the *MMACHC* gene.

#### 2.4.1.2 Transcobalamin 1 *(TCN1)*

The Transcobalamin 1 *(TCN1)* gene is located on chromosome 11, and codes for the vitamin B12 binding protein, Transcobalamin I (TCI; also called haptocorrin (HC) or R binder) [249-251]. TCI is involved in facilitating the entry of vitamin B12 into the cells, via receptor-mediated endocytosis [252]. Six studies have reported associations between variants within the *TCN1* gene and circulating vitamin B12 concentrations [205, 206, 233, 234, 253, 254].

Nongmaithem *et al.* [233] investigated the association between several nucleotide variations within the *TCN1* gene and vitamin B12 levels in a GWA study comprising 534 healthy children from Mysore, India. Carriers of the 'G' allele of the rs526934 variant were

found to have lower circulating vitamin B12 concentrations (β=-0.16 pmol/L, P=0.02) compared to 'A' allele carriers [233]. This finding was in accordance with the studies conducted in Chinese, Icelandic, Italian and individuals residing in the US (predominantly non-Hispanic white) [205, 206, 234, 254]. Furthermore, additional variants of the *TCN1* gene (rs34528912 and rs34324219) were observed to be associated with vitamin B12 status ($P < 0.05$) in individuals of Icelandic, Indian and Danish backgrounds [205, 233].

Although no functional data are available to confirm the functional effect of these SNPs on vitamin B12 concentrations, the results from these studies suggest that the SNPs may have important physiological consequences for the role of the TCN1 protein in relation to vitamin B12 levels.

#### 2.4.1.3 Fucosyltransferase 2 (*FUT2*)

The fucosyltransferase 2 *(FUT2* gene*),* also known as the Se gene (secretor), is located on chromosome 19. The *FUT2* gene codes for a secretor enzyme α(1,2) fucosyltransferase which fucosylates oligosaccharides producing H type 1 and 2 antigens. H antigens are precursors of ABO and Lewis b histo-blood group antigens that are expressed on mucosal surfaces [231]. Recent studies have shown suggestive associations between variants of *FUT2* with diabetes and body mass index [16, 255-257].

For the *FUT2* gene, seven SNPs including: rs281379, rs492602, rs516316, rs601338, rs602662, rs838133 and rs1047781 were previously reported to be associated with vitamin B12 levels [205, 206, 233, 234, 253, 254, 258-260]. To identify loci associated with plasma vitamin B12, a meta-analysis of three genome wide association scans (n=4763) was carried out in a Caucasian population residing in the US [234]. The SNP rs601338, also known as 428 G/A nonsecretor variant allele (W143X variant), was significantly associated with plasma vitamin B12 levels ($P=6.92 \times 10^{-15}$), with the allele 'A' being positively associated with plasma vitamin

B12 levels (*β*=0.06 pg/ml) [234]. This finding was further confirmed in another study looking at 37,283 Icelandic adults (P=2.40 x $10^{-95}$, *β*=0.162 pmol/L) [205], as well as in two Indian populations of children (*β*= 0.18 pmol/L – 0.25 pmol/L) [233]. Notably, the minor allele frequency (MAF) of rs601338 varies widely between ethnicities, contributing to genetic heterogeneity in *FUT2*-B12 associations. In previous reports by Grarup *et al.* [205] and Hazra *et al.* [260], the frequency of the minor allele 'G' for the associated SNP (rs601338) was between 38.4% and 49.0%, for Icelandic and Caucasian populations from the US, respectively. In contrast, the allele 'A' was found to be the minor allele in the Indian population (MAF=23.0%) [233]. The presence of the 'A' allele is associated with higher vitamin B12 concentrations, compared to 'G' allele carriers. This indicates that in the Indian population, a greater number of individuals carry the 'G' allele and hence could partly explain why they are expected to have a lower vitamin B12 status [258]. The *FUT2* rs601338 variant is less common in East Asians than Europeans [MAF= 3.5%; HapMap HCB (Han Chinese in Beijing, China) and MAF= 1.2%; HapMap JPT (Japanese in Tokyo, Japan)], and may explain why the locus has not been identified in Chinese individuals in previous studies [206]. Another common non-synonymous SNP rs1047781 (A385T) has been shown to be a potential functional variant associated with vitamin B12 status and a major FUT2 secretor defining SNP in East Asians, and has also been reported to reduce the expression of Fucosyltransferases [261, 262]. Lin *et al.* found that the 'T' allele of the SNP rs1047781 was significantly associated with higher vitamin B12 concentrations in 3,495 Chinese men (P=3.62 x $10^{-36}$, β = 70.21 pg/ml) [206]. This genetic marker is present only in East-Asians; hence, it could not be replicated in a study conducted in Icelandic individuals [205].

To date, three studies have shown an association between the SNP rs492602 and vitamin B12 concentrations [234, 253, 260]. The SNP rs492602 is in complete linkage disequilibrium (LD) with *FUT2* W143X (rs601338) ($r^2$= 1), as shown in the Nurses' Health

Study [260]. Hazra *et al*. [234] found that the 'A' allele of the SNP rs492602 variant was associated with lower vitamin B12 concentrations (β= -0.06 pg/ml, P= $1.30 \times 10^{-14}$) amongst 4,763 Caucasians from the US, this finding was similarly observed in a GWA study (2,696 women) by the same authors (β= -0.09 pg/ml, P= $5.36 \times 10^{-17}$) [260]. In a subsequent study in 3,114 Canadian adults, the 'G' allele was shown to be associated with a lower risk (P=$2.0 \times 10^{-4}$, Odds Ratio: 0.60, 95% CI 0.54-0.70) of vitamin B12 deficiency (< 148 pmol/L) [253].

Finally, the most commonly studied variant of the *FUT2* gene is the SNP rs602662. This SNP was also reported to be in LD with the SNPs rs601338 ($r^2 = 0.76$) and rs516316 ($r^2 = 0.83$) in Caucasian populations from the US and Iceland [205, 260]. Zinck, *et al*. [253], reported that 'A' allele carriers of the rs602662 variant were at a lower risk of vitamin B12 deficiency (< 148 pmol/L) (OR: 0.61, 95% CI 0.47-0.80, P=$3.0 \times 10^{-4}$) in a population of 3,114 Canadian adults [253]. Similarly, a higher vitamin B12 status was observed in carriers of the 'A' allele in four different studies looking at Caucasians ($\beta$= 0.04-43.27 pmol/L) [205, 234, 254, 260] and Indians ($\beta$= 0.10-0.25 pmol/L) [233, 258]. Furthermore, additional variants of the *FUT2* gene were observed to be associated with vitamin B12 levels ($P < 0.05$) in the following SNPs: rs1047781, rs516316, rs838133 and rs281379 [205, 206, 233].

It has been proposed that host genetic variation in the *FUT2* gene may alter the composition of the gut microbiome. Individuals, who are nonsecretors (homozygous for the non-functional *FUT2* phenotype), lack terminal fucose residues on mucin glycans [263, 264]. As a result, the gut microbial community of individuals with FUT2 deficiency may reduce in composition and diversity, as microbes cannot adhere or utilize host-derived glycans [264, 265]. Variations in the *FUT2* gene can potentially alter the susceptibility to *Helicobacter Pylori* (*H. pylori)* infection, and its related gastric-induced vitamin B12 malabsorption [266-271]. Gastric pathogens such as *H. pylori*, attach to α1,2-fucosylated glycan's on epithelial cells, or structures masked by fucosylation with the help of these H antigens in individuals with the

secretor status [266-271]. Infections with *H. pylori* in the human intestine, have been reported to interfere with the release of intrinsic factor needed for vitamin B12 absorption [271]. Interestingly, a study in Northern Portugal found that the SNP rs602662 'A' allele has been linked to a non-secretor status (null H antigens), and this may decrease the risk of bacterial infection from pathogens such as *H. pylori*, and explains why subjects who carry 'A' allele have a high vitamin B12 status [272]. Alternatively, independent of *H. pylori*-mediated gastritis, individuals who carried FUT2 secretor variants who were also heterozygous for a *GIF* (a fucosylated glycoprotein needed for vitamin B12 absorption) mutation, had lower vitamin B12 concentrations [273].

#### 2.4.1.4 Fucosyltransferase 6 (*FUT6)*

The fucosyltransferase 6 (*FUT6)* gene is located on chromosome 19, and encodes a Golgi stack membrane protein; involved in the formation of Sialyl-Lewis X, an E-selectin ligand [206]. These Lewis associated antigens are associated with *H. pylori* adherence to the gastric and duodenal mucosa [274, 275]. Overgrowth of *H. pylori* has been linked to vitamin B12 deficiency, as gastric bacteria reduces the secretion of IF which is needed to form the vitamin B12-IF complex [206, 271].

In light of the potential physiological link between the *FUT6* gene and vitamin B12 deficiency, three studies investigated the relationship between variants in the *FUT6* gene and vitamin B12 status. Lin *et al.* first observed [206] that the 'A' allele of the rs3760776 variant was associated with higher vitamin B12 levels ($\beta$=49.78 pg/ml, P=$3.68 \times 10^{-13}$) in a sample of 3,495 men of Chinese Han and Chinese descent [206]. Similarly, homozygous 'A' allele carriers of Icelandic ($\beta$=0.068 pmol/L, P=$4.4\times10^{-6}$) [205] and Indian ($\beta$=0.18-0.30 pmol/L) [233] populations had high serum vitamin B12 concentrations. Interestingly, this gene variant may have the potential to serve as a genetic marker for Type 2 diabetes [257].

Furthermore, additional variants of the *FUT6* gene (rs708686 [205, 233], rs78060698 [233], rs3760775 [233] and rs7788053 [205]) were observed to be associated with a higher vitamin B12 status in individuals of the Indian, Icelandic and Danish populations ($P < 0.05$). Bioinformatic analysis has shown that the *FUT3*, *FUT5* and *FUT6* genes form a cluster on chromosome 19p13.3 [276]. Interestingly, the SNPs: rs3760775, rs10409772, rs12019136, rs78060698, rs17855739, rs79744308, rs7250982, rs8111600 from this cluster were in LD with the *FUT6* SNP rs3760775 ($r^2 = 0.57 - 0.84$) in South Asian populations. Available data has shown differences in the LD structure between South Asian populations and individuals of East Asian and European origin [233]. The variation of LD patterns across ethnicities could account for the heterogeneity of vitamin B12 concentrations [277].

Nongmaithem *et al.* [233] noted that alternative allelic states of the SNP rs78060698 variant, may influence the binding affinity of HNF4α (a key regulator of FUT6 expression) to the FUT6 protein. FUT6 is responsible for synthesizing α(1,3) fucosylated glycans, which act as a biological interface for the host-microbial interaction [278]. It is plausible that the SNP rs78060698 maintains the structure of glycans, which in turn control intestinal host-microbial interactions leading to altered concentrations of vitamin B12 [233, 279]. Another hypothesis, is that genetic variants may disrupt the formation of fucosyltransferases which mediate the glycosylation of B12 binding proteins and their receptors, thus influencing vitamin B12 concentrations [233].

#### 2.4.1.5 Transcobalamin 2 (*TCN2)*

The *TCN2* gene also known as transcobalamin 2 is located on chromosome 22. This gene has the function of making a vitamin B12 binding protein called transcobalamin II (TC) found in human serum [280]. Data suggests that *TCN2* genetic variants are associated with Alzheimer's disease and clinical manifestations of autoimmune gastritis in individuals with

low vitamin B12 status [281, 282]. TC is involved with absorption and transporting vitamin B12 into the cell. Only 10-20% of vitamin B12 is attached to TC, the remainder is attached to holo-haptocorrin (transcobalamin 1) [253, 283, 284]. Five studies have reported associations between variants within the *TCN2* gene and vitamin B12 levels [205, 233, 253, 283, 285].

The most commonly reported *TCN2* polymorphism in Caucasian populations is the SNP rs1801198, where the C to G substitution at nucleotide 776 (TCN2 776C>G) results in an amino acid exchange of proline to arginine at codon 259 (P259R). In a candidate gene association study of 613 Irish men, a significant association was observed between the SNP rs1801198 and serum vitamin B12 levels ($P=0.01$). Individuals with the homozygous wildtype 'CC' genotype had lower vitamin B12 levels (mean: 243.5 pmol/L) compared to those with 'GG' genotype (mean: 279.7 pmol/L) [285]. In contrast, it was observed that Holo-transcobalamin (Holo-TC) concentrations were significantly associated with the SNP rs1801198, in a population of 122 individuals from Portugal, where the G allele carriers (median 54.2 pmol/L) had lower Holo-TC levels compared to the C variant ($P < 0.05$; median 66.3 pmol/L) [283]. Four other studies reported no significant associations between the SNP rs1801198 and vitamin B12 concentrations in Caucasian populations ($P>0.05$) [286-289]. It was found that the minor allele frequency (G allele) of the SNP rs1801198 ranged between 35% to 48% in Brazilian (36%) [289], Latino (35%) [287], Nordic (44%) [286, 288], Northern Irish (45%) [285] and Portuguese (48%) [283] individuals. Additional variants of the *TCN2* gene (rs757874, rs4820888, rs1131603 and rs5753231) were associated with vitamin B12 status ($P<0.05$) in individuals of Indian, Canadian, US, African American, and Scandinavian background [205, 233, 237, 253, 286].

It has been suggested that the 776GG homozygous variant encodes a protein with a lower binding affinity to vitamin B12 in comparison to the wildtype 'C' allele [287]. Additionally, other studies have indicated that variations in the TC protein reduce the binding

of vitamin B12 to TC or prevent the TC-R from recognising the vitamin B12-TC complex [231].

### 2.4.2 Genes that code for membrane transporters that actively facilitate membrane crossing

#### 2.4.2.1 Cubulin *(CUBN)*

Cubulin (*CUBN)* also known as the intestinal intrinsic factor receptor or intrinsic factor-cobalamin (IF-B12) receptor is located on chromosome 10. *CUBN* is expressed on the intestinal and kidney epithelial cells and is involved with the uptake of the intrinsic factor-vitamin B12 (vitaminB12-IF) complex [234, 290, 291]. *CUBN* polymorphisms have been associated with maternal neural tube defects risk, megaloblastic anaemia, coronary heart disease and gastric cancer in individuals with low vitamin B12 status [292-296].

Studies of the association between vitamin B12 status and the variants within *CUBN* have yielded conflicting results. Hazra *et al.* [234] was first to report an association between the 'G' allele of the rs1801222 (Ser253Phe) variant and higher vitamin B12 status (β= 0.05 pg/ml, P=$2.87\times10^{-9}$) in 4,763 individuals from the US population [234]. This association was confirmed in another study looking at 45,571 Icelandic and Danish individuals (β = 0.10 - 0.17 pmol/L; P=$3.3\text{ x}10^{-75}$) [205]. In contrast, a study in 3,114 Canadian individuals (85% Caucasian and 15% Non-Caucasian) showed that the 'G' allele of the rs1801222 variant was associated with a higher risk of vitamin B12 deficiency (OR: 1.61 pmol/L, 95% CI 1.24-2.09, P=$3.0\text{ x }10^{-4}$)[253]. Genotypic frequency of the risk conferring minor allele 'A' was compared between three different studies (Canadian, Nordic and individuals of European ancestry living in the USA). It was found that Canadian individuals carried the lowest frequency of the risk allele 'A', at 10% [253]. On the other hand, Hazra *et al.* [234] and Grarup *et al.* [205], observed that the minor allele frequency 'A' was 28.0% and 40.7% in Caucasian individuals residing in the

USA and Nordic populations, respectively. Interestingly, several other genetic variants within *CUBN* (rs4748353, rs11254363 and rs12243895) were found to be either positively or negatively associated with vitamin B12 levels in residents from China, [206] Canada [253], USA and Italy [254].

To date several hypotheses have attempted to explain how *CUBN* variants are involved with lower vitamin B12 concentrations. One hypothesis is that *CUBN* is co-expressed with the protein amnionless (*AMN*, Chromosome 14) forming the cubam complex [240]. Cubilin has additionally been suggested to function together with megalin (*LRP2*, chromosome 2) [297], thus any polymorphisms in either *AMN* or *LRP2* genes can affect B12 absorption leading to B12 malabsorption and deficiency. Another hypothesis is that polymorphisms affecting CUBN, decreases the transport and the absorption of vitamin B12 in the ileum [234]. Functional studies on rs11254363, rs1801222, rs12243895 and rs4748353 are required to explain how these variants affect the regulation of the *CUBN* gene.

#### 2.4.2.2 ATP-binding cassette Subfamily D Member 4 *(*ABCD4)

The ATP-binding cassette Subfamily D Member 4 *(ABCD4)* gene is located on chromosome 14. This gene codes for the ABCD4 protein, which is a membrane transporter involved in transporting vitamin B12 out of lysosomes [298]. It has been shown that polymorphisms of the *ABCD4* gene affect the functioning of the ABCD4 protein and the intracellular processing of vitamin B12 [241].

There has been only one study to date investigating the association between vitamin B12 status and *ABCD4* variants. Grarup *et al*. [205] examined 45,571 Nordic adults and 25,960 Icelandic adults in a GWA study [205], where the 'T' allele of the rs3742801 and 'C' allele of the rs4619337 variants were associated with higher vitamin B12 levels (β=0.045-0.093 pmol/L,

$P=5.3\times10^{-8}$ ; $\beta=0.05$, $P= 3.4 \times 10^{-8}$, respectively), suggesting an impact of this gene on vitamin B12 status.

Previous research has shown that the protein LMBD1 (which is responsible for the lysosomal export of vitamin B12), interacts with the ABCD4 protein. The mechanisms of interaction between LMBD1 and ABCD4 remain unclear, but it is believed that polymorphisms in human *LMBRD1* gene and *ABCD4* can prevent translocation of the vitamin B12 from the lysosome to the cytoplasm [241, 299].

#### 2.4.2.3 CD320 molecule (CD320)

The *CD320* gene also known as the '*CD320* molecule' gene is located on chromosome 19. This gene codes for the transcobalamin receptor (*TCblR*), which binds and engulfs holoTC by endocytosis [300]. At present, two SNPs: rs2336573 and rs8109720 have shown association with vitamin B12 levels [205, 237, 253].

The most commonly studied variant of the *CD320* gene is the rs2336573 variant, a missense polymorphism that results in an amino acid change from glycine to arginine, at the codon position 220. Zinck *et al.* found that the 'C' allele of the rs2336573 variant was associated with a lower risk (OR: 0.62, 95% CI 0.45-0.86, P=0.003) of vitamin B12 below adequate (< 220 pmol/L) among 3114 Canadian adults [253]. In contrast, an earlier study looking at a population of 45,571 adults from Iceland and Denmark found that the 'T' allele was associated with higher B12 levels ($\beta = 0.22 - 0.32$ pmol/L; $P=8.4\times10^{-59}$) [205]. A previous study has shown that this polymorphism is associated with the maternal risk of developing neural tube defects [292]. Cell culture models have shown that SNPs in the CD320 receptor can lead to a reduction in vitamin B12 uptake [300].

### 2.4.3 Involved in the catalysis of enzymatic reactions in the one carbon cycle

#### 2.4.3.1 Methylenetetrahydrofolate reductase (*MTHFR*)

The methylenetetrahydrofolate reductase (*MTHFR*) gene is located on chromosome 1 [301] and codes for a critical enzyme involved in homocysteine remethylation. MTHFR catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate in an irreversible reaction [243]. The two most well-known *MTHFR* gene polymorphisms are the C677T (rs1801133) and A1298C (rs1801131) variants. Both variants have been associated with reduced enzyme activity and an altered distribution of intracellular folate [302, 303].

The majority of candidate gene association studies have shown no association (P>0.05) with *MTHFR* gene polymorphisms (rs1801131 and rs1801133) and vitamin B12 concentrations in Brazilian [304, 305], North European [259], French [306], Norwegian [307] and Spanish [308] populations. However, Thuesen, *et al*. reported that 'T' allele carriers of the C677T genotype variant were associated with an increased prevalence of low serum vitamin B12 (OR 1·78; 95% CI 1·25, 2·54; P=0·003) in a population of 6,784 Danish adults [288]. There are no explanations to date, which have linked the biological mechanism of TT homozygosity and B12 deficiency. It could be postulated that the C677T polymorphism is associated with a decrease in intestinal absorption of vitamin B12 [309].

#### 2.4.3.2 Methioninesynthase reductase (*MTRR*)

The *MTRR* gene, also known as the 'methionine synthase reductase' gene is located on chromosome 5. This gene is responsible for maintaining adequate levels of activated vitamin B12 (methylcob(III)alamin), which maintains the enzyme methionine synthase in its active state [310]. Currently four SNPs: rs162036, rs162048, rs1532268 and rs3776455 have shown associations with vitamin B12 levels in healthy individuals [170].

The first SNP *MTRR* rs162036 (Lys350Arg) is a missense polymorphism [311], which was found to be associated with vitamin B12 levels (P=4.00 x $10^{-2}$) in 262 women of North European descent (No effect size available) [170]. The same authors also identified a significant association (P < 0.05) between the SNPs rs162048, rs1532268 and rs3776455 with vitamin B12 levels. This study provides the first evidence that *MTRR* polymorphisms (rs162036, rs162048, rs1532268 and rs3776455) significantly influence the circulating vitamin B12 concentrations.

### 2.4.4 Involved in cell cycle regulation

#### 2.4.4.1 Membrane Spanning 4-Domains A3 (*MS4A3)*

The Membrane Spanning 4-Domains A3 (*MS4A3*) gene is located on chromosome 11, and codes for the MS4A3 protein (also called HTm4). It has been suggested from limited studies that the MS4A3 protein may play a role in cell cycle regulation of hematopoietic cell development by inhibiting the G1/S cell cycle transition [244]. The only studied variant within this gene in relation to vitamin B12 concentrations is rs2298585, which was investigated in 3,495 men, all of Chinese origin. In this study [206], the 'T' allele of the rs2298585 variant was associated with higher serum vitamin B12 concentrations ($\beta$=71.80 pg/ml, P=2.64 x $10^{-15}$) [206]. Another study investigated this SNP in 37,283 Icelandic individuals, but found no statistical significance (β=0.214 pmol/L, P=0.075) [205].

It has been suggested that polymorphisms of the *MS4A3* gene may affect the cell-cycle regulation in the GI tract, thus affecting the renewal of intestinal and gastric epithelial cells leading to vitamin B12 malabsorption [312] . However, data from animal studies have demonstrated that MS4A3 is restricted to differentiating cells in the central nervous system and hematopoietic cells [313].

### 2.4.5 Mitochondrial protein

#### 2.4.5.1 Methylmalonic aciduria (cobalamin deficiency) cb1A type (*MMAA*)

The *MMAA* gene, also known as the 'methylmalonic aciduria (cobalamin deficiency) cb1A type', is located on chromosome 4q31.1-2 [314]. *MMAA* encodes a protein (MMAA) that may be involved in the translocation of vitamin B12 into the mitochondria [245]. In addition, MMAA could play an important role in the protection and reactivation of methylmalonyl-coA mutase (MCM) in vitro [246]. Three studies have reported associations between variants within the *MMAA* gene and vitamin B12 concentrations [170, 205, 233].

Andrew *et al*. was first to report that the SNP rs4835012 was significantly associated with vitamin B12 concentrations (P= $3.00 \times 10^{-2}$) in 262 Caucasian women of North European descent (no effect size available) [170]. More recently in a GWA study looking at 534 Indian children, the 'C' allele of the SNP rs2270655 was significantly associated with lower vitamin B12 concentrations ($\beta$ = -0.20 pmol/L, P= $2.00 \times 10^{-2}$) [233]. This association was confirmed in another study looking at 45,576 Danish and Icelandic adults ($\beta$ = -0.07 - -0.30, P=$2.20 \times 10^{-13}$) [205]. While these SNPs might be involved with determination of vitamin B12 concentrations, their precise biochemical role is unknown.

#### 2.4.5.2 Methylmalonyl-CoA mutase (MUT)

The *MUT* gene also known as the methylmalonyl-CoA mutase is located on chromosome 6. The *MUT* gene provides instructions for the formation of methylmalonyl-CoA mutase (MUT), which is a mitochondrial enzyme. MUT acts as a catalyst which isomerizes methylmalonyl-CoA to succinyl-CoA [315]. MUT requires 5-prime-deoxyadenosylcobalamin (AdoCbl), which is a form of B12 that works with MUT to form succinyl-CoA. Succinyl-CoA participates in the TCA cycle (tricarboxylic cycle) to yield energy [316]. The *MUT* gene is involved in homocysteine metabolism, and it is dependent on vitamin B12 for its function

[317]. Four studies have reported associations between variants within the *MUT* gene (chr6:49508102, rs1141321, rs9473555, rs6458690 and rs9381784) and vitamin B12 status [170, 205, 206, 234].

In a meta-analysis of data from 4,763 Caucasian individuals from the US, participants homozygous for the rs9473558 (now merged into rs1141321) ‘T’ allele ($\beta = -0.04$ pg/ml ,P= $4.05 \times 10^{-8}$) and *MUT* rs9473555 ‘C’ allele ( $\beta = -0.04$ pg/ml, P= $4.91 \times 10^{-8}$) were inversely associated with plasma vitamin B12 levels [234]. These findings were confirmed in other studies involving Icelandic ($\beta$= -0.061 pmol/L; β=-0.062 pmol/L, respectively) [205] and Chinese populations (β= -30.34 pg/ml; β= -31.0 pg/ml, respectively) [206].

#### 2.4.5.3 Citrate lyase beta like (*CLYBL*)

The citrate lyase beta like (*CLYBL*) gene is located at chromosome 13 and codes for a human mitochondrial protein. The functions of CLYBL include metal ion binding, carbon-carbon lyase activity and citrate (pro-3s)-lyase activity [206]. Approximately 5% of humans have a stop codon polymorphism in *CLYBL* which is associated with low levels of plasma vitamin B12, but the mechanistic link of this to vitamin B12 is currently unknown [318].

The association between the *CLYBL* variant rs41281112 and vitamin B12 levels has been studied in two different populations. Lin *et al.* [206] found that the ‘T’ allele was associated with lower serum vitamin B12 levels among 3,495 men of Chinese Han and Chinese descent ($\beta$=-83.60 pg/ml, P= $9.23\times10^{-10}$) [206]. Similarly, Grarup *et al.* [205] found that the ‘T’ allele of the SNP rs41281112 variant was associated with lower serum vitamin B12 levels ($\beta$=-0.29- -0.17 pmol/L, P=$8.9 \times 10^{-35}$) in 45,571 adults, all of Icelandic and Danish origin [205].

At present, molecular functioning studies have elucidated that the polymorphism rs41281112 (G<A) changes the amino acid from Arginine to a stop codon resulting in a loss

of protein expression [318]. As a result, Lin *et al.* [206] proposed that the rs41281112 variant interferes with the binding of *CLYBL* protein to metal ions, potentially leading to a lower uptake of vitamin B12 [206].

### 2.4.6 Other genes

Our review also identified that SNPs in actin like 9 (*ACTL9,*rs2340550) [206], serum paraoxonase/arylesterase 1 *(PON1, rs391757)*[253], cystathionine beta synthase *(CBS, rs2124459)[253]*, carbamoyl-phosphate synthase 1 (*CPS1*, rs1047891) [205] and DNA methyltransferase gene/ tRNA aspartic acid methyltransferase 1 (*DNMT2/TRDMT1,* rs56077122[205] and rs2295809[253]) genes were associated with vitamin B12 status in Canadian, Chinese, Danish and Icelandic populations. The SNPs in the intergenic regions [rs583228, rs10515552, rs12377462 [206], rs117456053, rs62515066 and Chr6:88792234 [205] were found to be associated with vitamin B12 status, however, plausible underlying biological mechanism as to why these SNPs were associated with vitamin B12 concentrations have not been identified.

### 2.4.7 Ethnic-specific genetic differences in B12 deficiency

In the past, vitamin B12 deficiency within populations in the Indian subcontinent, Mexico, Central and South America and certain regions of Africa was solely attributed to dietary habits/low consumption of meat [3]. We now know that genetic factors also influence vitamin status in individuals [319]. Indian populations have a high prevalence of vitamin B12 deficiency, typically attributed to the high number of vegetarians present in the population. However, non-vegetarians in India have been observed to have lower vitamin B12 concentrations compared to Caucasian populations [258, 320]. In addition, a recent systematic review showed that B12 deficiency is common during pregnancy in other populations where vegetarianism is rare [68]. Poor dietary intake, low bioavailable B12 in meat products (i.e.,

food processing and reheating of food) as well as a possible underlying genetic predisposition to vitamin B12 status could be the reasons for such observation in non-vegetarian populations [9, 321].

Although several studies have explored the association of SNPs with vitamin B12 status, only a limited number of genetic loci have been reported to support the presence of ethnic differences in vitamin B12 status in non-European populations [206, 233]. We can assume four genetic mechanisms which possibly account for these differences: 1) difference in effect allele frequencies 2) genetic heterogeneity across different ethnic groups 3) variance in LD structure, and 4) gene-gene and gene-environment interactions [322]. A key example of ethnic specificity has been demonstrated in the *FUT2* gene, whereby different mutations leading to nonsecretor status have been identified (the secretor status of *FUT2* gene is associated with a low vitamin B12 status) [323]. The 428G→A polymorphism (rs601338) is characteristic for the nonsecretor allele in Europeans and appears in about 20% of the Caucasian population [324]. In South-East and East-Asians populations, the SNP rs601338 is rare and the more common *FUT2* missense mutation rs1047781 is associated with nonsecretor status [325].

Genetic variants associated with circulating vitamin B12 have been studied in the following populations: African American (n=1) [237], Brazilian (n=4) [289, 304, 305, 326], Canadian (n=1) [253], Caucasian (n=4) [234, 237, 259, 260], Chinese (n=1)[206], Danish (n=2) [205, 288], European ancestry (n=1) [170], French (n=1) [306], Icelandic (n=1) [205], Indian (n=2) [233, 258], Italian ancestry and residents of the USA (n=1) [254], Latino (n=2) [287, 308], Northern Irish (n=1) [285], Norwegian (n=2) [286, 307] and Portuguese (n=1) [283]. To date, the majority of genetic association studies of vitamin B12 status have been performed in Caucasian populations, and a few have reported associations in high-risk populations such as Mexico and India [258, 327]. More studies exploring a wider range of ethnicities with large sample sizes may help to identify novel SNPs that may be associated with

vitamin B12 status. Studying the genetic structure of chromosomal regions that are associated with variability in vitamin B12 levels in different populations, can help us understand the evolutionary aspects of B12 associations and their relationship with environmental exposures. It is important that before any diet-related recommendations based on genotypes are given at the population level, associations between the SNPs and various health outcomes need to be confirmed [222].

## 2.5 Conclusion

In summary, our review has identified significant associations of vitamin B12 status with 59 B12-related SNPs from 19 genes. Among these genes; five were co-factors or regulators for the transport of vitamin B12 (*FUT2*, *FUT6*, *MMACHC*, *TCN1* and *TCN2*); three were membrane transporters actively facilitating the membrane crossing of vitamin B12 (*ABCD4*, *CUBN* and *CD320*); three were involved in the catalysis of enzymatic reactions in the one carbon cycle (*CBS, MTHFR* and *MTRR*); one was involved in cell cycle regulation (MS4A3); three were mitochondrial proteins (*CLYBL*, *MMAA* and *MUT*) and lastly four genes had an unknown function (*ACTL9*, *CPS1*, *DNMT2*/*TRDMT1* and *PON1*). Our review highlights the complex nature of the B12 genetics where several genes/ SNPs from various parts of B12 metabolic pathway contribute to the susceptibility to vitamin B12 deficiency. Identification of gene variants involved in this metabolic pathway using large scale genetic association studies in diverse ethnic populations would contribute to our understanding of the pathophysiology of B12 deficiency and help in discovering biomarkers of vitamin B12-related chronic diseases.

**Authors' contribution**

**Shelini Surendran extracted and interpreted the genetic variants related to vitamin B12 status and wrote the manuscript.** The data collected in this review was double checked by VKS and IAS. VKS conceived and designed the review and assisted in interpreting the results.

All authors were involved in revising the manuscript critically for intellectual content. All authors have approved the final version of the manuscript.

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# Chapter 3

## The influence of one-carbon metabolism gene polymorphisms and gene-environment interactions on homocysteine, vitamin B12, folate and lipids in a Brazilian adolescent population

For this study, I developed an analysis plan before I undertook the statistical analysis. I screened and validated the dataset to perform statistical analysis. I performed the entire statistical analysis using the SPSS software; I undertook a literature review as part of the introduction to the study and wrote the manuscript. I revised the manuscript based on the comments from all the co-authors before the manuscript was submitted to the Journal of Diabetology. I was also involved in drafting the responses to the comments from the reviewers.

**Published (The Published version of the paper is attached as an appendix at the end of the thesis)**

Shelini Surendran, Carla C. Morais, Dulcinéia SP Abdalla, Shatwan IA, Julie A. Lovegrove, Cristiane Cominetti, Karani S. Vimaleswaran*, Maria A. Horst*. The influence of one-carbon metabolism gene polymorphisms and gene-environment interactions on homocysteine, vitamin B12, folate and lipids in a Brazilian adolescent population. Journal of Diabetology; ISSN 2543-3288 (**Published**)

*Equally contributed

## 3.1 Abstract:

**Background:** Several single nucleotide polymorphisms (SNPs) have been associated with the metabolism of vitamin B12, folic acid, homocysteine and lipids. However, the interaction between SNPs involved in the one-carbon metabolism pathway and macronutrient intake on cardiovascular risk factors in the Brazilian population has not yet been investigated. Hence, this study investigated the association of ten SNPs involved in the one-carbon metabolism pathway with vitamin B12, folic acid, homocysteine and lipid levels, and examined the interaction of these SNPs with lifestyle factors (dietary and physical activity levels) in adolescents with cardiovascular risk.

**Methods:** A total of 113 adolescents (10-19 years old), from a public school in the city of Goiânia, Goiás, Brazil underwent anthropometric, biochemical, food consumption evaluations and genetic tests.

**Results:** After adjusting for potential confounders, SNPs rs4633 (catechol-O-methyltransferase, *COMT*), rs602662 (fucosyltransferase 2, *FUT2*) and rs1801394 (5-methyltetrahydrofolate-homocysteine methyltransferase reductase, *MTRR*) showed significant associations with folic acid (P=0.042), vitamin B12 (P=0.009) and oxidized-low density lipoprotein (ox-LDL) (P=0.041) concentrations, respectively. The *COMT* SNP rs4680 showed a significant interaction with carbohydrate intake on ox-LDL concentrations ($P_{interaction}$=0.005). In addition, the *FUT2* SNP rs602662, showed a significant interaction with protein intake on homocysteine concentrations ($P_{interaction}$=0.007). However, after correction for multiple testing, none of these associations and interactions were statistically significant.

**Conclusion:** For the first time, we provide evidence for the interactions between *COMT* SNP rs4680 and carbohydrate intake on ox-LDL levels and the *FUT2* SNP rs602662 and protein intake on homocysteine concentrations, respectively. However, given that our findings did not

reach Bonferroni significance, replication of our results in a larger sample size is required to confirm our findings.

## 3.2 Introduction:

Cardiovascular disease (CVD) has remained the leading cause of mortality in Brazil since the latter part of the 1960s [328, 329]. Although effective tobacco control policies and access to improved healthcare have led to drastic improvements in cardiovascular health, an upward trend in unhealthy eating habits and physical inactivity has been observed in the Brazilian population [329]. Smoking, obesity, hypertension, hyperlipidaemia, and insulin resistance have long been recognized as major risk factors for CVDs [330]; however the aetiology of CVD is not yet fully understood [331]. There has recently been renewed interest in the relationship between elevated homocysteine levels and the development of CVD [24].

Epidemiological studies have shown that hyperhomocysteinaemia is a well-known independent risk factor for atherosclerotic vascular disease and hypercoagulability states [24]. It is known to mediate adverse effects on vascular endothelium and smooth muscle cells [332]. In addition, hyperhomocysteinaemia reduces high-density lipoprotein (HDL) synthesis [333] and enhances the synthesis of lipoprotein A [334]. Some studies have indicated that up to 25% of coronary events may be attributed to the increase in homocysteine levels [335], which have been shown to inversely correlate with B-complex vitamins, such as folate and vitamin B12. Although B vitamins have a role in reducing blood homocysteine concentrations, the effect of these vitamins on cardiovascular function remain unclear [4]. A few studies have indicated that high folate and vitamin B12 status are associated with a reduced risk of coronary heart disease [336, 337]. Therefore, maintaining the concentrations of homocysteine, vitamin B12, folate and lipids within the body is of grave importance.

The one carbon metabolism pathway is a network of biochemical reactions involved in the transfer of single-carbon units (CH3 or methyl group), controlled by different enzymes and

nutritional cofactors [338]. Cells require one-carbon units for nucleotide synthesis and methylation reactions. Currently, common variants in genes of the one-carbon metabolism pathway have been reported to influence concentrations of homocysteine, folate, vitamin B12 and lipids [234]. A few studies have examined whether the association between genetic variants involved in the one-carbon metabolism pathway and homocysteine concentrations are modified by lifestyle factors such as diet [339, 340]. However, no studies, to date, have examined the interaction between one-carbon metabolism-related genes and lifestyle factors on vitamin B12, folate and lipid concentrations. Hence, seven genes involved in the one carbon metabolism were selected for our study [betaine-homocysteine S-methyltransferase (*BHMT*), catechol-o-methyl transferase (*COMT*), fucosyltransferase 2 (*FUT2)*, methylenetetrahydrofolate reductase (*MTHFR)*, 5-methyltetrahydrofolate-homocysteine methyltransferase or methionine synthase (*MTR),* 5-methyltetrahydrofolate-homocysteine methyltransferase reductase or methionine synthase reductase (*MTRR)* and transcobalamin 2 (*TCN2)*].

In this study, the aim was to examine whether 10 SNPs involved in the one-carbon metabolism pathway are associated with vitamin B12, homocysteine, folic acid and lipid-related outcomes, and whether dietary intake and physical activity levels have a modifying effect on these associations. Interaction and association analyses were carried out in 113 adolescents with cardiovascular risk factors from the city of Goiânia, Goiás, Brazil.

## 3.3 Materials and methods

### 3.3.1 Study Participants

This cross-sectional study was conducted in a public school in the city of Goiânia, Goiás, Brazil, between March to May 2014. A total of 454 students were initially enrolled into the study, and 201 students were found to be eligible for participation. After screening through lifestyle, socioeconomic and clinical history, only 113 adolescents (aged 10-19 years) were selected to answer a food frequency record and provided a blood sample for biochemical and DNA analysis. Full details of the methodology have been explained previously [341]. **Table 11** shows the characteristics of the study participants.

**Table 11: The characteristics of study participants stratified by sex**

| | All (N=113) | Boys (N=47) | Girls (N=66) | P value* |
|---|---|---|---|---|
| Age (yrs) | 13.87 ± 2.37 | 13.32 ± 2.35 | 14.26 ± 2.32 | **0.037** |
| Height (m) | 1.62 ± 0.11 | 1.63 ± 0.14 | 1.61 ± 0.08 | 0.205 |
| Weight (kg) | 63.53 ± 17.59 | 66.36 ± 20.13 | 61.51 ± 15.37 | 0.169 |
| BMI (kg/m$^2$) | 24.01 ± 4.92 | 24.33 ± 5.07 | 23.79 ± 4.84 | 0.567 |
| Vitamin B12 (pg/ml) | 519.80 ± 232.15 | 534.62 ± 252.96 | 509.24 ± 217.50 | 0.569 |
| Homocysteine (μmol/l) | 7.04 ± 2.99 | 7.90 ± 2.91 | 6.42 ± 2.92 | **0.009** |
| Folic acid (ng/ml) | 11.02 ± 3.27 | 10.78 ± 3.62 | 11.20 ± 3.01 | 0.519 |
| Triacylglycerol (mg/dl) | 94.05 ± 54.16 | 99.00 ± 59.00 | 90.53 ± 50.61 | 0.415 |

| | | | | |
|---|---|---|---|---|
| Total Cholesterol (mg/dl) | 155.42 ± 26.34 | 150.47 ± 24.51 | 158.95 ± 27.20 | 0.091 |
| HDL (mg/dl) | 46.29 ± 11.79 | 43.60 ± 11.47 | 48.21 ± 11.72 | **0.040** |
| LDL (mg/dl) | 90.28 ± 21.00 | 87.04 ± 18.19 | 92.59 ± 22.64 | 0.167 |
| VLDL (mg/dl) | 18.85 ± 10.82 | 19.83 ± 11.75 | 18.15 ± 10.15 | 0.419 |
| Oxidized-LDL (U/L) | 6.42 ± 13.69 | 5.92 ± 11.47 | 6.77 ± 15.12 | 0.749 |
| Total energy (Kcal/day) | 2521.63 ± 585.84 | 3010.08 ± 594.92 | 2173.79 ± 213.40 | **<0.0001** |
| Carbohydrate intake (energy %) | 47.70 ± 20.59 | 40.86 ± 19.56 | 52.56 ± 20.05 | **0.003** |
| Fat intake (energy %) | 25.36 ± 13.22 | 22.84 ± 11.58 | 27.16 ± 14.09 | 0.087 |
| Protein intake (energy %) | 16.99 ± 8.38 | 14.56 ± 6.94 | 18.72 ± 8.92 | **0.006** |

*Abbreviations: BMI Body mass index*

*Data presented as Mean ± SD*

*[*]P values are showing the differences in mean values between Boys and Girls.*

*P values were calculated by using Independent t test*

Participants were selected on the basis that they were overweight/ obese and/or were previously diagnosed with dyslipidaemia, but not with CVD [341]. The presence of dyslipidaemia was identified by the use of specific medications or when the interviewees reported having hypercholesterolemia or hypertriglyceridemia, previously diagnosed by a physician. Individuals were not included in the study if they were previously diagnosed with CVD, they used lipid-lowering drugs and they were supplemented with folic acid, cobalamin and/or pyridoxine and/or nutritional treatment.

The present study was approved by the Federal University of Goiás (addendum in protocol number 422.329, 07/10/2013). Written informed consent was obtained from students whose age was above 18 years, and for those whose age was less than 18 years consent was obtained from their parents or guardians. Participants were allowed to leave the study at will and opt out from any of the procedures. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

### 3.3.2 Anthropometric and biochemical measurements

Details of anthropometric measurements have been described previously [341]. In brief, at baseline, all participants were measured for weight, height, and waist circumference (WC) using standard study protocols [341]. The Body Mass Index (BMI) was estimated as weight (in kg) divided by the square of body height (m). BMI was classified according to the WHO (2007) classification for BMI/age according to sex [342]. Individuals below the 15th percentile were considered below normal weight, those between the 15th and 85th percentiles were classified as normal-weight, those who fit between the 85th and 97th percentiles were classified as overweight, and those above the 97th percentile were considered obese.

For the determination of biochemical parameters, blood samples (12 ml) were collected by peripheral venous puncture in the morning, after a 12 hour fast. The blood samples were used to measure homocysteine, vitamin B12, folic acid, lipid profile [including oxidized-low density lipoprotein (ox-LDL)] concentrations, and for DNA extraction. Vitamin B12 and homocysteine concentrations were analysed using a chemiluminescence method. HDL-cholesterol (HDL-C) was determined after precipitation of the LDL and very-low-density lipoprotein (VLDL) fractions. The Friedewald formula was applied to obtain the measurement of LDL and VLDL cholesterol [343]. Plasma ox-LDL levels were measured using commercially available sandwich enzyme-linked immunosorbent assay (Mercodia AB, Uppsala, Sweden) [344].

### 3.3.3 Assessment of Dietary intake and physical activity

Study participants undertook a food consumption record, where all foods and beverages consumed over two days were reported in a diary. Prior to completing the record, participants were given oral and written instructions to help them record all the food and beverages that they had consumed e.g. place of consumption and preparation method. This method was used to collect participant's usual food intake, highlighting household measures and portion sizes. After completion of the record, a trained member of research staff reviewed the record with the respondent. All information provided by the participants was double-checked for accuracy. Energy and nutrient intake from the recorded data was calculated based on the Avanutri ® software (Avanutri Informática Ltda, Rio de Janeiro, Brazil), with emphasis on lipids, B12 and folic acid. Wherever appropriate, nutrient intake values were adjusted to energy by the nutrient (energy-adjusted) residual method [345].

The Global Physical Activity Questionnaire (GPAQ), short form was used to assess physical activity. Individuals were divided into physically active and inactive individuals.

### 3.3.4 SNP Selection and Genotyping

Ten common SNPs involved in the one carbon metabolism pathway were selected based on the published reports [254, 340, 346-349]: rs1801133 (677C>T) and rs1801131 (1298A>C) of *MTHFR*; rs1805087 (2756A>G) of *MTR*; rs1801394 (66A>G) of *MTRR*; rs1801198 (776G>C) of *TCN2*; rs4680 (158G>A) and rs4633 of *COMT*; rs3797546 and rs492842 of *BHMT*; and rs602662 of *FUT2*. The *MTHFR* rs1801133 and *MTHFR* rs1801131 SNPs [1-4] are essential variants known to influence circulating homocysteine. Whilst variations in the *BHMT* gene may contribute to hyperhomocysteinemia [5], it is unknown whether the SNPs rs3797546 and rs492842 alter homocysteine levels. Previous studies indicate that the SNPs *MTR* rs1805087, *MTRR* rs1801394 [14], *MTHFR* rs1801133 and *MTHFR* rs1801131 [1-4] are

associated with folate concentrations. Furthermore, genome-wide significant associations with serum B12 have been reported for the SNPs *TCN2* rs1801198 [6] and *FUT2* rs602662 [4, 7]. The most commonly studied, *MTHFR* SNP rs1801133 has shown associations with total cholesterol, HDL-C, and LDL-C [8, 9]. The *COMT* gene is known to be involved in cardiovascular, endocrine and sympathetic pathways [350-352]. The *COMT* rs4680 SNP is an extensively studied polymorphism, which has shown associations with triacylglycerol (TAG) [10, 11], total cholesterol, and LDL-C levels [12]. Currently, no studies have examined the association between *COMT* rs4633 with incident CVD or metabolic traits.

DNA was then extracted from peripheral leukocytes in the blood, using a commercial kit (Roche TM Diagnostics GmbH, Mannheim, Germany) following the manufactures guidelines accordingly. The purity and concentration of the DNA samples were assessed using a Nanodrop ® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, N.C., USA). The 10 SNPs involved in the one carbon metabolism were genotyped by using real-time polymerase chain reaction using the QuantStudio TM OpenArray TaqMan TM platform (Life Technologies, Foster City, Calif., USA) with personalised cards for 12K Flex system QuantStudio ® (Life Technologies) with validated TaqMan Assay. The frequency of each SNP in this study sample was in agreement with the Hardy-Weinberg equilibrium ($P$>0.05) (**Table 12**).

**Table 12: Genotype distribution of SNPs involved in the one carbon-metabolism pathway**

| **Gene symbol** | **SNP rs number** | **Major allele/ Minor allele** | **Common homozygotes (%)** | **Heterozygotes (%)** | **Rare homozygotes (%)** | **Minor allele frequency** | **HWE P value** |
|---|---|---|---|---|---|---|---|
| *MTHFR* | rs1801131 | A/C | 56 (49.9) | 43 (38.1) | 13 (11.5) | 0.31 | 0.29 |
| *MTHFR* | rs1801133 | C/T | 55 (48.7) | 41 (36.3) | 12 (10.6) | 0.30 | 0.31 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *MTR* | rs1805087 | A/G | 77 (68.1) | 32 (28.3) | 2 (1.8) | 0.16 | 0.52 |
| *MTRR* | rs1801394 | A/G | 45 (39.8) | 49 (43.4) | 16 (14.2) | 0.37 | 0.66 |
| *TCN2* | rs1801198 | G/C | 60 (53.1) | 33 (29.2) | 10 (8.8) | 0.26 | 0.10 |
| *COMT* | rs4680 | G/A | 35 (31) | 48 (42.5) | 24 (21.2) | 0.45 | 0.33 |
| *COMT* | rs4633 | C/T | 44 (38.9) | 51 (45.1) | 16 (14.2) | 0.37 | 0.84 |
| *BHMT* | rs3797546 | T/C | 67 (59.3) | 27 (23.9) | 7 (6.2) | 0.20 | 0.08 |
| *BHMT* | rs492842 | T/C | 35 (31) | 43 (38.1) | 27 (23.9) | 0.46 | 0.07 |
| *FUT2* | rs602662 | G/A | 34 (30.1) | 52 (46.0) | 24 (21.2) | 0.45 | 0.62 |

*Abbreviations: HWE, Hardy Weinberg Equilibrium; MTHFR, methylene tetrahydrofolate reductase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase;TCN2, transcobalamin 2; COMT, catechol-O-methyltransferase; BHMT, betaine-homocysteine methyltransferase; FUT2, fucosyltransferase 2*

### 3.3.5 Statistical Analysis

Statistical analyses were carried out using the SPSS software (version 22; SPSS Inc., Chicago, IL, USA). Data distribution was verified by the Shapiro-Wilk test. Individuals with BMI of ≥ 25 kg/m$^2$ were categorised as obese and those with a BMI of < 25 kg/m$^2$ were classified as non-obese. Descriptive statistics for continuous variables are shown as means and standard deviation (SD). The mean differences between continuous variables and the genotypes were analysed by the independent sample t test.

Linear regression was used to examine the association of the SNPs involved in the one carbon metabolism pathway with vitamin B12, folic acid, homocysteine and lipid concentrations (TAG, HDL-cholesterol, LDL-cholesterol, and ox-LDL). The interaction between the SNPs and dietary factors on determining vitamin B12, folic acid, homocysteine

and lipid concentrations were determined by including the interaction term (SNP*diet) in the general linear regression models. Models were adjusted for age, sex, BMI and total energy intake, wherever appropriate. The dominant model was applied only for those SNPs which had a frequency of rare homozygotes less than ≤19%. Correction for multiple testing was applied using Bonferroni correction [adjusted *P* value for association was <0.00071 (10 SNPs * 7 outcomes (B12, folic acid, homocysteine, TAG, HDL-C, LDL-C, and ox-LDL concentrations) =70 tests)] and for interaction <0.00018 (10 SNPs * 7 outcomes (B12, folic acid, homocysteine, TAG, HDL-C, LDL-cholesterol, and ox-LDL concentrations)* 4 lifestyle factors= 280 tests). All data are expressed as mean ± SD.

#### 3.3.6 Power calculation

Given that there were no previously reported effect sizes, we were unable to perform a power calculation. However, based on the effect sizes that were observed for the associations, we performed a retrospective power calculation using the QUANTO software, Version 1.2.4 (May 2009). Power calculations were carried out in the form of least detectable effects based on the assumption of significance levels and powers of 5 and 80%, respectively. At 80% power, the minimum detectable effects ranged from beta 7.5 U/L (ox-LDL) for a SNP with MAF of 15% to beta 8.5 U/L for a SNP with MAF 50% for a sample size of 113 individuals.

### 3.4 Results

#### 3.4.1 Characteristics of the participants

The clinical characteristics of the studied population are shown in **Table 11**. The sample consisted of 47 boys and 66 girls. The mean age ± SD of the student group was 13.32 ± 2.35 years for boys and 14.26 ± 2.32 years for girls. When the metabolite means were categorized by sex, plasma homocysteine and HDL concentrations were found to show significant differences between boys and girls (*P*=0.009, *P*=0.040, respectively). In the study population, dietary intake of carbohydrate (energy %) and protein intake (energy %) was higher in girls

than boys (*P*=0.003, *P*=0.006 respectively), while there was no significant difference observed (*P*=0.087) in dietary intake of fat (energy %) between girls and boys (**Table 11**).

### 3.4.2 Association between SNPs and vitamin B12, folic acid, homocysteine and lipid traits

When analysing associations between 10 SNPs involved in genes related to the one-carbon metabolism cycle and biochemical indexes, we found that *COMT* rs4633 was significantly associated with folic acid ($P_{association}$=0.042). Folic acid was significantly lower in CC common homozygous individuals (10.25 ± 2.99 ng/ml) than in pooled TT and CC individuals (11.67 ± 3.29 ng/ml) ($P_{association}$=0.042) (**Table 13**). Furthermore, homozygosity for the G allele at the *FUT2* rs602662 SNP was significantly associated with lower vitamin B12 concentrations compared with the wild-type group where vitamin B12 concentrations was 24.27% lower in GG individuals than in AA individuals ($P_{association}$=0.009) (**Table 13**). In addition to these findings, the minor allele (G) of the *MTRR* rs1801394 SNP, was significantly associated with elevated ox-LDL levels ($P_{association}$=0.041) (**Table 13).** After Bonferroni correction, none of the results were considered statistically significant (*P*>0.00071).

**Table 13: Association between SNPs involved in the one-carbon metabolism pathway and vitamin B12, homocysteine, folic acid and lipid traits**

| SNPs | MAF | Vitamin B12 (pg/ml) | Homocysteine (µmol/l) | Folic acid (ng/ml) | High-density lipoprotein cholesterol (mmol/l) | Low-density lipoprotein cholesterol (mmol/l) | Triglycerides (mmol/l) | Oxidized-low density lipoprotein cholesterol (U/L) |
|---|---|---|---|---|---|---|---|---|
| ***MTHFR* gene (rs1801131)** | 0.31 | | | | | | | |
| **AA** | | 524.23 ± 223.49 | 6.90 ± 2.96 | 11.26 ± 3.34 | 47.18 ± 12.84 | 87.55 ± 21.54 | 95.05 ± 62.54 | 7.90 ± 18.65 |
| **A/C** | | 520.21 ± 241.74 | 7.19 ± 3.07 | 10.87 ± 3.20 | 45.68 ± 10.60 | 92.54 ± 20.21 | 92.73 ± 45.32 | 5.07 ± 5.67 |
| **Dominant model** | | | | | | | | |
| **(AA vs AC+ CC)** | | 0.916 | 0.895 | 0.682 | 0.708 | 0.180 | 0.739 | 0.209 |
| **P value** | | | | | | | | |
| ***MTHFR* gene (rs1801133)** | 0.30 | | | | | | | |
| **CC** | | 562.36 ± 238.63 | 6.80 ± 3.00 | 11.51 ± 3.18 | 46.04 ± 10.33 | 90.67 ± 22.87 | 86.33 ± 46.07 | 4.28 ± 5.21 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **C/T** | | 491.42 ± 224.74 | 7.37 ± 3.00 | 10.75 ± 3.37 | 46.25 ± 13.14 | 89.08 ± 19.51 | 100.26 ± 61.36 | 8.95 ± 19.12 |
| **Dominant model (CC vs CT+ TT) P value** | | 0.058 | 0.100 | 0.158 | 0.468 | 0.519 | 0.300 | 0.106 |
| ***MTR* gene (rs1805087)** | 0.16 | | | | | | | |
| **AA** | | 523.97 ± 221.74 | 7.22 ± 3.29 | 11.03 ± 3.31 | 46.64 ± 12.73 | 89.42 ± 20.73 | 92.83 ± 58.11 | 6.70 ± 14.34 |
| **A/G** | | 518.59 ± 518.59 | 6.77 ± 2.21 | 11.08 ± 3.27 | 45.82 ± 9.78 | 91.71 ± 22.29 | 95.44 ± 46.26 | 6.01 ± 12.72 |
| **Dominant model (AA vs AG+ GG) P value** | | 0.815 | 0.301 | 0.919 | 0.886 | 0.517 | 0.893 | 0.836 |
| ***MTRR* gene (rs1801394)** | 0.37 | | | | | | | |
| **AA** | | 560.53 ± 249.40 | 6.61 ± 2.58 | 10.96 ± 3.42 | 46.29 ± 9.67 | 90.78 ± 23.02 | 93.98 ± 62.30 | 3.13 ± 3.81 |
| **A/G** | | 494.11 ± 218.14 | 7.35 ± 3.27 | 11.14 ± 3.20 | 46.38 ± 13.09 | 89.60 ± 19.91 | 93.28 ± 48.75 | 8.95 ± 17.46 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Dominant model** | | | | | | | | |
| **(AA vs AG + GG)** | | 0.265 | 0.394 | 0.508 | 0.827 | 0.814 | 0.958 | **0.041** |
| **P value** | | | | | | | | |
| ***TCN2* gene (rs1801198)** | 0.26 | | | | | | | |
| **GG** | | 523.88 ± 221.89 | 6.99 ± 2.60 | 11.34 ± 3.26 | 46.93 ± 12.74 | 90.70 ± 22.25 | 91.88 ± 50.19 | 5.99 ± 13.23 |
| **G/C** | | 530.67 ± 263.13 | 6.94 ± 3.16 | 10.67 ± 3.14 | 44.95 ± 9.83 | 90.09 ± 19.48 | 98.53 ± 61.99 | 7.82 ± 15.69 |
| **Dominant model** | | | | | | | | |
| **(GG vs GC + CC)** | | 0.982 | 0.751 | 0.231 | 0.213 | 0.776 | 0.463 | 0.497 |
| **P value** | | | | | | | | |
| ***COMT* gene (rs4680)** | 0.45 | | | | | | | |
| **GG** | | 495.54 ± 234.10 | 6.98 ± 3.20 | 10.02 ± 2.81 | 44.00 ± 10.67 | 86.71 ± 20.17 | 102.14 ± 65.53 | 5.14 ± 6.30 |
| **GA** | | 546.98 ± 249.81 | 6.88 ± 2.32 | 11.56 ± 3.24 | 45.58 ± 11.29 | 87.71 ± 22.27 | 95.27 ± 57.18 | 7.07 ± 17.47 |
| **AA** | | 515.50 ± 181.22 | 7.08 ± 3.94 | 11.59 ± 3.61 | 49.21 ± 13.83 | 96.83 ± 17.83 | 81.13 ± 27.52 | 7.20 ± 14.86 |
| **Additive model P value** | | 0.825 | 0.843 | 0.094 | 0.258 | 0.186 | 0.376 | 0.658 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ***COMT* gene (rs4633)** | 0.37 | | | | | | | |
| **CC** | | 511.77 ± 248.82 | 6.98 ± 3.28 | 10.25 ± 2.99 | 45.05 ± 10.87 | 87.91 ± 19.92 | 99.84 ± 61.18 | 4.99 ± 6.00 |
| **C/T** | | 532.07 ± 221.84 | 7.00 ± 2.77 | 11.67 ± 3.29 | 47.25 ± 12.36 | 90.99 ± 21.46 | 89.36 ± 49.63 | 7.54 ± 17.11 |
| **Dominant model (CC vs CT + TT) P value** | | 0.907 | 0.740 | **0.042** | 0.524 | 0.337 | 0.364 | 0.146 |
| ***BHMT* gene (rs3797546)** | 0.20 | | | | | | | |
| **TT** | | 536.31 ± 233.97 | 7.04 ± 3.18 | 11.51 ± 3.33 | 46.21 ± 11.33 | 89.57 ± 21.17 | 94.55 ± 56.16 | 6.68 ± 15.25 |
| **T/C** | | 523.41 ± 224.50 | 6.53 ± 2.03 | 10.54 ± 2.90 | 46.59 ± 12.48 | 92.18 ± 21.76 | 99.29 ± 54.96 | 6.74 ± 12.72 |
| **Dominant model (TT vs TC + CC) P value** | | 0.971 | 0.151 | 0.231 | 0.461 | 0.495 | 0.811 | 0.791 |
| ***BHMT* gene (rs492842)** | 0.46 | | | | | | | |
| **TT** | | 554.89 ± 254.53 | 6.65 ± 2.66 | 11.92 ± 3.21 | 47.57 ± 11.81 | 85.91 ± 24.16 | 90.83 ± 43.39 | 6.47 ± 12.79 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **TC** | | 492.35 ± 196.18 | 7.64 ± 3.30 | 10.14± 3.16 | 45.77 ± 10.46 | 93.84 ± 17.92 | 97.05 ± 55.94 | 8.74 ± 18.36 |
| **CC** | | 469.70 ± 201.74 | 6.91 ± 3.14 | 11.27 ± 3.35 | 44.26 ± 11.06 | 89.37 ± 21.64 | 99.07 ± 68.56 | 3.34 ± 4.95 |
| **Additive model P value** | | 0.293 | 0.602 | 0.095 | 0.872 | 0.179 | 0.915 | 0.220 |
| ***FUT2* gene (rs602662)** | 0.45 | | | | | | | |
| **GG** | | 471.41 ± 193.77 | 6.93 ± 3.35 | 11.47 ± 3.31 | 44.68 ± 10.01 | 84.50 ± 20.31 | 90.12 ± 54.27 | 8.21 ± 13.26 |
| **GA** | | 494.73 ± 220.75 | 7.18 ± 2.46 | 10.69 ± 3.19 | 47.21 ± 13.38 | 90.52 ± 20.81 | 93.40 ± 54.93 | 4.99 ± 13.69 |
| **AA** | | 622.50 ± 268.60 | 6.84 ± 3.64 | 11.15 ± 3.40 | 46.29 ± 10.37 | 97.79 ± 21.98 | 104.17 ± 56.13 | 7.42 ± 15.24 |
| **Additive model P value** | | **0.009** | 0.677 | 0.620 | 0.622 | 0.063 | 0.664 | 0.374 |

*Values are given as mean ± SD. P values for differences between genotypes were obtained using linear regression model adjusted for age, sex and BMI. Adjusted P value after correction for multiple testing was 0.00071.*

*Abbreviations: MAF, minor allele frequency;MTHFR, methylene tetrahydrofolate reductase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase;TCN2, transcobalamin 2; COMT, catechol-O-methyltransferase; BHMT, betaine-homocysteine methyltransferase; FUT2, fucosyltransferase 2; SNPs, Single-nucleotide polymorphisms; SD, Standard deviation*

### 3.4.3 Interaction between SNPs and B12, folic acid, homocysteine

An interaction was observed between the *BHMT* SNP rs492842 and dietary fat intake on vitamin B12 levels (*P*=0.034). In addition, further interactions were found between the *FUT2* SNP rs602662 with dietary protein intake (*P*= 0.007) and carbohydrate intake (*P*= 0.031) on homocysteine concentrations (**Table 14**). We found that rare AA homozygotes of the *FUT2* SNP rs602662 had higher homocysteine levels (Mean ± SE: 8.038 ± 0.896 µmol/l) compared to the GG allele carriers (Mean ± SE: 5.857 ± 1.039 µmol/l) among those in the highest tertile of protein intake (Mean ± SE: 148.618± 5.777 g/day); however, the difference in the means of homocysteine concentrations between the genotype groups in the highest tertile of protein intake was not statistically significant (*P*=0.227), which could be because of the small sample size.

**Table 14: Interaction between SNPs and dietary factors on vitamin B12, homocysteine and Folic acid traits**

| ***P values for the interaction between SNPs and dietary factors on vitamin B12*** | | |
|---|---|---|
| Interaction between SNP * rs1801131 fat energy intake | Interaction between SNP rs1801131* protein energy intake | Interaction between SNP rs1801131* carbohydrate energy intake |
| 0.685 | 0.095 | 0.074 |
| Interaction between SNP rs1801133* fat energy intake | Interaction between SNP rs1801133* protein energy intake | Interaction between SNP rs1801133* carbohydrate energy intake |
| 0.429 | 0.067 | 0.115 |
| Interaction between SNP rs1805087* fat energy intake | Interaction between SNP rs1805087* protein energy intake | Interaction between SNP rs1805087* carbohydrate energy intake |
| 0.368 | 0.539 | 0.206 |
| Interaction between SNP rs1801394* fat energy intake | Interaction between SNP rs1801394* protein energy intake | Interaction between SNP rs1801394* carbohydrate energy intake |
| 0.733 | 0.070 | 0.743 |

| Interaction between SNP rs1801198* fat energy intake | Interaction between SNP rs1801198* protein energy intake | Interaction between SNP rs1801198* carbohydrate energy intake |
|---|---|---|
| 0.789 | 0.109 | 0.631 |
| Interaction between SNP rs4680* fat energy intake | Interaction between SNP rs4680* protein energy intake | Interaction between SNP rs4680* carbohydrate energy intake |
| 0.662 | 0.265 | 0.559 |
| Interaction between SNP rs4633* fat energy intake | Interaction between SNP rs4633* protein energy intake | Interaction between SNP rs4633* carbohydrate energy intake |
| 0.455 | 0.490 | 0.799 |
| Interaction between SNP rs3797546* fat energy intake | Interaction between SNP rs3797546* protein energy intake | Interaction between SNP rs3797546* carbohydrate energy intake |
| 0.353 | 0.979 | 0.281 |
| Interaction between SNP rs492842* fat energy intake | Interaction between SNP rs492842* protein energy intake | Interaction between SNP rs492842* carbohydrate energy intake |
| **0.034** | 0.678 | 0.331 |
| Interaction between SNP rs602662* fat energy intake | Interaction between SNP rs602662* protein energy intake | Interaction between SNP rs602662* carbohydrate energy intake |
| 0.087 | 0.144 | 0.533 |
| ***P values for the interaction between SNPs and dietary factors on homocysteine*** | | |
| Interaction between SNP * rs1801131 fat energy intake | Interaction between SNP rs1801131* protein energy intake | Interaction between SNP rs1801131* carbohydrate energy intake |
| 0.806 | 0.803 | 0.625 |
| Interaction between SNP rs1801133* fat energy intake | Interaction between SNP rs1801133* protein energy intake | Interaction between SNP rs1801133* carbohydrate energy intake |
| 0.975 | 0.621 | 0.433 |
| Interaction between SNP rs1805087* fat energy intake | Interaction between SNP rs1805087* protein energy intake | Interaction between SNP rs1805087* carbohydrate energy intake |
| 0.123 | 0.922 | 0.389 |

| Interaction between SNP rs1801394* fat energy intake | Interaction between SNP rs1801394* protein energy intake | Interaction between SNP rs1801394* carbohydrate energy intake |
|---|---|---|
| 0.252 | 0.645 | 0.456 |
| Interaction between SNP rs1801198* fat energy intake | Interaction between SNP rs1801198* protein energy intake | Interaction between SNP rs1801198* carbohydrate energy intake |
| 0.869 | 0.212 | 0.341 |
| Interaction between SNP rs4680* fat energy intake | Interaction between SNP rs4680* protein energy intake | Interaction between SNP rs4680* carbohydrate energy intake |
| 0.062 | 0.189 | 0.054 |
| Interaction between SNP rs4633* fat energy intake | Interaction between SNP rs4633* protein energy intake | Interaction between SNP rs4633* carbohydrate energy intake |
| 0.596 | 0.359 | 0.133 |
| Interaction between SNP rs3797546* fat energy intake | Interaction between SNP rs3797546* protein energy intake | Interaction between SNP rs3797546* carbohydrate energy intake |
| 0.713 | 0.614 | 0.209 |
| Interaction between SNP rs492842* fat energy intake | Interaction between SNP rs492842* protein energy intake | Interaction between SNP rs492842* carbohydrate energy intake |
| 0.232 | 0.227 | 0.606 |
| Interaction between SNP rs602662* fat energy intake | Interaction between SNP rs602662* protein energy intake | Interaction between SNP rs602662* carbohydrate energy intake |
| 0.334 | **0.007** | **0.031** |
| ***P values for the interaction between SNPs and dietary factors on folic acid*** | | |
| Interaction between SNP * rs1801131 fat energy intake | Interaction between SNP rs1801131* protein energy intake | Interaction between SNP rs1801131* carbohydrate energy intake |
| 0.378 | 0.642 | 0.774 |
| Interaction between SNP rs1801133* fat energy intake | Interaction between SNP rs1801133* protein energy intake | Interaction between SNP rs1801133* carbohydrate energy intake |
| 0.595 | 0.587 | 0.722 |

| Interaction between SNP rs1805087* fat energy intake | Interaction between SNP rs1805087* protein energy intake | Interaction between SNP rs1805087* carbohydrate energy intake |
|---|---|---|
| 0.834 | 0.887 | 0.498 |
| Interaction between SNP rs1801394* fat energy intake | Interaction between SNP rs1801394* protein energy intake | Interaction between SNP rs1801394* carbohydrate energy intake |
| 0.641 | 0.826 | 0.327 |
| Interaction between SNP rs1801198* fat energy intake | Interaction between SNP rs1801198* protein energy intake | Interaction between SNP rs1801198* carbohydrate energy intake |
| 0.845 | 0.759 | 0.547 |
| Interaction between SNP rs4680* fat energy intake | Interaction between SNP rs4680* protein energy intake | Interaction between SNP rs4680* carbohydrate energy intake |
| 0.610 | 0.495 | 0.228 |
| Interaction between SNP rs4633* fat energy intake | Interaction between SNP rs4633* protein energy intake | Interaction between SNP rs4633* carbohydrate energy intake |
| 0.721 | 0.248 | 0.050 |
| Interaction between SNP rs3797546* fat energy intake | Interaction between SNP rs3797546* protein energy intake | Interaction between SNP rs3797546* carbohydrate energy intake |
| 0.188 | 0.394 | 0.754 |
| Interaction between SNP rs492842* fat energy intake | Interaction between SNP rs492842* protein energy intake | Interaction between SNP rs492842* carbohydrate energy intake |
| 0.084 | 0.971 | 0.447 |
| Interaction between SNP rs602662* fat energy intake | Interaction between SNP rs602662* protein energy intake | Interaction between SNP rs602662* carbohydrate energy intake |
| 0.521 | 0.775 | 0.115 |

*P values were obtained by using a general linear model adjusted for age, sex and BMI. Abbreviations: SNPs, Single-nucleotide polymorphisms; BMI, Body mass index*

### 3.4.4 Interaction between SNPs and dietary factors on lipid concentrations

Interactions were observed between the *COMT* SNPs (rs4680 and rs4633) and dietary carbohydrate intake on HDL-C concentrations ($P$=0.011 and $P$=0.036, respectively). Furthermore, an interaction was found between the *COMT* SNP (rs4680) and dietary carbohydrate intake on ox-LDL concentrations ($P$=0.005) (**Table 15**). However, none of the interactions between the SNPs and dietary intake on lipid outcomes reached statistical significance after correction for multiple testing.

**Table 15: Interaction between SNPs and dietary factors on lipid traits**

| ***Interaction between SNPs and dietary factors on HDL-C*** | | |
|---|---|---|
| Interaction between SNP * rs1801131 fat energy intake | Interaction between SNP rs1801131* protein energy intake | Interaction between SNP rs1801131* carbohydrate energy intake |
| 0.964 | 0.402 | 0.899 |
| Interaction between SNP rs1801133* fat energy intake | Interaction between SNP rs1801133* protein energy intake | Interaction between SNP rs1801133* carbohydrate energy intake |
| 0.393 | 0.471 | 0.994 |
| Interaction between SNP rs1805087* fat energy intake | Interaction between SNP rs1805087* protein energy intake | Interaction between SNP rs1805087* carbohydrate energy intake |
| 0.651 | 0.298 | 0.499 |
| Interaction between SNP rs1801394* fat energy intake | Interaction between SNP rs1801394* protein energy intake | Interaction between SNP rs1801394* carbohydrate energy intake |
| 0.896 | 0.712 | 0.676 |
| Interaction between SNP rs1801198* fat energy intake | Interaction between SNP rs1801198* protein energy intake | Interaction between SNP rs1801198* carbohydrate energy intake |
| 0.414 | 0.822 | 0.649 |
| Interaction between SNP rs4680* fat energy intake | Interaction between SNP rs4680* protein energy intake | Interaction between SNP rs4680* carbohydrate energy intake |
| 0.898 | 0.536 | **0.011** |

| Interaction between SNP rs4633* fat energy intake | Interaction between SNP rs4633* protein energy intake | Interaction between SNP rs4633* carbohydrate energy intake |
|---|---|---|
| 0.846 | 0.620 | **0.036** |
| Interaction between SNP rs3797546* fat energy intake | Interaction between SNP rs3797546* protein energy intake | Interaction between SNP rs3797546* carbohydrate energy intake |
| 0.274 | 0.162 | 0.555 |
| Interaction between SNP rs492842 fat energy intake | Interaction between SNP rs492842* protein energy intake | Interaction between SNP rs492842* carbohydrate energy intake |
| 0.604 | 0.960 | 0.513 |
| Interaction between SNP rs602662* fat energy intake | Interaction between SNP rs602662* protein energy intake | Interaction between SNP rs602662* carbohydrate energy intake |
| 0.650 | 0.123 | 0.813 |
| ***Interaction between SNPs and dietary factors on LDL-C*** | | |
| Interaction between SNP * rs1801131 fat energy intake | Interaction between SNP rs1801131* protein energy intake | Interaction between SNP rs1801131* carbohydrate energy intake |
| 0.529 | 0.467 | 0.798 |
| Interaction between SNP rs1801133* fat energy intake | Interaction between SNP rs1801133* protein energy intake | Interaction between SNP rs1801133* carbohydrate energy intake |
| 0.640 | 0.656 | 0.737 |
| Interaction between SNP rs1805087* fat energy intake | Interaction between SNP rs1805087* protein energy intake | Interaction between SNP rs1805087* carbohydrate energy intake |
| 0.456 | 0.933 | 0.876 |
| Interaction between SNP rs1801394* fat energy intake | Interaction between SNP rs1801394* protein energy intake | Interaction between SNP rs1801394* carbohydrate energy intake |
| 0.487 | 0.384 | 0.222 |
| Interaction between SNP rs1801198* fat energy intake | Interaction between SNP rs1801198* protein energy intake | Interaction between SNP rs1801198* carbohydrate energy intake |
| 0.127 | 0.664 | 0.250 |

| Interaction between SNP rs4680* fat energy intake | Interaction between SNP rs4680* protein energy intake | Interaction between SNP rs4680* carbohydrate energy intake |
|---|---|---|
| 0.509 | 0.709 | 0.299 |
| Interaction between SNP rs4633* fat energy intake | Interaction between SNP rs4633* protein energy intake | Interaction between SNP rs4633* carbohydrate energy intake |
| 0.743 | 0.915 | 0.067 |
| Interaction between SNP rs3797546* fat energy intake | Interaction between SNP rs3797546* protein energy intake | Interaction between SNP rs3797546* carbohydrate energy intake |
| 0.594 | 0.097 | 0.306 |

| Interaction between SNP rs492842* fat energy intake | Interaction between SNP rs492842* protein energy intake | Interaction between SNP rs492842* carbohydrate energy intake |
|---|---|---|
| 0.380 | 0.392 | 0.402 |
| Interaction between SNP rs602662* fat energy intake | Interaction between SNP rs602662* protein energy intake | Interaction between SNP rs602662* carbohydrate energy intake |
| 0.399 | 0.462 | 0.610 |
| ***Interaction between SNPs and dietary factors on Triglycerides*** | | |
| Interaction between SNP * rs1801131 fat energy intake | Interaction between SNP rs1801131* protein energy intake | Interaction between SNP rs1801131* carbohydrate energy intake |
| 0.970 | 0.792 | 0.504 |
| Interaction between SNP rs1801133* fat energy intake | Interaction between SNP rs1801133* protein energy intake | Interaction between SNP rs1801133* carbohydrate energy intake |
| 0.938 | 0.798 | 0.266 |
| Interaction between SNP rs1805087* fat energy intake | Interaction between SNP rs1805087* protein energy intake | Interaction between SNP rs1805087* carbohydrate energy intake |
| 0.648 | 0.362 | 0.245 |
| Interaction between SNP rs1801394* fat energy intake | Interaction between SNP rs1801394* protein energy intake | Interaction between SNP rs1801394* carbohydrate energy intake |
| 0.176 | 0.285 | 0.857 |

| Interaction between SNP rs1801198* fat energy intake | Interaction between SNP rs1801198* protein energy intake | Interaction between SNP rs1801198* carbohydrate energy intake |
|---|---|---|
| 0.490 | 0.719 | 0.317 |
| Interaction between SNP rs4680* fat energy intake | Interaction between SNP rs4680* protein energy intake | Interaction between SNP rs4680* carbohydrate energy intake |
| 0.290 | 0.408 | 0.923 |
| Interaction between SNP rs4633* fat energy intake | Interaction between SNP rs4633* protein energy intake | Interaction between SNP rs4633* carbohydrate energy intake |
| 0.185 | 0.220 | 0.770 |
| Interaction between SNP rs3797546* fat energy intake | Interaction between SNP rs3797546* protein energy intake | Interaction between SNP rs3797546* carbohydrate energy intake |
| 0.127 | 0.741 | 0.457 |
| Interaction between SNP rs492842* fat energy intake | Interaction between SNP rs492842* protein energy intake | Interaction between SNP rs492842* carbohydrate energy intake |
| 0.237 | 0.216 | 0.989 |
| Interaction between SNP rs602662* fat energy intake | Interaction between SNP rs602662* protein energy intake | Interaction between SNP rs602662* carbohydrate energy intake |
| 0.360 | 0.082 | 0.817 |
| ***Interaction between SNPs and dietary factors on LDL-ox*** | | |
| Interaction between SNP * rs1801131 fat energy intake | Interaction between SNP rs1801131* protein energy intake | Interaction between SNP rs1801131* carbohydrate energy intake |
| 0.161 | 0.962 | 0.582 |
| Interaction between SNP rs1801133* fat energy intake | Interaction between SNP rs1801133* protein energy intake | Interaction between SNP rs1801133* carbohydrate energy intake |
| 0.399 | 0.972 | 0.908 |
| Interaction between SNP rs1805087* fat energy intake | Interaction between SNP rs1805087* protein energy intake | Interaction between SNP rs1805087* carbohydrate energy intake |
| 0.493 | 0.126 | 0.784 |

| Interaction between SNP rs1801394* fat energy intake | Interaction between SNP rs1801394* protein energy intake | Interaction between SNP rs1801394* carbohydrate energy intake |
|---|---|---|
| 0.235 | 0.781 | 0.672 |
| Interaction between SNP rs1801198* fat energy intake | Interaction between SNP rs1801198* protein energy intake | Interaction between SNP rs1801198* carbohydrate energy intake |
| 0.832 | 0.100 | 0.489 |
| Interaction between SNP rs4680* fat energy intake | Interaction between SNP rs4680* protein energy intake | Interaction between SNP rs4680* carbohydrate energy intake |
| 0.353 | 0.348 | **0.005** |
| Interaction between SNP rs4633* fat energy intake | Interaction between SNP rs4633* protein energy intake | Interaction between SNP rs4633* carbohydrate energy intake |
| 0.217 | 0.372 | 0.984 |
| Interaction between SNP rs3797546* fat energy intake | Interaction between SNP rs3797546* protein energy intake | Interaction between SNP rs3797546* carbohydrate energy intake |
| 0.846 | 0.227 | 0.270 |
| Interaction between SNP rs492842* fat energy intake | Interaction between SNP rs492842* protein energy intake | Interaction between SNP rs492842* carbohydrate energy intake |
| 0.624 | 0.466 | 0.690 |
| Interaction between SNP rs602662* fat energy intake | Interaction between SNP rs602662* protein energy intake | Interaction between SNP rs602662* carbohydrate energy intake |
| 0.743 | 0.298 | 0.112 |

*P values were obtained by using a general linear model adjusted for age, sex and BMI. Abbreviations: SNPs, Single-nucleotide polymorphisms; BMI, Body mass index*

### 3.4.5 Gene-physical activity interactions on vitamin B12, folic acid, homocysteine and lipid profile

In addition to the genetic component of metabolic traits and markers of one-carbon metabolites, physical inactivity could be an important contributor that could interact with an

individual's genetic predisposition. Our results showed that the *MTHFR* SNP rs1801131 showed a significant interaction with physical activity on folic acid concentrations ($P_{interaction}$=0.034). Folate plays a critical role in the methylation pathway and is involved in the methylation of DNA, creatine and acetylcholine, all of which are important for physical activity. It is important to note that physical activity levels may interact with folate metabolism by increasing intestinal folate absorption or stimulating the methionine synthase enzyme due to an increased metabolic demand and the associated possible increase in turnover of methylated molecules required for exercise [353]. In addition, the three SNPs individually: *MTHFR* SNP rs1801133, *BHMT* rs492842 and *FUT2* rs602662 all showed a significant interaction with physical activity on triglyceride concentrations ($P_{interaction}$=0.030, 0.004 and 0.014, respectively). Finally, the *BHMT* SNP rs3797546 showed an interaction with physical activity on ox-LDL levels. However, these interactions were not statistically significant after correction for multiple testing ($P_{interaction}$ >0.00018) **(Table 16).** To date all the gene-physical activity interactions observed in this study are novel, thus we are unable to compare our findings with previous literature. It important that in the future, replications of these findings are made preferably in an independent larger cohort with adequate statistical power utilising more direct measures of physical activity, in order to confirm or refute our findings.

**Table 16: P values for the interaction between SNPs and physical activity levels on vitamin B12, homocysteine, folic acid and lipid traits**

| Gene | rs number | Model | B12 | Homocysteine | Folic acid | HDL | LDL | Triglycerides | LDL-ox |
|---|---|---|---|---|---|---|---|---|---|
| *MTHFR* | rs1801131 | Dominant | 0.758 | 0.640 | **0.034** | 0.570 | 0.764 | 0.139 | 0.496 |
| *MTHFR* | rs1801133 | Dominant | 0.589 | 0.810 | 0.404 | 0.446 | 0.541 | **0.030** | 0.760 |
| *MTR* | rs1805087 | Dominant | 0.607 | 0.560 | 0.940 | 0.440 | 0.890 | 0.345 | 0.134 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *MTRR* | rs1801394 | Dominant | 0.106 | 0.325 | 0.238 | 0.956 | 0.915 | 0.701 | 0.563 |
| *TCN2* | rs1801198 | Dominant | 0.414 | 0.321 | 0.941 | 0.517 | 0.726 | 0.186 | 0.887 |
| *COMT* | rs4680 | Additive | 0.543 | 0.058 | 0.783 | 0.537 | 0.663 | 0.113 | 0.681 |
| *COMT* | rs4633 | Dominant | 0.221 | 0.253 | 0.993 | 0.828 | 0.400 | 0.056 | 0.597 |
| *BHMT* | rs3797546 | Dominant | 0.146 | 0.274 | 0.255 | 0.811 | 0.250 | 0.209 | **0.050** |
| *BHMT* | rs492842 | Additive | 0.947 | 0.281 | 0.423 | 0.513 | 0.483 | **0.004** | 0.677 |
| *FUT2* | rs602662 | Additive | 0.613 | 0.100 | 0.458 | 0.147 | 0.724 | **0.014** | 0.491 |

*P values were obtained by using a general linear model adjusted for age, sex and BMI. Abbreviations: SNPs, Single-nucleotide polymorphisms; BMI, Body mass index*

### 3.4.6 Discussion

To our knowledge, this is the first genetic epidemiological study to investigate the interactions between SNPs involved in the one-carbon metabolism pathway and environmental/lifestyle factors on vitamin B12, folic acid, homocysteine and lipid levels (HDL-C, LDL, TAG and ox-LDL) in the Brazilian adolescent population. Our study provides evidence for novel interactions between SNP rs4680 (*COMT* gene) and carbohydrate intake on ox-LDL levels and the SNP rs602662 (*FUT2* gene) and protein intake on homocysteine concentrations in Brazilian adolescents. Given that ox-LDL and hyperhomocysteinaemia are well-known independent risk factors for atherosclerotic vascular disease [24, 354], our findings have significant public health implications.

Genes involved in one carbon metabolism are of particular interest because of their role in CVDs [355]. From the 10 SNPs which were investigated in this study, association of the SNP rs4633 at the *COMT* gene with folic acid concentrations (*P*=0.042), the SNP rs602662 at

the *FUT2* gene with vitamin B12 levels (*P*=0.009) and finally the SNP rs1801394 at the *MTRR* gene with ox-LDL concentrations (*P*=0.041) were observed. Even though the findings were not significant after Bonferroni correction, the association between the *FUT2* SNP rs602662 and vitamin B12 concentrations is in accordance with previous studies [205, 233, 234, 253, 254, 258, 260]. Since the current sample size is relatively small, further studies utilizing a larger sample size is required to confirm the observed associations.

To date, only one study has shown a gene-diet interaction on ox-LDL concentrations in a population from the Attica region in Greece [356]. In this study, there was an interaction of the *MTHFR* SNP rs1801133 with the Mediterranean diet on ox-LDL concentrations. A high adherence to the Mediterranean diet was found to be associated with decreased ox-LDL concentrations in T allele carriers of SNP rs1801133 [356]. Further to this, many studies have reported that *MTHFR* variants (C677T and A1298C) are linked to higher homocysteine levels, when folate consumption is low [357, 358]. In the present study, we identified significant gene-diet interactions between SNP rs4680 at the *COMT* gene and carbohydrate intake on ox-LDL concentrations and the SNP rs602662 at the *FUT2* gene and protein intake on homocysteine concentrations. However, further stratification of participants based on their consumption of low, medium and high dietary carbohydrate/protein did not show a statistically significant association between the SNP and the outcome in any of the tertiles, which could account for the small sample size. This is the first study to provide evidence for gene-diet interactions at the *COMT* and *FUT2* gene loci, on ox-LDL and homocysteine concentrations, respectively, and hence, we do not have any previous studies to compare our findings.

Total carbohydrate intake has increased considerably in Brazil in the last few decades [359]. Data from two population-based surveys conducted in women over 35 years of age from Rio de Janeiro, reported that the carbohydrate intake has increased from 352g (95% CI 325 to 382) in 1995 to 437 g (95% CI 415 to 458) in 2005 [359]. Interestingly, our study in this

Brazilian adolescent population has identified an interaction between *COMT* SNP rs4680 and carbohydrate intake on ox-LDL concentrations. Despite our study being the first to report this gene-diet interaction, previous studies have shown that carbohydrate restricted diets can promote weight loss and is associated with reduced cardiovascular disease risk [360]. However, the exact mechanism by which the *COMT* SNP rs4680 interacts with dietary carbohydrate to influence ox-LDL concentrations is unknown and requires further studies to understand the mechanism contributing to this association. Furthermore, in our study we observed an interaction of the *FUT2* SNP rs1805087 with protein consumption on homocysteine levels. However, we have no previous studies to confirm and validate this novel finding. The findings in this paper suggest that that the inheritance of ox-LDL and homocysteine levels are complex, where several genes/polymorphisms are likely to contribute to the alteration of ox-LDL or homocysteine levels through gene-gene and gene-diet interactions. More in depth research implementing animal studies, nutrigenomics and metabolomics are needed to clarify the effects of SNPs and carbohydrate on ox-LDL concentrations, and protein on homocysteine concentrations, respectively.

One of the main limitations of our study is the small sample size. Given that there are no previously reported effect sizes for the *FUT2* and *COMT* SNP-diet interactions on blood homocysteine and ox-LDL concentrations, we were unable to calculate the statistical power of our study. Our retrospective power calculation showed that the minimum detectable effects for ox-LDL levels ranged from beta 7.5 U/L (ox-LDL) for a SNP with MAF of 15% to beta 8.5 U/L for a SNP with MAF 50%. Hence, if the actual effect sizes were lower than this, our study would be underpowered. However, our study did find significant associations and gene-diet interactions despite the small sample size; but the findings requires a replication given that the significant *P* values did not reach the Bonferroni corrected P value. Another limitation is that our study was cross-sectional, and therefore we were unable to examine the causal relationship

between the SNP-diet interactions on blood homocysteine and ox-LDL concentrations. Therefore, randomized controlled trials with prospective genotyping are required to explore the causality using genetic markers. Given that our study relied on a usual food record, we cannot negate the possibility of misreporting and measurement error. A further limitation of the current study is the lack of a control group, as only individuals with cardiovascular risk factors were included. On the other hand, the main strength of our study is that we examined the effect of ten SNPs on vitamin B12, folic acid, homocysteine concentrations and lipid traits during adolescence, a critical period where lifestyle habits are usually followed through to adulthood. By studying this population, we were able to identify different genotypes of interest, which could be further investigated to improve the understanding of the role of these micronutrients in relation to the prevention of hyperhomocysteiemia and increased ox-LDL concentrations. Additionally, little is known about gene-diet interactions which influence ox-LDL concentrations; thus, our study adds to the limited body of research.

### 3.4.7 Conclusion

Our study shows an interaction between *COMT* SNP rs4680 and carbohydrate intake on ox-LDL levels among adolescents with cardiovascular risk factors. Furthermore, a borderline interaction was observed between *FUT2* SNP rs602662 and protein intake on homocysteine concentrations. After correction for multiple testing, none of the SNP-environment interactions on homocysteine, folate, B12 or lipid concentrations were detected. Hence, our findings warrant confirmation in larger, well characterized and well powered prospective studies/ randomized controlled trials, before any public health recommendations and personalised nutrition advice can be developed for the adolescent Brazilian population.

**Author contributions: Shelini Surendran performed the statistical analysis and drafted the manuscript**; MAH and CC were responsible for the study conception and provided guidance to the research; CCM conducted data and sample collection; KSV designed the gene-environment interaction study; JAL, MAH, CCM, DSPA, IS and CC critically reviewed the manuscript; VKS conceived the nutrigenetics study and guide guidance to the statistical analysis; All authors contributed to and approved the final version of the manuscript.

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# Chapter 4

## A genetic approach to examine the relationship between vitamin B12 status and metabolic traits in a South Asian population

I was the lead co-ordinator for this study. I was responsible for writing up the documents required to gain ethical approval to conduct the study both in the University of Colombo, Sri Lanka and the University of Reading, UK. I was involved in recruiting participants for the study and organizing the locations of the study visits. I spent three months in 2017 in Colombo, Sri Lanka to conduct this study. I was primarily involved in data collection, which involved collecting anthropometric measurements, conducting food frequency questionnaires and physical activity questionnaires. I was also involved in transporting the blood samples to LGC genomics for genetic analysis in the United Kingdom.

For this study, I developed an analysis plan before I undertook the statistical analysis. I screened and validated the dataset to perform statistical analysis. I performed the entire statistical analysis using the SPSS software; I undertook a literature review as part of the introduction to the study and wrote the manuscript. I revised the manuscript based on the comments from all the co-authors before the manuscript was submitted to the International Journal of Diabetes in Developing Countries. I was also involved in drafting the responses to the comments from the reviewers.

**Published (The Published paper is attached as an appendix at the end of the thesis)**

Surendran S, Alsulami S[1], Lankeshwara R, Jayawardena R, Wetthasinghe K Sarkar S, Ellahi B, Lovegrove JA, Anthony DJ, Vimaleswaran KS (2019). A genetic approach to examine the relationship between vitamin B12 status and metabolic traits in a South Asian population. International Journal of Diabetes in Developing Countries (**Published**).

## 4.1 Abstract

**Background:** Observational studies in South Asian populations have suggested an association between vitamin B12 status and metabolic traits; however, the findings have been inconclusive. Hence, the aim of the present study was to use a genetic approach to explore the relationship between metabolic traits and vitamin B12 status in a Sri Lankan population and to investigate whether these relationships were modified by dietary intake.

**Methods:** A total of 109 Sinhalese adults (61 men and 48 women aged 25-50 years), from Colombo city underwent anthropometric, biochemical, dietary intake analysis and genetic tests. Genetic risk scores (GRS) based on 10 metabolic single nucleotide polymorphisms (SNPs) (metabolic-GRS) and 10 vitamin B12 SNPs (B12-GRS) were constructed.

**Results:** The B12-GRS was significantly associated with serum vitamin B12 (P=0.008), but not with metabolic traits (P>0.05); whereas, the metabolic-GRS had no effect on metabolic traits (P>0.05) and vitamin B12 concentrations (P>0.05). An interaction was observed between B12-GRS and protein energy intake (%) on waist circumference (P=0.002). Interactions were also seen between the metabolic-GRS and carbohydrate energy intake (%) on waist to hip ratio (P=0.015).

**Conclusion:** Our findings suggest that a genetically lowered vitamin B12 concentration may have an impact on central obesity in the presence of a dietary influence; however, our study failed to provide evidence for an impact of metabolic-GRS on lowering B12 concentrations. Given that our study has a small sample size, further large studies are required to confirm our findings.

## 4.2 Introduction

In recent years, the incidence of obesity in Sri Lanka has increased markedly [361]. The prevalence of being overweight or obese in Sri Lankan adults is 34.4% (25.2% and 9.2%, in 2005 and 2006 respectively), with an upward trend being observed [361, 362]. Obesity increases the risk for certain health conditions, such as insulin resistance, diabetes mellitus and hypertension [363]. South Asians have been observed to exhibit increased visceral fat and waist circumference (WC), hyperinsulinemia and insulin resistance; this has been termed the 'South Asian phenotype' [364]. Despite a known genetic contribution, the increase in obesity has been largely associated with changes in lifestyle habits [365, 366]. It is imperative that modifiable risk factors for obesity and associated metabolic problems are identified, especially if they can be easily addressed.

Vitamin B12 is a micronutrient that has been identified as a modifiable risk factor associated with the progression of metabolic disorders. In humans, vitamin B12 acts as an essential co-enzyme involved in DNA synthesis and cellular energy production [161]. Subclinical deficiency of vitamin B12 has been linked to higher levels of homocysteine; this may have important consequences in the progression of chronic diseases, by inducing oxidative stress and inflammation [367]. Vitamin B12 deficiency has also been linked to many other complications including an increased risk of obesity [7, 84, 85], diabetes [67, 69, 70] and cardiovascular disease [115]. Currently, one study has investigated the effect of genetically instrumented vitamin B12 concentrations on body mass index (BMI) in individuals with European ancestry; however, there were no associations between the vitamin B12 genetic risk score (GRS) and BMI [16].

Genetic studies have implicated several gene loci in the predisposition to vitamin B12 deficiency, but no study has yet been carried out in the Sri Lankan population [14]. The mechanisms by which obesity and its comorbidities are related to vitamin B12 deficiency are

poorly understood. Hence, we conducted a gene-based approach to explore the relationship between metabolic traits and vitamin B12 status in a Sinhalese cohort and investigated whether these relationships were modified by dietary intake in the Genetics of Obesity and Diabetes (GOOD) study.

## 4.3 Methodology

### 4.3.1 Study Participants

The Genetics of Obesity and Diabetes (GOOD) study is a cross-sectional study that was conducted in the city of Colombo, Sri Lanka, between April to August 2017. Healthy adults between the ages of 25-50 years were enrolled into the study. Exclusion criteria were: having a previous history of type 2 diabetes, cardiovascular disease or hypertension, having a BMI of more than 40 $kg/m^2$ or being classed morbidly obese by a physician, being blood related to other participants in the study, having any communicable disease, being pregnant or lactating, taking dietary or vitamin supplements and taking medications that affect lipid metabolism or hypertension (**Figure 7**).

The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Ethical Review Committee of the University of Colombo (EC-17-107) and the University of Reading Research Ethics Committee (17/25). All participants provided informed written consent before participating.

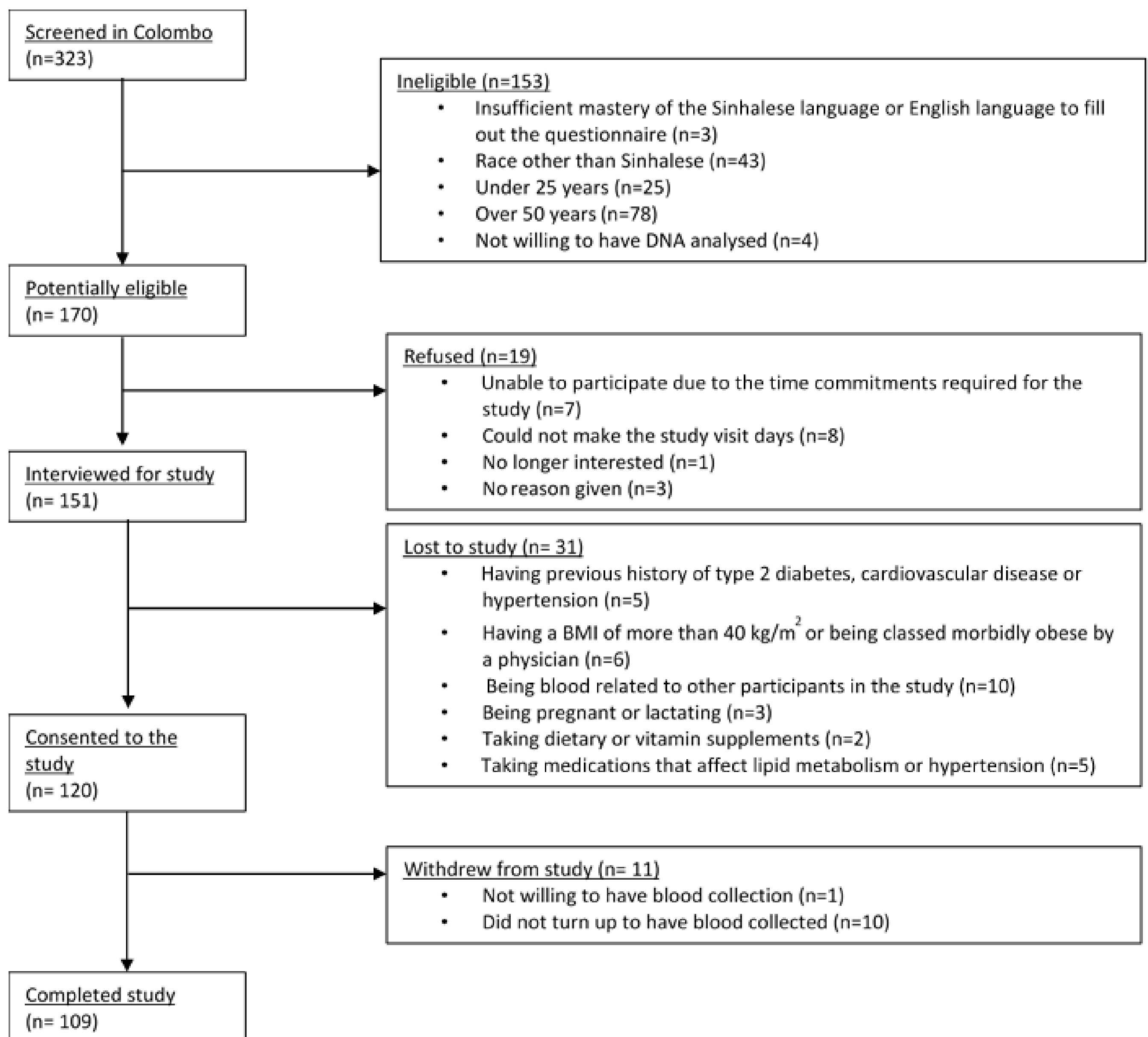


**Figure 7: Flowchart of the subject recruitment process**

### 4.3.2 Anthropometric Measures

Body weight was measured to the nearest 100 grams using an electronic scale (Seca 815, Seca GmbH. Co. kg, Germany) and height was measured to the nearest millimeter using a stadiometer (Seca 217, Seca GmbH. Co. kg, Germany). The BMI calculation was based on the body weight (kg) divided by the square of body height (m). Waist circumference and hip

circumference was measured using a metal tape (Lufkin W606PM®, Parsippany, NJ, USA). Body fat percentage was estimated using a hand-held bio-electrical impedance analysis technique (Omron Body Fat Monitor BF306, Omron, Milton Keynes, United Kingdom).

### 4.3.3 Biochemical Analysis

Blood samples (10 ml) were collected by a trained phlebotomist in the morning, after a 12 hour overnight fast. Fasting serum insulin and vitamin B12 levels were determined using the chemiluminescent microparticle immunoassay method on an Architect i1000 analyser (Abbott Laboratories, IL, USA). Fasting plasma glucose concentrations were measured using the glucose hexokinase method using the Beckman Coulter AU5800 analyser (Beckman Coulter®, California, United States). Glycated haemoglobin (HbA1c) was estimated by high-performance liquid chromatography using the BioRad D10 HPLC analyser (Biorad, Hercules, CA, USA).

### 4.3.4 Dietary intake analysis

Dietary intakes were assessed using a previously validated and published [368] interviewer administered food frequency questionnaire (FFQ) containing 85 food items. In brief, participants were asked to estimate the usual frequency (number of times per day, week or month/never) and the portion sizes of various food items. The recorded data was analysed with the NutriSurvey 2007 database (EBISpro, Germany) to estimate energy as well as macro- and micronutrient consumption[345].

"The Global Physical Activity Questionnaire" (GPAQ), developed by the World Health Organization (WHO) was used to measure physical activity [369]. Individuals were classified as vigorously active, when they both exercised and engaged in demanding work activities, and moderately active, when the participants either exercised or carried out heavy physical work. The remaining study participants were classified into the sedentary group.

### 4.3.5 SNP selection and Genotyping

We selected 10 metabolic disease-related SNPs (associated with obesity and diabetes): Fat mass and obesity-associated [*FTO*]- rs9939609 and rs8050136, Melanocortin 4 Receptor [*MC4R*]- rs17782313 and rs2229616, Transcription factor 7-like 2 [*TCF7L2*]- rs12255372 and rs7903146, Potassium voltage-gated channel subfamily J member 11 [*KCNJ11*]- rs5219, Calpain 10 [*CAPN10*]- rs3792267, rs2975760 and rs5030952) for our analysis based on previously published candidate gene association and genome-wide association (GWA) studies for metabolic disease-related traits [370-378].

The 10 vitamin B12-related SNPs (Methylenetetrahydrofolate reductase [*MTHFR*]- rs1801133, Carbamoyl-phosphate synthase 1 [*CPS1*]- rs1047891, Cubulin [*CUBN*]- rs1801222, CD320 molecule [*CD320*]- rs2336573, Transcobalamin 2 [*TCN2*]- rs1131603, Citrate lyase beta like [*CLYBL*]- rs41281112, Fucosyltransferase 2 [*FUT2*]- rs602662, Transcobalamin 1 [*TCN1*]- rs34324219, Fucosyltransferase 6 [*FUT6*]- rs778805 and Methylmalonyl-CoA mutase [*MUT*]- rs1141321) were chosen on the basis of the recent review article by Surendran et al. [14].

Blood samples for the measurement of DNA were transported in dry ice to the UK. Genomic DNA was extracted from a 5 ml whole blood sample from each participant and genotyping was performed at LGC Genomics (http://www.lgcgroup.com/services/genotyping), which employs the competitive allele-specific PCR-KASP® assay.

The Hardy-Weinberg equilibrium (HWE) *P* values were computed for the following 20 SNPs. The SNPs *FUT2* rs602662 and *CAP10* rs3792267 deviated from HWE; however, these SNPs were not excluded from analysis. The *FUT2* SNP rs602662 previously departed from HWE in a GWA study conducted in India; the authors ruled out that the deviation was not due to a genotyping error and still used this SNP for analysis in their study [233]. In addition, the

KASP$^{TM}$ genotyping technology used in our study has been independently assessed to be over 99.8% accurate. Validation of the KASP$^{TM}$ genotyping was conducted at LGC genomics, where the genotyping results were assessed by two project managers separately to confirm that the data was accurate, and this ruled out genotyping artefacts as possible reasons for deviation from HWE. The reasons for deviation from HWE could be due to population or racial grouping substructure (Sub-grouping), non-random mating, linkage disequilibrium (incomplete mixing of different ancestral population) or chance findings [379].

### 4.3.6 Statistical Analysis

The SPSS statistical package (version 22; SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Allele frequencies were estimated by gene counting (**Table 17).** The normality of variable distribution was verified by the Shapiro-Wilk test, and data not normally distributed were log transformed prior to analysis. We performed an independent t-test to compare the means of the quantitative variables between men and women. Comparison of the means between the two groups was analysed by the Chi Square test for categorical outcomes.

**Table 17: Genotype distribution of vitamin B12 related SNPs and metabolic disease-related SNPs**

| Gene | rs number | Major allele | Minor allele | Common Homozygotes (%) | Heterozygotes (%) | Rare Homozygotes (%) | Minor allele frequency | HWE P value |
|---|---|---|---|---|---|---|---|---|
| *MTHFR* | rs1801133 | C | T | 89 (81.7) | 19 (17.4) | 1 (0.9) | 0.100 | 0.990 |
| *CPS1* | rs1047891 | C | A | 56 (51.9) | 44 (40.7) | 8 (7.4) | 0.278 | 0.873 |
| *CUBN* | rs1801222 | C | T | 78 (72.2) | 29 (26.9) | 1 (0.9) | 0.144 | 0.338 |
| *CD320* | rs2336573 | C | T | 99 (90.8) | 10 (9.2) | 0 (0) | 0.046 | 0.616 |
| *TCN2* | rs1131603 | T | C | 107 (98.2) | 2 (1.8) | 0 (0) | 0.009 | 0.923 |
| *CLYBL* | rs41281112 | C | T | 105 (96.3) | 4 (3.7) | 0 (0) | 0.018 | 0.845 |
| *FUT2* | rs602662 | G | A | 60 (55.6) | 30 (27.8) | 18 (16.7) | 0.306 | **0.000** |
| *TCN1* | rs34324219 | C | A | 107 (98.2) | 2 (1.8) | 0 (0) | 0.009 | 0.923 |
| *FUT6* | rs778805 | C | T | 29 (26.6) | 53 (48.6) | 27 (24.8) | 0.491 | 0.776 |
| *MUT* | rs1141321 | G | A | 28 (25.7) | 60 (55.0) | 21 (19.3) | 0.470 | 0.271 |
| *CAPN10* | rs3792267 | G | A | 79 (72.5) | 24 (22.0) | 6 (5.5) | 0.165 | **0.035** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *CAPN10* | rs2975760 | T | C | 66 (60.6) | 38 (34.9) | 5 (4.6) | 0.220 | 0.874 |
| *CAPN10* | rs5030952 | C | T | 101 (92.7) | 8 (7.3) | 0 (0) | 0.037 | 0.691 |
| *KCNJ11* | rs5219 | C | T | 49 (45.0) | 45 (41.3) | 15 (13.8) | 0.344 | 0.373 |
| *TCF7L2* | rs12255372 | G | T | 57 (52.3) | 45 (41.3) | 7 (6.4) | 0.271 | 0.633 |
| *TCF7L2* | rs7903146 | C | T | 45 (41.3) | 54 (49.5) | 10 (9.2) | 0.340 | 0.274 |
| *FTO* | rs9939609 | T | A | 48 (44.0) | 47 (43.1) | 14 (12.8) | 0.344 | 0.641 |
| *MCR* | rs17782313 | T | C | 48 (44.0) | 50 (45.9) | 11 (10.1) | 0.330 | 0.700 |
| *FTO* | rs8050136 | C | A | 48 (44.0) | 47 (43.1) | 14 (12.8) | 0.340 | 0.641 |
| *MC4R* | rs2229616 | G | A | 99 (91.7) | 9 (8.3) | 0 (0) | 0.042 | 0.651 |

*MAF; minor allele frequency, HWE; Hardy Weinberg Equilibrium,* $X^2$*; Chi-Squared value*

A schematic representation of the study design is presented in **figure 8**. The unweighted, risk-allele GRS method was calculated for each participant as the sum of risk allele counts across each SNP, which predicted vitamin B12 status or metabolic disease risk. The B12-GRS was generated from the SNPs in the genes *MTHFR, CPS1, CUBN, CD320, TCN2, CLYBL, FUT2, TCN1, FUT6, MUT*, which have been shown to be associated with vitamin B12 concentrations. Furthermore, another unweighted GRS was created using allele markers previously reported to be associated with metabolic disease traits. The metabolic-GRS was generated from the SNPs in the genes *CAP10*, *KCNJ11*, *TCF7L2*, *FTO* and *MC4R*. A value of 0,1 or 2 was assigned to each SNP, which denotes the number of risk alleles on that SNP. These values were then calculated by adding the number of risk alleles across each SNP. The average number of risk alleles per person for the B12-GRS was 8.69 (SD = 1.70), which ranged from 5 to 15. The sample was stratified, by the median, into a "low genetic risk group," for those with a GRS ≤ 9 risk alleles (n = 79), and into a "high genetic risk group," for those with a GRS ≥ 10 risk alleles (n = 30). For the metabolic-GRS, the average number of risk alleles per person was 7.00 (SD = 2.28), which ranged from 1 to 13. The sample was stratified, into a "low genetic risk group," for those with a GRS ≤ 8 risk alleles (n = 88), and into a "high genetic risk group," for those with a GRS ≥ 9 risk alleles (n = 21). Linear regression was used to examine the association of the two GRS scores with the biochemical and anthropometric outcomes (glucose, insulin, HbAC1, vitamin B12, body fat %, BMI, WC and WHR). The interaction between the two GRS scores and dietary factors on biochemical and anthropometric outcomes was determined by including interaction terms (GRS*diet) in the regression model. Models were adjusted for age, sex, BMI, and total energy intake, wherever appropriate.

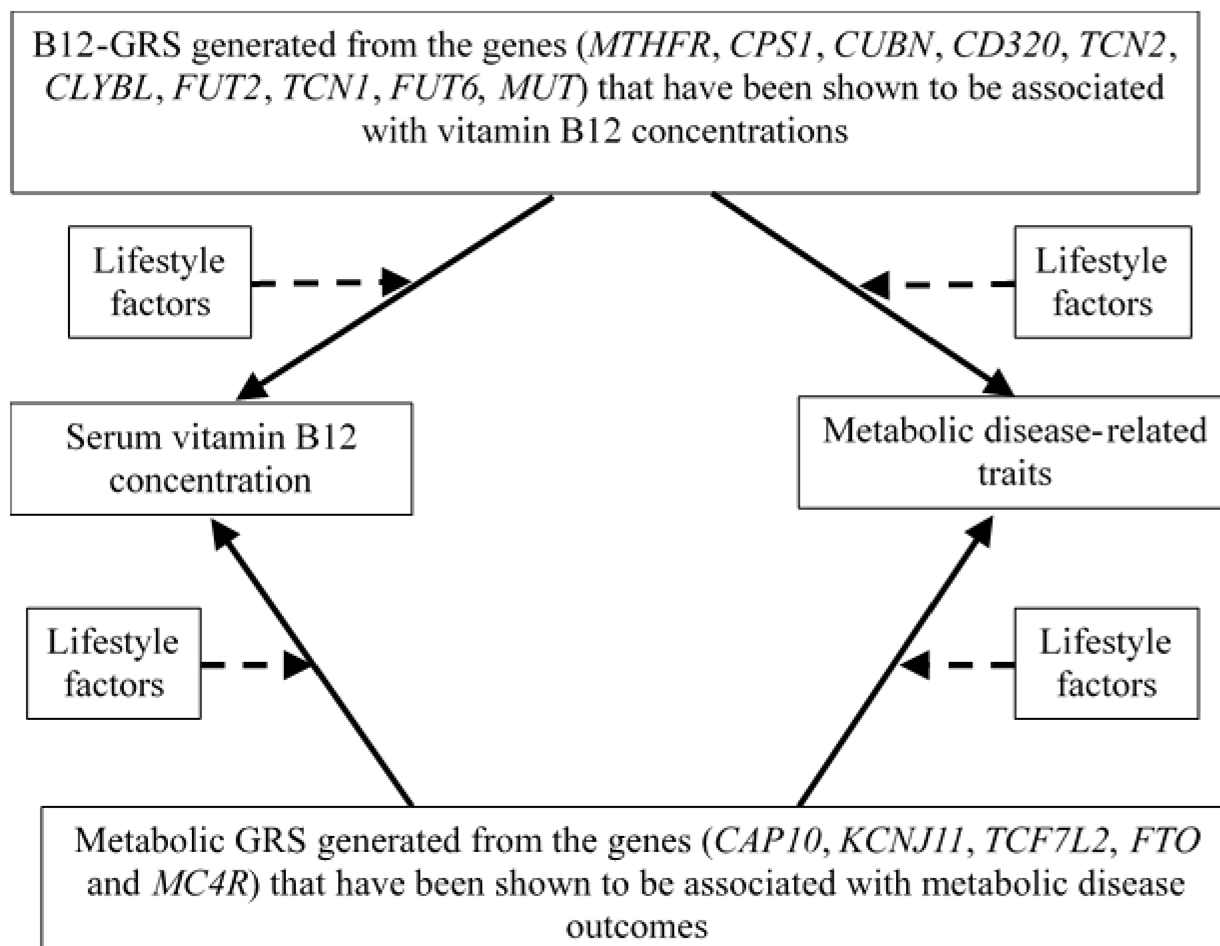


**Figure 8: Diagram representing the study design**

*The diagram shows four possible associations, and four possible interactions. One-sided arrows with unbroken lines represent genetic associations and one-sided arrows with broken lines represent interactions between a lifestyle factor and GRS on serum vitamin B12/ metabolic traits. We tested the association between the metabolic-GRS and vitamin B12 concentrations and metabolic disease-related traits. We then tested the associations between the B12 –GRS and vitamin B12 status and metabolic disease related traits. Lastly, we tested whether these genetic associations were modified by lifestyle factors (macronutrient intake and physical activity levels).*

Correction for multiple testing was applied using Bonferroni correction [adjustment P value for association analysis was <0.00313 [2 GRS * 8 biochemical and anthropometric outcomes (Fasting blood glucose, fasting insulin, glycated haemoglobin, vitamin B12, Fat %, BMI, WC and WHR)=16 test)] and for interaction < 0.00078 [2 GRS * 8 biochemical and anthropometric * 4 lifestyle factors (dietary carbohydrate energy %, dietary protein energy %, dietary fat energy % and physical activity levels))= 64]. Given that there are no studies on GRS and no previously reported effect sizes for the South Asians, we were unable to perform a power calculation.

## 4.4 Results

### 4.4.1 Characteristics of the participants

In this study, 109 participants (mean age, 38.34 ± 6.92 years; BMI, 24.58 ± 4.12 kg/m$^2$) were included. **Table 18** illustrates the main characteristics of the study participants stratified according to sex. No significant difference between men and women were observed in the levels of fasting glucose, insulin, HbAC1 and plasma vitamin B12 (P>0.05).

**Table 18: Anthropometric and biochemical characteristics of men and women participants (n=109, Men 61: women 48)**

| | **Total (n=109)** | **Men (n=61)** | **Women (n=48)** | **P value*** |
|---|---|---|---|---|
| Age (yrs) | 38.24 ± 6.92 | 37.34 ± 6.97 | 39.38 ± 6.77 | 0.129 |
| Height (cm) | 164.97 ± 9.15 | 170.95 ± 6.18 | 157.36 ± 6.16 | **<0.0001** |
| Weight (kg) | 67.07 ± 13.05 | 71.76 ± 11.81 | 61.11 ± 12.17 | **<0.0001** |
| BMI (kg/m$^2$) | 24.58 ± 4.12 | 24.51 ± 3.52 | 24.68 ± 4.80 | 0.844 |
| Waist circumference (cm) | 83.73 ± 17.97 | 89.83 ± 14.04 | 75.99 ± 19.52 | **<0.0001** |
| Hip circumference (cm) | 91.16 ± 17.78 | 92.27 ± 13.83 | 89.75 ± 21.87 | 0.488 |
| WHR | 0.92 ± 0.11 | 0.98 ± 0.08 | 0.85 ± 0.11 | **<0.0001** |
| Fat (%) | 27.25 ± 7.37 | 23.52 ± 5.12 | 32.00 ± 7.08 | **<0.0001** |
| Obesity cases** | 40.37% | 37.70% | 43.75% | 0.523 |
| Fasting Blood Glucose (mg/dL) | 85.64 ± 12.64 | 87.41 ± 15.41 | 83.40 ± 7.40 | 0.100 |
| Fasting Blood Insulin (pmol/L) | 68.55 ± 49.97 | 71.77 ± 59.12 | 64.46 ± 35.28 | 0.451 |
| Fasting Blood HbA1C (mmol/mol) | 35.62 ± 5.91 | 35.20 ± 5.99 | 36.16 ± 5.84 | 0.402 |
| Fasting Blood B12 (pmol/L) | 380.65 ± 132.83 | 389.80 ± 135.00 | 369.02 ± 130.52 | 0.420 |
| Physical Activity Levels (Low %/ moderate %/ high %) | 72.5/ 19.3/ 8.3 | 70.5/19.7/9.8 | 75.0/18.8/6.3 | 0.777 |

| | | | | |
|---|---|---|---|---|
| Total energy (kcal/d) | 2097.92 ± 456.01 | 2173.68 ± 427.82 | 2001.65 ± 476.72 | **0.050** |
| Protein (energy %) | 11.29 ± 2.31 | 11.25 ± 2.41 | 11.33 ± 2.20 | 0.853 |
| Fat (energy %) | 21.87 ± 5.31 | 21.64 ± 5.22 | 22.16 ± 5.45 | 0.613 |
| Carbohydrate (energy %) | 69.62 ± 8.80 | 69.89 ± 10.29 | 69.28 ± 6.52 | 0.721 |
| Dietary fibre (g) | 16.78 ± 8.18 | 17.24 ± 8.46 | 16.20 ± 7.85 | 0.513 |
| Polyunsaturated fatty acids (g) | 3.32 ± 1.69 | 3.36 ± 1.66 | 3.27 ± 1.75 | 0.779 |

*Abbreviations: BMI Body mass index; SD indicates standard deviations; WHR, waist to hip ratio*

*Data presented as Mean ± SD*

** P < 0.05, statistically significant differences in mean values between men/women, unadjusted*

***Obesity cases refers to the percentage of individuals with a BMI of over 25.*

### 4.4.2 Association between B12-GRS and Obesity GRS with biochemical and anthropometric measurements

A significant association between B12-GRS and serum vitamin B12 was observed ($P = 0.008$) (**Table 19 and Figure 9**); However, this finding was not significant after correction for multiple testing. No associations between the B12-GRS and metabolic traits ($P > 0.05$) were observed (**Table 19**). Furthermore, no associations between the metabolic-GRS and vitamin B12 or metabolic traits ($P>0.05$) were observed (**Table 20**).

**Table 19: Association between the B12-GRS with obesity traits, biochemical traits and anthropometric measurements**

*Values are given as mean ± standard deviation.*

| SNPs | Log Glucose (mg/dL) | Log Insulin (pmol/L) | Log HbA1C (mmol/mol) | Vitamin B12 (pmol/L) | Fat (%) | Log BMI | Log WC | WHR |
|---|---|---|---|---|---|---|---|---|
| ≤ 9 risk alleles | 1.931 ± 0.046 | 1.773 ± 0.261 | 1.545 ± 0.070 | 402.101 ± 129.265 | 27.098 ± 7.757 | 1.388 ± 0.074 | 1.912 ± 0.123 | 0.921 ± 0.116 |
| ≥ 10 risk alleles | 1.923 ± 0.068 | 1.722 ± 0.201 | 1.549 ± 0.068 | 324.167 ± 127.344 | 27.651 ± 6.331 | 1.377 ± 0.065 | 1.900 ± 0.131 | 0.919 ± 0.111 |
| P value | 0.782 | 0.553 | 0.652 | **0.008** | 0.576 | 0.515 † | 0.785 | 0.525 |

*P values for differences between ≤9 and ≥10 risk alleles were obtained using linear regression model adjusted for age, sex and BMI.*

*† P values were obtained by using a general linear model adjusted for age and sex*

*Abbreviations: HbAC1 glycated haemoglobin; BMI body mass index; WC waist circumference; WHR waist to hip ratio*

**Table 20: Association between the metabolic-GRS and obesity traits, biochemical traits and anthropometric measurements**

*Values are given as mean ± standard deviation.*

| SNPs | Log Glucose (mg/dL) | Log Insulin (pmol/L) | Log HbA1C (mmol/mol) | Vitamin B12 (pmol/L) | Fat (%) | Log BMI | WC | WHR |
|---|---|---|---|---|---|---|---|---|
| ≤ 8 risk alleles | 1.929 ± 0.056 | 1.760 ± 0.243 | 1.545 ± 0.070 | 382.227 ± 139.729 | 26.651 ± 7.375 | 1.384 ± 0.072 | 1.912 ± 0.123 | 0.928 ± 0.119 |
| ≥ 9 risk alleles | 1.930 ± 0.035 | 1.753 ± 0.267 | 1.551 ± 0.070 | 374.048 ± 101.471 | 29.761 ± 6.949 | 1.387 ± 0.069 | 1.893 ± 0.136 | 0.887 ± 0.087 |
| P value | 0.550 | 0.777 | 0.772 | 0.962 | 0.247 | 0.732 † | 0.722 | 0.796 |

*P values for differences between ≤8 and ≥9 risk alleles were obtained using linear regression model adjusted for age, sex and BMI.*

*† P values were obtained by using a general linear model adjusted for age and sex*

*Abbreviations: HbAC1 glycated haemoglobin; BMI body mass index; WC waist circumference; WHR waist to hip ratio*

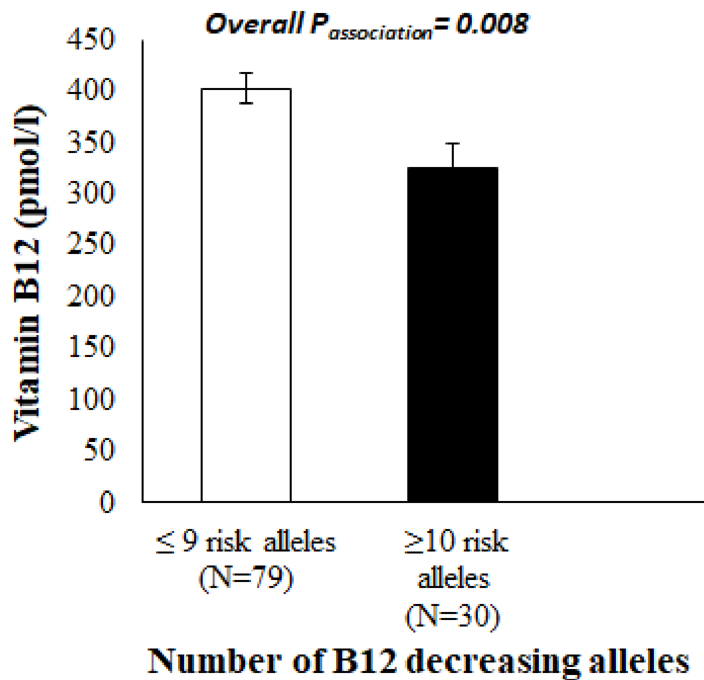


**Figure 9:** ***Association between the B12-GRS and serum vitamin B12 levels***

*Vitamin B12 decreasing alleles ranged from 5 to 15. Individuals with ≤9 or ≥10 alleles were grouped to obtain a reasonable number of individuals in each group. Error bars indicate Standard error.*

### 4.4.3 Interaction between the B12-GRS and dietary factors on biochemical and anthropometric measurements

An interaction was found between the B12-GRS and protein energy (%) on log transformed WC (P=0.002). However, further stratification of participants based on their consumption of low, medium and high dietary protein (energy %) did not show statistically significant associations between the GRS and the outcome in any of the tertiles, which could account for the small sample size (**Table 21**).

**Table 21: Interaction between the B12-GRS and lifestyle factors on anthropometric measurements**

| ***Interaction between the GRS and lifestyle factors on Log waist circumference (cm)*** | | | |
|---|---|---|---|
| Interaction between B12-GRS * fat energy % | Interaction between B12-GRS * protein energy % | Interaction between B12-GRS * carbohydrate energy % | Interaction between B12-GRS * Physical activity levels |
| 0.002 ± 0.004<br>(0.727) | 0.037 ± 0.011<br>**(0.002)** | -0.003 ± 0.003<br>(0.344) | -0.051 + 0.037<br>(0.173) |
| Interaction between metabolic-GRS * fat energy % | Interaction between metabolic-GRS * protein energy % | Interaction between metabolic-GRS * carbohydrate energy % | Interaction between metabolic-GRS * Physical activity levels |
| -0.007 ± 0.006<br>(0.212) | -0.024 ± 0.009<br>**(0.011)** | 0.007 ± 0.003<br>(**0.031**) | 0.020 ± 0.044<br>(0.654) |
| ***Interaction between the GRS and dietary factors on waist to hip ratio*** | | | |
| Interaction between B12-GRS * fat energy % | Interaction between B12-GRS * protein energy % | Interaction between B12-GRS * carbohydrate energy % | Interaction between-B12 GRS * Physical activity levels |
| 0.002 ± 0.004<br>(0.660) | 0.013 ± 0.010<br>(0.196) | -0.003 ± 0.002<br>(0.241) | 0.018 ± 0.032<br>(0.584) |
| Interaction between metabolic-GRS * fat energy % | Interaction between metabolic-GRS * protein energy % | Interaction between metabolic-GRS * carbohydrate energy % | Interaction between metabolic-GRS * Physical activity levels |
| -0.009 ± 0.005<br>(0.079) | -0.012 ± 0.008<br>(0.158) | 0.007 ± 0.003<br>(**0.015**) | 0.038 ± 0.039<br>(0.323) |
| ***Interaction between the GRS and lifestyle factors on Log BMI*** | | | |

| Interaction between B12-GRS * fat energy % | Interaction between B12-GRS * protein energy % | Interaction between B12-GRS * carbohydrate energy % | Interaction between B12-GRS * Physical activity levels |
|---|---|---|---|
| -0.002 ± 0.003<br>(0.539†) | 0.009 ± 0.008<br>(0.259†) | -0.001 ± 0.002<br>(0.762†) | 0.015 ± 0.023<br>(0.513†) |
| **Interaction between metabolic-GRS * fat energy %** | **Interaction between metabolic-GRS * protein energy %** | **Interaction between metabolic-GRS * carbohydrate energy %** | **Interaction between metabolic-GRS * Physical activity levels** |
| -0.004 ± 0.004<br>(0.245†) | -0.004 ± 0.006<br>(0.480†) | 0.002 ± 0.002<br>(0.322†) | -0.005 ± 0.028<br>(0.851†) |

*Values are beta coefficients ± standard errors. P values are inserted in brackets*

*P values were obtained by using a general linear model adjusted for age, sex and BMI*

† *P values were obtained by using a general linear model adjusted for age and sex*

### 4.4.4 Interaction between the metabolic-GRS and dietary factors on biochemical and anthropometric measurements

We observed a significant interaction between the metabolic-GRS and carbohydrate energy intake (%) on waist to hip ratio ($P_{interaction}$ = 0.015) (**Figure 10 and Table 21**). Individuals who carried 8 or less risk alleles for metabolic disease had 7.47 % lower WHR measurements (cm) in the highest tertile of carbohydrate energy intake (%) (Mean ± S.D: 78.00 ± 7.90%) compared to those with 9 or more risk alleles (P= 0.035) (**Table 21**).

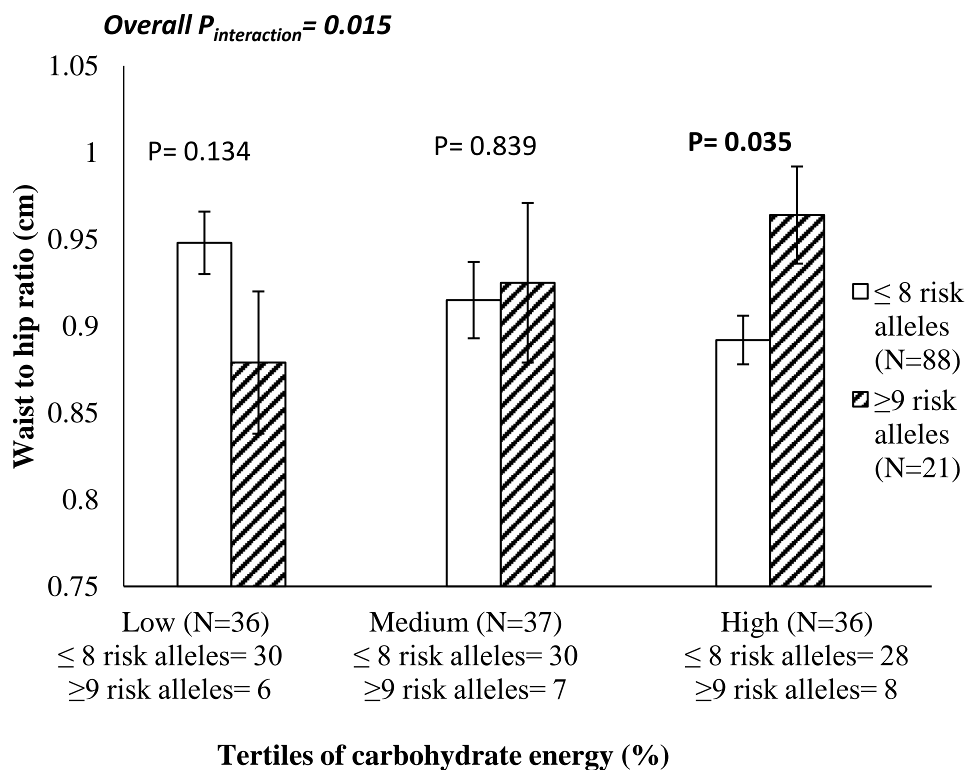


**Figure 10: Interaction between the metabolic-GRS and carbohydrate energy intake (%) on waist-to-hip ratio (cm) ($P_{interaction}$ = 0.015).**

*Among those who consumed a high carbohydrate diet, individuals who carried 9 or more risk alleles had significantly higher levels of waist-to-hip ratios compared to individuals carrying 8 or less risk alleles (P = 0.035). Error bars indicate Standard error.*

Interactions were also seen between the metabolic-GRS and carbohydrate energy (%) on log fasting insulin concentrations (P=0.011) and log WC (P=0.031), and the metabolic-GRS and protein energy (%) on log fasting insulin levels and (P= 0.032) and log WC (P=0.011) (**Table 21 and Table 22**).

**Table 22: Interaction between the B12-GRS and metabolic-GRS and lifestyle factors on biochemical outcomes**

| ***Interaction between the GRS and lifestyle factors on Log glucose (mg/dL)*** | | | |
|---|---|---|---|
| Interaction between B12-GRS * fat energy % | Interaction between B12-GRS * protein energy % | Interaction between B12-GRS * carbohydrate energy % | Interaction between the B12-GRS * Physical activity levels |
| 0.002 ± 0.002 (0.416) | -0.004 ± 0.005 (0.437) | -0.000 ± 0.001 (0.866) | 0.035 ± 0.016 (0.053) |
| Interaction between metabolic-GRS * fat energy % | Interaction between metabolic-GRS * protein energy % | Interaction between metabolic-GRS * carbohydrate energy % | Interaction between metabolic-GRS * Physical activity levels |
| 0.001 ± 0.003 (0.609) | 0.004 ± 0.004 (0.295) | -0.002 ± 0.002 (0.258) | -0.013 ± 0.020 (0.518) |
| ***Interaction between the GRS and lifestyle factors on Log insulin (pmol/L)*** | | | |
| Interaction between B12-GRS * fat energy % | Interaction between B12-GRS * protein energy % | Interaction between B12-GRS * carbohydrate energy % | Interaction between B12-GRS * Physical activity levels |
| -0.005 ± 0.008 (0.545) | -0.009 ± 0.022 (0.681) | 0.003 ± 0.005 (0.591) | -0.035 ± 0.067 (0.600) |
| Interaction between metabolic-GRS * fat energy % | Interaction between metabolic-GRS * protein energy % | Interaction between metabolic-GRS * carbohydrate energy % | Interaction between metabolic-GRS * Physical activity levels |
| 0.018 ± 0.010 | 0.037 ± 0.017 | -0.016 ± 0.006 | -0.076 ± 0.080 |

| | | | |
|---|---|---|---|
| (0.076) | **(0.032)** | **(0.011)** | (0.345) |
| ***Interaction between the GRS and lifestyle factors on Log HbA1C (pmol/L)*** | | | |
| Interaction between B12-GRS * fat energy % | Interaction between B12-GRS * protein energy % | Interaction between B12-GRS * carbohydrate energy % | Interaction between B12-GRS * Physical activity levels |
| -0.000 ± 0.003<br>(0.954) | -0.007 ± 0.007<br>(0.299) | 0.001 ± 0.002<br>(0.711) | 0.019 ± 0.022<br>(0.387) |
| Interaction between metabolic-GRS * fat energy % | Interaction between metabolic-GRS * protein energy % | Interaction between metabolic-GRS * carbohydrate energy % | Interaction between metabolic-GRS * Physical activity levels |
| 0.001 ± 0.003<br>(0.802) | 0.001 ± 0.006<br>(0.810) | -0.001 ± 0.002<br>(0.543) | -0.041 ± 0.026<br>(0.116) |
| ***Interaction between the GRS and lifestyle factors on vitamin B12 (pmol/L)*** | | | |
| Interaction between B12-GRS * fat energy % | Interaction between B12-GRS * protein energy % | Interaction between B12-GRS * carbohydrate energy % | Interaction between B12-GRS * Physical activity levels |
| -2.377 ± 5.093<br>(0.642) | 13.481 ± 13.969<br>(0.337) | -0.722 ± 3.337<br>(0.829) | -46.714 ± 43.442<br>(0.285) |
| Interaction between metabolic-GRS * fat energy % | Interaction between metabolic-GRS * protein energy % | Interaction between metabolic-GRS * carbohydrate energy % | Interaction between metabolic-GRS * Physical activity levels |
| -6.189 ± 6.856<br>(0.369) | 7.017 ± 11.531<br>(0.544) | -0.806 ± 4.181<br>(0.848) | 36.492 ± 53.889<br>(0.500) |
| ***Interaction between the GRS and lifestyle factors on body fat (%)*** | | | |
| Interaction between B12-GRS * fat energy % | Interaction between B12-GRS * protein energy % | Interaction between B12-GRS * carbohydrate energy % | Interaction between the B12-GRS * Physical activity levels |
| 0.031 ± 0.124 | -0.326 ± 0.342 | 0.049 ± 0.082 | -0.548 ± 1.068 |

| (0.804) | (0.343) | (0.550) | (0.609) |
|---|---|---|---|
| Interaction between metabolic-GRS * fat energy % | Interaction between metabolic-GRS * protein energy % | Interaction between metabolic-GRS * carbohydrate energy % | Interaction between metabolic-GRS * Physical activity levels |
| 0.084 ± 0.160<br>(0.601) | 0.060 ± 0.273<br>(0.826) | -0.037 ± 0.099<br>(0.709) | 0.637 ± 1.270<br>(0.617) |

*Values are beta coefficients ± standard errors. P values were inserted in brackets*

*P values were obtained by using a general linear model adjusted for age, sex and BMI*

*Abbreviations: HbAC1 glycated haemoglobin; BMI body mass index*

#### 4.4.5 Interaction between the B12-GRS and physical activity on biochemical and anthropometric measurements

No statistically significant interactions were observed between the two GRSs (vitamin B12 and metabolic) and physical activity on biochemical and anthropometric measurements **(Table 21 and Table 22).** After correction for multiple testing, none of these gene-diet and gene-physical activity interactions remained statistically significant.

### 4.5 Discussion:

To our knowledge, this is the first study to use a genetic approach to explore the relationship between metabolic traits and vitamin B12 status in a South Asian population. Our study confirmed the strength of the association between B12-GRS and B12 concentrations and demonstrated the impact of genetically instrumented B12 concentrations on waist circumference, an indicator of central obesity, through the influence of dietary protein intake. Furthermore, our study has also showed a significant effect of metabolic-GRS on waist to hip ratio through the influence of high carbohydrate intake. Given that the total daily intake of

protein is low and carbohydrate is high in Sri Lankan adults [380], our findings, if replicated in future studies, might carry significant public health implications in terms of revising the food-based dietary guidelines which could prevent central obesity and the associated CVD-related outcomes.

In this study, we constructed a GRS consisting of ten vitamin B12 decreasing SNPs in genes involved in vitamin B12 metabolism [14]. The B12-GRS was associated with vitamin B12 levels, suggesting that it would be an ideal instrument for vitamin B12 status. Given the lack of association between the B12-GRS and metabolic disease traits in our study, we were unable to provide evidence for linear decreases in vitamin B12 concentrations having substantive effects on metabolic disease traits. However, we found a significant interaction between the B12-GRS and protein energy (%) on log WC. Interestingly, individuals who carried 9 or less alleles had lower WC when consuming a high protein diet compared to those consuming a low protein diet. Although no statistically significant differences in WC were observed between the alleles of the B12-GRS, the impact of the B12-GRS on WC was observed only under the influence of a high protein diet. Further investigations are required to confirm this finding to determine the clinical significance and potential applications as part of weight management interventions.

At present, carbohydrates constitute the majority of the energy intake among South Asian countries such as Sri Lanka (~71.2%) [380]; in contrast, the consumption of carbohydrates is lower in Western countries (~45 %) [381]. Furthermore, high carbohydrate intake has been associated with an increased risk of diabetes in a South Indian population [382], and an increase in WC among pre-menopausal (20-45 years) Sri Lankan women [383]. In the present study, we found a significant interaction between the metabolic-GRS and carbohydrate energy percentage on waist-to-hip ratio, where the individuals carrying more than 9 risk alleles had a higher waist-to-hip ratio among those in the highest tertile of carbohydrate energy percentage.

There are no previous reports of the risk variants used in our GRS, but Goni et al [384] found that carbohydrates (total and complex) interacted with a GRS of 16 obesity/lipid metabolism polymorphisms to modify the effect on body fat mass in 711 individuals of Caucasian ancestry. In our study, we only observed interactions of the metabolic-GRS on WC and waist-to-hip ratio, which suggests that effects are likely to be on central obesity as opposed to common obesity.

South Asians have a higher risk of developing obesity related non-communicable diseases relative to white Caucasians despite lower BMI levels; this has been termed the 'South Asian phenotype'. The distinctive features of this phenotype include a higher WC, abdominal adiposity combined with insulin resistance, and a greater predisposition to diabetes [364]. The role of vitamin B12 in promoting this adverse phenotype has been suggested by Yajnik et al., who demonstrated that offspring born to mothers with a low vitamin B12 and high folate status had a greater risk of developing insulin resistance during childhood [69]. According to Yajnik et al., vitamin B12 deficiency prevents the generation of tetrahydrofolate from 5-methyltetrahydrofolate in the one-carbon metabolism cycle; as a result, homocysteine levels accumulate leading to altered lean tissue deposition and reduced protein synthesis [69]. Furthermore, vitamin B12 is involved in the conversion of methylmalonyl-CoA to succinyl-CoA by the enzyme methylmalonyl-CoA mutase (adenosyl-B12 as a cofactor). Subsequently, vitamin B12 deficiency results in elevated methylmalonyl-CoA, inhibiting the mitochondrial enzyme carnitine palmitoyltransferase, which may promote lipogenesis and insulin resistance [69, 385].

No studies to date, have investigated interactions between the two GRSs and physical activity on metabolic traits and B12 concentrations in Asian Sri Lankans. Although, 60% of Sri Lankan adults are reported to be highly physically active [386], no significant interactions were found between the two GRSs and physical activity on metabolic traits, which could be

due to a small sample size and measurement bias associated with self-reported physical activity questionnaire. The strengths of our study include the use of a validated food frequency questionnaire [368] to measure macronutrient intake, the comprehensive measurements of lifestyle factors, and the use of GRSs which increased the statistical power of our study [387]. Nevertheless, some limitations need to be acknowledged. The first limitation concerns the relatively small sample size of the study; however, we were still able to identify significant gene-diet interactions. Furthermore, we used Bonferroni correction to correct for multiple testing and this can often lead to larger power, specifically where studies have a small sample size and a small number of disease-associated markers. This is also true for when studies have a large allele frequency difference due to a small sample size [388]. Secondly, information about the type of oil used for frying, the estimation of different dietary fat components (monounsaturated or saturated fatty acids) and vitamin B12 intake was not collected. This could have limited our in-depth analysis of interactions of specific macronutrients and vitamins with the two GRSs. Furthermore, the study was limited to Sinhalese adults in Colombo, and the conclusions may not be applicable to other ethnic groups in Sri Lanka. Finally, none of the genetic associations or gene-lifestyle interactions were statistically significant after correction for multiple testing; however, given that this is the first study using a genetic approach to establish a relationship between vitamin B12 status and metabolic disease outcomes in South Asians, we have taken into consideration of the significant findings; hence, further large studies are required to replicate our findings.

## 4.6 Conclusion

In summary, our study suggests that a genetically lowered vitamin B12 concentration may have an impact on central obesity in the presence of a dietary influence; however, our study failed to show an impact of the metabolic-GRS on lowering B12 concentrations through a dietary influence. Our study also showed a significant effect of the metabolic-GRS on waist to

hip ratio, another indicator of central obesity, through the influence of a high carbohydrate intake. However, after correction for multiple testing, none of these findings were statistically significant. Hence, further replication studies are highly warranted on large samples to confirm or refute our findings.

**Author contributions:**

**Shelini Surendran obtained ethical approval to conduct this study, was responsible for the study conception, collected the data, performed statistical analysis and wrote the manuscript**. SS, VKS, DJA and RL were responsible for the study conception. KW provided guidance to the research; SS and RL conducted data and sample collection; SS, RJ and SA were involved in the dietary data analysis; SS and SaS were involved in the physical activity data analysis; KSV designed the gene-diet interaction study and was involved in drafting the manuscript; SS, VKS, JAL and RJ critically reviewed the manuscript; All authors contributed to and approved the final version of the manuscript.

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# Chapter 5

## Evidence for the association between *FTO* gene variants and vitamin B12 concentrations in an Asian Indian population

For this study, I developed an analysis plan before I undertook the statistical analysis. I screened and validated the dataset to perform statistical analysis. I performed the entire statistical analysis using the SPSS software; I undertook a literature review as part of the introduction to the study and wrote the manuscript. I revised the manuscript based on the comments from all the co-authors before the manuscript was submitted to the Journal of Genes and Nutrition.

**Published (The published paper is attached as an appendix at the end of the thesis)**

Shelini Surendran, Ramamoorthy Jayashri, Lauren Drysdale, Dhanasekaran Bodhini, Nagarajan Lakshmipriya, Coimbatore Subramaniyam Shanthirani, Vasudevan Sudha, Julie A. Lovegrove, Ranjit M. Anjana, Viswanathan Mohan, Venkatesan Radha, Rajendra Pradeepa, Karani S. Vimaleswaran (2019). Evidence for the association between *FTO* gene variants and vitamin B12 concentrations in an Asian Indian population. Genes & Nutrition (**Published**).

### 5.1 Abstract

**Background:** Low vitamin B12 concentrations have been associated with major clinical outcomes, including adiposity, in Indian populations. The Fat mass and obesity associated gene (*FTO*) is an established obesity-susceptibility locus; however, it remains unknown whether it influences vitamin B12 status. Hence, we investigated the association of two previously studied

*FTO* polymorphisms with vitamin B12 concentrations and metabolic disease-related outcomes and examined whether these associations were modified by dietary factors and physical activity.

**Methods:** A total of 176 individuals with type 2 diabetes, 152 with pre-diabetes and 220 normal glucose-tolerant individuals were randomly selected from the Chennai Urban Rural Epidemiology Study. Anthropometric, clinical and biochemical investigations, which included body mass index (BMI), waist circumference, vitamin B12, homocysteine and folic acid were measured. A validated food frequency questionnaire was used for dietary assessment and self-reported physical activity measures were collected. An unweighted genetic risk score (GRS) was calculated for two *FTO* single nucleotide polymorphisms (rs8050136 and rs2388405) by summation of the number of risk alleles for obesity. Interaction analyses were performed by including the interaction terms in the regression model.

**Results:** The GRS was significantly associated with increased BMI (P=0.009) and risk of obesity (P=0.023). Individuals carrying more than one risk allele for the GRS had 13.13% lower vitamin B12 concentrations, compared to individuals carrying zero risk alleles (P=0.018). No associations between the GRS and folic acid and homocysteine concentrations were observed. Furthermore, no statistically significant GRS-diet or GRS-physical activity interactions with vitamin B12, folic acid, homocysteine or metabolic-disease outcomes were observed.

**Conclusion:** The study shows for the first time that a genetic risk score using two *FTO* SNP's is associated with lower vitamin B12 concentrations; however, we did not identify any evidence for the influence of lifestyle factors on this association. Further replication studies in larger cohorts are warranted to investigate the association between the GRS and vitamin B12 concentrations.

## 5.2 Introduction

Obesity and its related comorbidities are leading causes of mortality and morbidity worldwide [389]. It is estimated that >12% of the Indian population is either overweight or obese [390]. Epidemiological studies have documented that the increased accessibility of low-cost, high-calorie and nutrient-poor foods, were among the major driving forces for the epidemic of obesity [391-393]. This has led to a substantial increase in the prevalence of obesity-associated metabolic problems, such as type 2 diabetes mellitus (T2DM), dyslipidaemia and hypertension in India [394]. Furthermore, several studies have also demonstrated that obesity is associated with substantial nutrient deficiencies, including vitamin B12 [6, 395, 396].

Vitamin B12 deficiency is a major public health problem in India and a recent cross-sectional study conducted in 630 healthy adults in a South Indian population, reported that 35% of adults were vitamin B12 deficient [397]. An adequate vitamin B12 concentration is essential for growth, development and health. In addition, it is essential for DNA synthesis, haematological development and maintenance of the myelin nerve sheaths [398-400]. The primary causes of vitamin B12 deficiency are age, consumption of vegetarian diets and the inability to absorb vitamin B12 from food (via genetic defects or disease) [14, 190]. To date, several studies have indicated that vitamin B12 status may be influenced by excess body weight [7, 401]. However a pooled analysis of 19 studies found no evidence for an inverse relationship between vitamin B12 and BMI levels and reported that the majority of observational studies had a high risk of bias and heterogeneity [93]. In the light of these findings, using a genetic approach to explain the genetic mechanisms for obesity and its link with vitamin B12 concentrations could be a better option, in terms of reducing any influence from unmeasured confounding factors.

Genome-wide association studies have identified several genetic variants related to obesity and type 2 diabetes risk [402, 403].To date, the Fat mass and obesity associated (*FTO*)

gene has been identified as the strongest common genetic predictor of obesity [11]. Individuals, who are homozygous for *FTO* risk alleles, are on average, at 1.67-fold increased odds of obesity and three kg heavier in comparison to individuals without any risk alleles [404]. While several studies have reported the association between the *FTO* gene on measures of body weight and composition, various dietary parameters and physical activity levels have also been shown to contribute [405-407]. Recently, a cross-sectional study in an Indian population showed that physical activity and dietary intake may modify the association between the *FTO* gene variants and obesity-related traits [408].We used *FTO* gene variants as instruments to establish the relationship between obesity and B12 status and tested whether this relationship was modified by lifestyle factors. The two main objectives of this study were firstly to determine whether the *FTO* single nucleotide polymorphisms (SNPs), rs8050136 and rs2388405, were associated with obesity traits, vitamin B12, folic acid, and homocysteine and secondly whether these associations were modified by diet and physical activity levels in Asian Indians.

## 5.3 Methodology

### 5.3.1 Study population

A total of 900 unrelated study subjects were randomly recruited from the Chennai Urban Rural Epidemiology Study (CURES) follow-up study, which is an epidemiological study conducted on a representative population of Chennai, (formerly Madras) in southern India. The methodology of the study is published elsewhere [409, 410] and is briefly outlined here (**Figure 11**). In Phase 1 of CURES, 26,001 (aged ≥20 years) individuals were recruited based on a systematic random sampling technique. In the baseline survey, of the 26,001 individuals screened, all the individuals with diabetes (Phase 2, $n$ = 1,382) and 1 in every 10 individuals (Phase 3, $n$ = 2,207) underwent further detailed investigations, and these constituted the cohort for the follow-up study ($n$ = 3,589). From these 3,589 individuals, 900 individuals, 300 normal glucose-tolerant (NGT), 300 prediabetic and 300 T2DM

individuals were randomly selected for this study. Only 548 individuals had samples available for genetic analysis (220 NGT, 152 prediabetic and 176 T2DM individuals). Individuals were excluded from participation if they were known cases of type I diabetes, had diabetes secondary to other causes, e.g., chronic pancreatitis, if they were 80 years of age or were taking vitamin B12 supplements. **Table 23** shows the characteristics of the study participants.

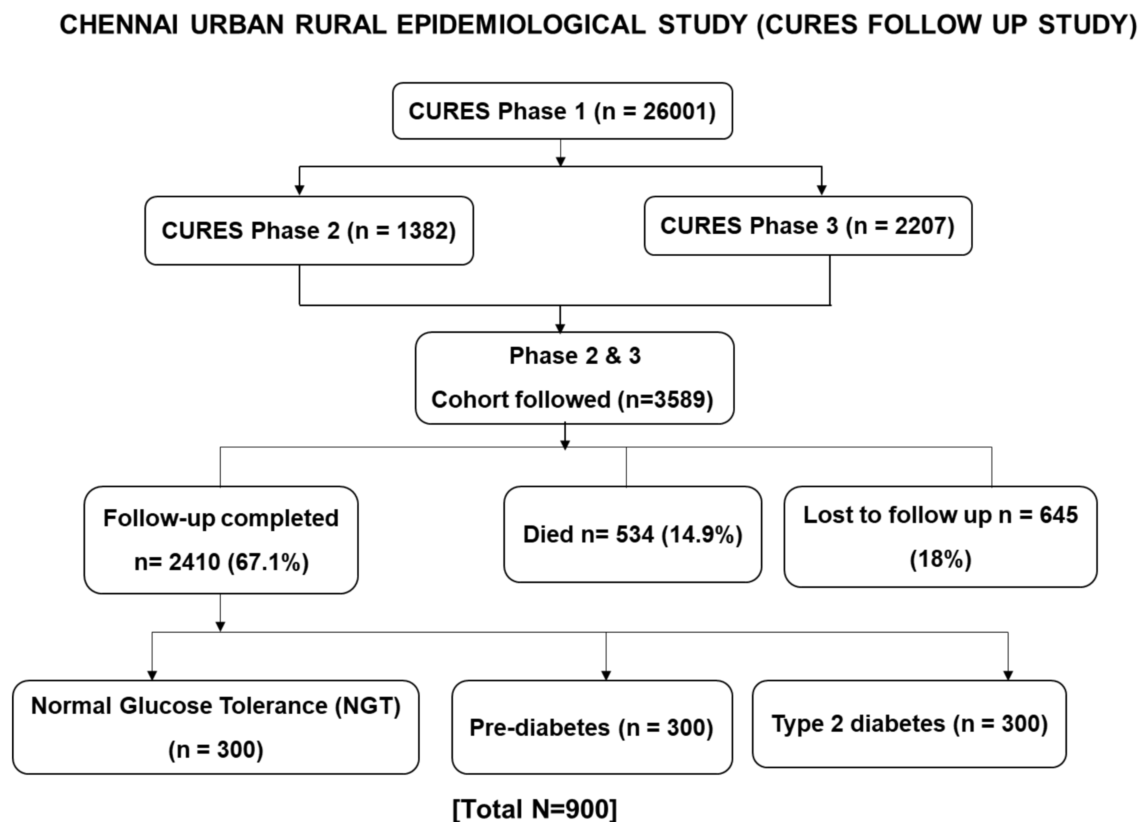


**Figure 11: Flow diagram describing the selection of study participants**

**Table 23: Baseline characteristics of the CURES study participants: Comparison of non-obese and obese individuals**

| Characteristics | n | Non-Obese individuals | n | Obese individuals | P value * |
|---|---|---|---|---|---|
| Age (yrs) | 320 | 52.2 ± 13.0 | 579 | 48.8 ± 10.8 | **<0.001** |
| BMI ($kg/m^2$) | 320 | 21.9 ± 2.0 | 579 | 29.4 ± 4.0 | **<0.001** |
| WC (cm) | 320 | 80.5 ± 8.3 | 578 | 93.1 ± 9.7 | **<0.001** |
| Hip (cm) | 320 | 88.5 ± 6.2 | 578 | 102.6 ± 9.5 | **<0.001** |
| WHR | 320 | 0.91 ± 0.09 | 578 | 0.91 ± 0.09 | 0.991 |
| Fasting plasma glucose (mg/dl) | 299 | 118 ± 55 | 553 | 116 ± 42 | 0.618 |
| Fasting serum insulin (µIU/ml) | 320 | 8.1 ± 6.1 | 579 | 9.9 ± 6.3 | **<0.001** |

| | | | | | |
|---|---|---|---|---|---|
| Glycated Haemoglobin (%) | 320 | 6.5 ± 1.8 | 579 | 6.6 ± 1.6 | 0.414 |
| Vitamin B12 levels (pg/mL) | 320 | 425 ± 263 | 579 | 412 ± 273 | 0.495 |
| Homocysteine (µmol/L) | 320 | 14.0 ± 8.9 | 579 | 13.6 ± 9.0 | 0.511 |
| Folic (ng/ml) | 320 | 8.73 ± 5.71 | 579 | 8.29 ± 5.79 | 0.276 |
| Total energy intake (kcal) | 194 | 2526 ± 791 | 335 | 2600 ± 753 | 0.280 |
| Protein energy % | 194 | 11.4 ± 1.1 | 335 | 11.4 ± 1.1 | 0.889 |

| | | | | | |
|---|---|---|---|---|---|
| Carbohydrate energy % | 194 | 63.6 ± 6.3 | 335 | 64.8 ± 5.8 | **0.033** |
| Fat Energy % | 194 | 24.1 ± 4.5 | 335 | 23.7 ± 4.7 | 0.400 |
| Total Fibre (g) | 194 | 31.1 ± 10.3 | 335 | 32.2 ± 11.1 | 0.227 |
| Physical Activity Level | 186 | Low (78.0%)<br>Medium (20.4%)<br>High (1.6%) | 280 | Low (83.2%)<br>Medium (15.0%)<br>High (1.8%) | **0.009[a]** |

*Data shown are represented as means ± SD*

*P values were calculated by using the Independent t test*

** P values for the differences in the means/proportions between non-obese and obese individuals*

*[a]P values were calculated by using the Chi Squared test*

*Abbreviations: CURES Chennai Urban Rural Epidemiological Study; BMI Body mass index; WC waist circumference; WHR waist to hip ratio*

The Madras Diabetes Research Foundation Institutional Ethics Committee granted the ethical approval and informed consent was obtained from the study participants. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki (ICH GCP).

### 5.3.2 Phenotype measurements

Anthropometric measurements including weight, height and waist circumference were measured using standardized techniques. The body mass index (BMI) was calculated using the formula, weight (kg)/height ($m^2$) and obesity was classified as BMI $\geq 25$ according to WHO Asia Pacific Guidelines for Asians (The Asia Pacific perspective 2000). Fasting plasma glucose (glucose oxidase–peroxidase method), was measured using Hitachi-912 Autoanalyzer (Hitachi, Mannheim, Germany). Glycated haemoglobin (HbA1c) was estimated by high-performance liquid chromatography using a Variant™ machine (Bio-Rad, Hercules, CA, USA). Serum insulin, serum vitamin $B_{12}$ and folic acid concentration was estimated using the electrochemiluminescence using a Roche e601Cobas immunoassay analyser (Roche Diagnostics, Indianapolis, Indiana, USA). The intra- and inter-assay coefficients of variation for vitamin $B_{12}$ assay were 0.95% and 4.08%. Serum homocysteine was measured using enzymatic assay using the Beckman Coulter AU2700 (Fullerton, CA, USA) Biochemistry analyser.

### 5.3.3 Dietary assessments and physical activity:

Dietary intakes were assessed using a previously validated and published [35] interviewer administered semi-quantitative food frequency questionnaire (FFQ) containing 222 food items to estimate food intake over the past year. The length of the interview ranged from 20 and 30 min during which participants were asked to recall their usual portion size and usual frequency (number of times per day, week, month or year/never) of foods listed within the FFQ

over the year. Common household measures such as household cups, bowls, ladles, spoons (for the cooked foods like vegetables), wedges, circles of different diameter and visual atlas of different sizes of fruits (small, medium, large) were shown to assist the individuals in estimating portions. A detailed description of the development of FFQ and the data on reproducibility and validity had been published previously [411]. The recorded data was analysed with the EpiNu® software to estimate energy as well as macronutrient and dietary fibre intake.

A validated self-report questionnaire was used to measure physical activity questionnaire [412]. Based on exercise, leisure time activities and job-related activities respondents were categorized into three groups indicating activity level (vigorously active, moderately active and sedentary). Individuals were graded as vigorously active if they did leisure time exercise and had physically demanding work, whereas individuals who either exercised or had physically demanding work were categorized as moderately active. All others were categorized as sedentary.

### 5.3.4 SNP selection and genotyping

Genetic variants within the *FTO* gene have shown consistent and strong associations with obesity [11]. Evidence suggests that the *FTO* gene confers an increased risk of obesity by approximately 1.20-fold, and a corresponding increase in BMI by 0.39 kg/m$^2$ per minor allele [413]. The BMI-increasing allele in the *FTO* gene is less prevalent in Asian (~ 30%) and African populations (~ 12%), than in European ancestry populations (~ 42%). However, the effect of the risk alleles on BMI variance is somewhat similar in the Asian (0.2%), African (0.1%) and European populations (0.3%) [413-415].

Of particular interest are intronic SNPs, which may harbour ‘intronic enhancers’ that may exert functional effects and contain potential transcriptional factor binding sites.

Furthermore, some of these intronic variants have been shown to increase disease risk or modulate the genotype-phenotype relationship [416]. The SNP rs8050136 of the *FTO* gene has shown consistent and strong associations with obesity and type 2 diabetes [11, 417]. Additionally, the SNP rs2388405 was previously selected for analysis in a case-control study conducted in a Chinese population, due to its possibility of being an 'intronic enhancer' [418] and also in a study in a Han Chinese population [419] and a Caucasian population [420]. Hence, we selected these two intronic SNPs of the *FTO* gene with a known minor allele frequency (MAF) >15% in the South Asian population: rs8050136 (intron 1, MAF = 29%; HapMap South Asian population) and rs2388405 (intron 4, MAF = 40%; HapMap South Asian population).

The standard Phenol-chloroform method was used to extract DNA from whole blood [421]. The SNPs rs8050136 and rs2388405 were genotyped by polymerase chain reaction on a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA) using the primers "F: 5'TTT GTT TTG GCT TTC TGC AGT CT3', R: CAA AAA CCA CAG GCT CAG A3' and F: 5'TCT GTG GGA ATC TCC GCT TTC AGT, R: 5'GAG CCC TTG CGC ATT GCC AG3' respectively. The PCR products were digested with MluCI (rs8050136) and ScaI (rs2388405) restriction enzymes (New England Biolabs, Inc., Beverly, MA) and the digested products were resolved by a 3 % agarose gel electrophoresis. Based on the analysis of 200 blind duplicates (20 %), there was 100% concordance in the genotyping. Furthermore, a few variants were confirmed by direct sequencing with an ABI 310 genetic analyser (Foster City, CA).

### 5.3.5 Statistical analysis:

The SPSS statistical package (version 22; SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Allele frequencies were estimated by gene counting. The Chi-square test was used to compare the proportions of genotypes or alleles. The genotypic frequencies

in all participants showed no significant departure from the Hardy Weinberg Equilibrium (HWE) ($P > 0.05$) for the *FTO* rs8050136 (MAF: 0.13 and HWE: P=0.749) and rs2388405 (MAF: 0.09 and HWE: P=0.259) SNPs.

Generalised obesity was defined according to the World Health Organization Asia Pacific Guidelines for Asians as non-obese (BMI < 25 kg/m2 ) and obese (BMI ≥ 25 kg/m2 ) [422].We performed an independent t-test to compare the means of the quantitative variables between individuals with normal-glucose tolerance (NGT) vs pre-diabetes and NGT vs T2D). Comparison of the proportion of individuals engaging in different types of physical activity levels (vigorously active, moderately active and sedentary) between NGT individuals vs pre-diabetes and NGT individual's vs T2D was analysed by the Chi Square test.

The unweighted, risk-allele GRS method was calculated for each participant by summation of the number of risk alleles for obesity. The GRS was generated from the SNPs rs8050136 and rs2388405 of the *FTO* gene. A value of 0, 1 or 2 was assigned to each SNP, which denotes the number of risk alleles for obesity on that SNP. These values were then calculated by adding the number of risk alleles across each SNP. The risk allele score was then divided into individuals carrying 0 risk allele vs more than 1 risk alleles. Association analyses between the GRS and continuous and categorical variables were carried out by linear and logistic regression models, respectively. Linear and logistic regression models were also used for interaction analyses between GRS and dietary factors (continuous variables) / physical activity (categorical variable) on continuous and categorical outcomes respectively, where the interaction terms were included into the models and were adjusted for age, BMI, sex, T2D, T2D medication and total energy intake when appropriate.

Correction for multiple testing was applied using Bonferroni correction [adjustment P value for association analysis was <0.0083 [1 GRS * 6 biochemical and metabolic traits

(vitamin B12, Homocysteine, folic acid, obesity, BMI, waist circumference) = 6 tests)] and for interaction <0.0017 [1 GRS* 6 biochemical and metabolic traits * 5 lifestyle factors (dietary carbohydrate energy %, dietary protein energy %, dietary fat energy %, dietary fiber intake (g) and physical activity levels)= 30 tests]. Given that there are no studies on GRS and no previously reported effect sizes for the South Asians, we were unable to perform a power calculation for the present study.

## 5.4 Results

### 5.4.1 Characteristics of the participants

Based on the clinical and biochemical characteristics of the individuals from the CURES study as illustrated in **Table 23**, individuals with obesity (n=579) had higher fasting plasma insulin (<0.001) compared to non-obese individuals (n=320). We also observed that obese individuals consumed higher quantities of dietary carbohydrate (energy %) than non-obese individuals (P=0.033). Additionally, a significant difference in physical activity levels between non-obese individuals and obese individuals was observed (P=0.009). The baseline characteristics which compares individuals with NGT, pre-diabetes and T2D is shown in **Table 24**.

**Table 24: Baseline characteristics of the CURES study participants: Comparison of NGT, Pre-diabetics and T2D individuals**

| Characteristic | n | Participants with normal glucose tolerance (NGT) | n | Pre-diabetics | P value * | n | Participants with type 2 diabetes (T2D) | P value ** |
|---|---|---|---|---|---|---|---|---|
| Age (yrs) | 300 | 48.2 ± 11.9 | 300 | 48.4 ± 11.7 | 0.849 | 300 | 53.6 ± 11.0 | **<0.001** |
| BMI ($kg/m^2$) | 300 | 25.8 ± 5.0 | 300 | 27.4 ± 5.2 | <0.001 | 299 | 26.9 ± 4.6 | **0.010** |
| WC (cm) | 300 | 85.9 ± 11.4 | 300 | 89.6 ± 11.1 | <0.001 | 298 | 90.3 ± 10.1 | **<0.001** |
| Hip (cm) | 300 | 96.4 ± 11.0 | 300 | 98.7 ± 11.6 | 0.015 | 298 | 97.6 ± 9.6 | 0.174 |
| WHR | 300 | 0.89 ± 0.09 | 300 | 0.91 ± 0.09 | 0.022 | 298 | 0.93 ± 0.08 | **<0.001** |
| Fasting plasma glucose (mg/dl) | 274 | 90 ± 6 | 283 | 103 ± 18 | <0.001 | 296 | 154 ± 61 | **<0.001** |
| Fasting serum insulin (µIU/ml) | 300 | 8.3 ± 5.6 | 300 | 8.1 ± 5.6 | 0.757 | 300 | 11.3 ± 6.9 | **<0.001** |
| Glycated Haemoglobin (%) | 300 | 5.7 ± 0.6 | 300 | 5.9 ± 0.6 | <0.001 | 300 | 8.1 ± 2.0 | **<0.001** |
| Vitamin B12 levels (pg/mL) | 300 | 450 ± 332 | 300 | 409 ± 246 | 0.086 | 300 | 389 ± 211 | **0.008** |
| Homocysteine | 300 | 13.1 ± 6.1 | 300 | 13.3 ± 7.4 | 0.650 | 300 | 14.9 ± 11.7 | **0.020** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| (µmol/L) | 300 | 13.1 ± 6.1 | 300 | 13.3 ± 7.4 | 0.650 | 300 | 14.9 ± 11.7 | **0.020** |
| Folic | 300 | 10.16 ± 6.35 | 300 | 7.99 ± 6.17 | <0.001 | 300 | 7.17 ± 4.11 | **<0.001** |
| (ng/ml) | 300 | 10.16 ± 6.35 | 300 | 7.99 ± 6.17 | <0.001 | 300 | 7.17 ± 4.11 | **<0.001** |
| Total energy intake (kcal) | 248 | 2581 ± 750 | 124 | 2588 ± 807 | 0.932 | 157 | 2548 ± 767 | 0.675 |
| Protein energy % | 248 | 11.3 ± 1.2 | 124 | 11.3 ± 1.1 | 0.912 | 157 | 11.4 ± 1.1 | 0.328 |
| Carbohydrate energy % | 248 | 64.1 ± 6.5 | 124 | 64.8 ± 5.5 | 0.284 | 157 | 64.4 ± 5.5 | 0.657 |
| Fat Energy % | 248 | 24.0 ± 4.8 | 124 | 23.5 ± 4.4 | 0.299 | 157 | 23.9 ± 4.5 | 0.879 |
| Total Fibre (g) | 248 | 31.9 ± 10.7 | 124 | 31.1 ± 10.8 | 0.510 | 157 | 32.2 ± 11.0 | 0.832 |
| Physical Activity Level | 228 | Low (77.6%) | 105 | Low (82.9%) | 0.230 | 133 | Low (85.7%) | 0.143[a] |
| | | Medium (21.1%) | | Medium (14.3%) | | | Medium (12.3%) | |
| | | High (1.3%) | | High (2.9%) | | | High (1.5%) | |

*Data shown are represented as means ± SD*

*P values were calculated by using the Independent t test*

**P values for the differences in the means/ proportions between NGT and pre-diabetic individuals*

*** P values for the differences in the means/ proportions between NGT and T2D individuals*

*[a]P values were calculated by using the Chi Squared test*

*Abbreviations: CURES Chennai Urban Rural Epidemiological Study; BMI Body mass index; WC waist circumference; WHR waist to hip ratio*

### 5.4.2 Association between GRS and obesity-related phenotypes

We were able to identify an association between GRS and BMI (P=0.009). Individuals who carried more than one risk allele had higher BMI levels (Mean ± SE: 27.55 ± 4.98) compared to individuals with zero risk alleles (Mean ± SE: 26.43 ± 5.03) (**Table 25 and Figure 12**).

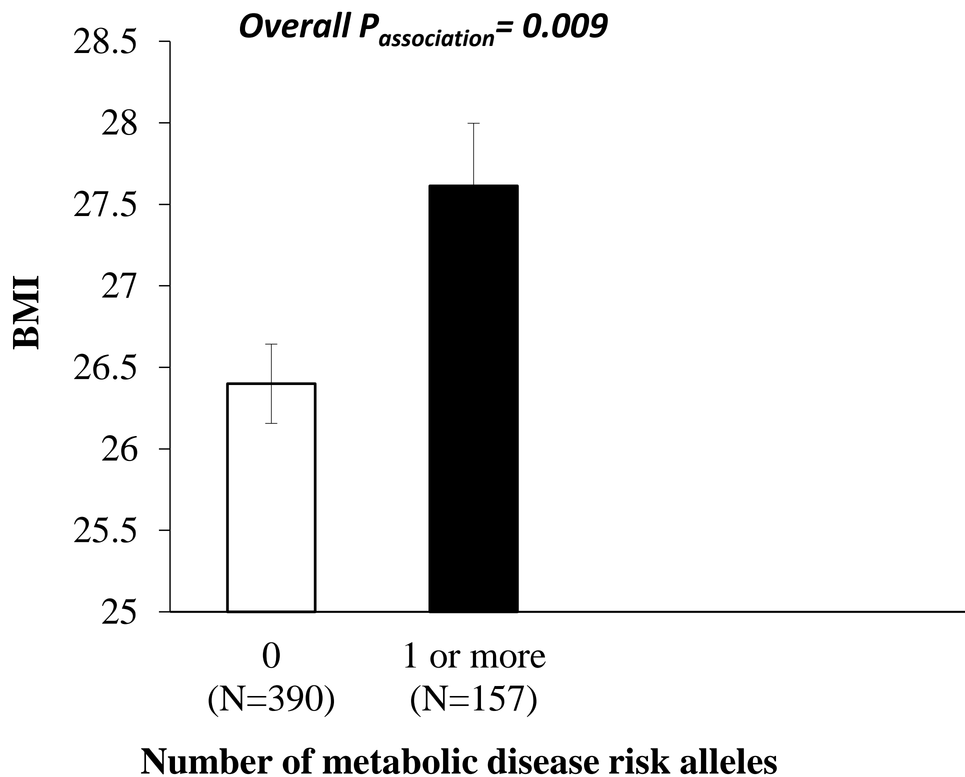


**Figure 12: Association between the GRS and BMI**

*Obesity risk increasing alleles ranged from 0 to 3. The white bars indicate individuals with 0 risk alleles and the black bars indicate individuals carrying ≥1 alleles. Individuals who carried 1 or more risk alleles had significantly higher BMI compared to individuals carrying 0 risk alleles (P = 0.009)]. Error bars indicate Standard error.*

**Table 25: Association between the FTO-GRS with vitamin B12, folic acid, homocysteine and obesity traits**

| Risk alleles in all participants | n | Vitamin B12 (pg/mL) | n | Homocysteine (µmol/L) | n | Folic acid (ng/ml) | n | BMI (kg/m$^2$) | n | WC (cm) | n | Odds Ratio (95% CI) of Obesity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 380 | 410 ± 202 | 390 | 13.2 ± 7.7 | 390 | 8.89 ± 5.92 | 390 | 26.4 ± 5.0 | 390 | 87.6 ± 11.1 | 194 | 1.63 (1.07-2.49) |
| ≥1 | 154 | 356 ± 189 | 157 | 14.8 ± 8.9 | 157 | 7.89 ± 5.48 | 157 | 27.6 ± 5.0 | 156 | 90.0 ± 11.6 | 353 | |
| P value[a] | | **0.018** | | 0.077 | | 0.147 | | **0.009†** | | 0.747 | | ***0.023** |

| Risk alleles in NGT individuals | n | Vitamin B12 (pg/mL) | n | Homocysteine (µmol/L) | n | Folic acid (ng/ml) | n | BMI (kg/m$^2$) | n | WC (cm) | n | Odds Ratio (95% CI) of Obesity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 151 | 416 ± 199 | 157 | 12.5 ± 5.7 | 157 | 10.45 ± 6.42 | 157 | 25.7 ± 5.0 | 157 | 85.0 ± 11.2 | 157 | 1.71 (0.92 - 3.21) |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ≥1 | 61 | 327 ± 167 | 63 | 13.9 ± 5.9 | 63 | 8.99 ± 6.02 | 63 | 27.2 ± 5.4 | 63 | 88.5 ± 12.6 | 63 | |
| P value[b] | | **0.004** | | 0.102 | | 0.143 | | **0.054††** | | 0.694 | | 0.092** |

*Values are given as mean ± standard deviation.*

*[a]P values for differences between 0 and 1 risk alleles were obtained using linear regression model adjusted age, BMI, Type 2 diabetes status, Type 2 diabetes medication and sex*

*† P values were obtained by using a general linear model adjusted for age, Type 2 diabetes status, Type 2 diabetes medication and sex*

** P values were adjusted for age, sex and Type 2 diabetes status using binary logistic regression*

*[b]P values for differences between 0 and 1 risk alleles were obtained using linear regression model adjusted age, BMI and sex*

*†† P values were obtained by using a general linear model adjusted for age and sex*

*** P values were adjusted for age and sex using binary logistic regression*

*Abbreviations: BMI body mass index; WC waist circumference; WHR waist to hip ratio; NGT normal glucose tolerant*

There was a significant association between the GRS and obesity ($P_{association}$=0.023), where individuals carrying more than one risk allele had 1.6 times increased risk of obesity compared to those carrying zero risk alleles (**Table 25**). However, after Bonferroni correction, none of these associations remained statistically significant. Moreover, no statistically significant associations were observed between GRS and waist circumference (P=0.747) (**Table 25**).

### 5.4.3 Association between the GRS and vitamin B12, homocysteine and folic acid levels

We found that the GRS was significantly associated with vitamin B12 concentrations (P=0.018) (**Table 25 and Figure 13**), and individuals carrying more than one risk allele had 13.1% lower vitamin B12 concentrations (Mean ± SE: 355 ± 189 pg/mL), compared to individuals carrying zero risk alleles (Mean ± SE: 410 ± 202 pg/mL). However, this finding was not significant after correction for multiple testing.

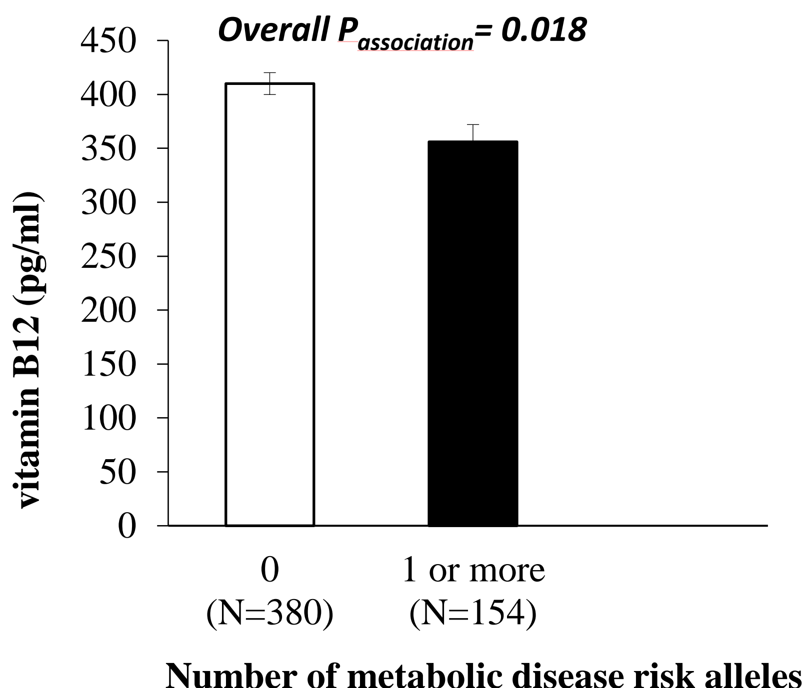


**Figure 13: Association between the GRS and serum vitamin B12 concentrations**

*Obesity risk increasing alleles ranged from 0 to 3. The white bars indicate individuals with 0 risk alleles and the black bars indicate individuals carrying ≥1 alleles. Individuals who carried 1 or more risk alleles had significantly lower B12 concentrations compared to individuals carrying 0 risk alleles (P = 0.018)]. Error bars indicate Standard error.*

There were no statistically significant associations between GRS and homocysteine or folic acid concentrations (**Table 25**).

### 5.4.4 Interaction between the GRS and lifestyle factors on vitamin B12, folic acid, homocysteine and obesity traits

None of the lifestyle factors (dietary intake (carbohydrate, protein, fat, fibre) or physical activity) significantly interacted with the GRS on biochemical and anthropometric measurements after correction for multiple testing **(Tables 26 and 27).**

**Table 26: Interaction between the FTO-GRS and lifestyle factors on vitamin B12, folic acid, homocysteine and obesity** traits

| | BMI (kg/m$^2$) | WC (cm) | Vitamin B12(pg/mL) | Homocysteine (µmol/L) | Folic acid (ng/ml) |
|---|---|---|---|---|---|
| **Interaction between the GRS and carbohydrate energy (%)** | -0.08 ± 0.09 | 0.02 ± 0.11 | 1.40 ± 3.55 | -0.03 ± 0.13 | 0.01 ± 0.10 |
| P value | †0.387 | 0.882 | 0.694 | 0.830 | 0.952 |
| **Interaction between the GRS and Fat energy (%)** | 0.23 ± 0.12 | 0.18 ±0.15 | 0.98 ± 4.87 | -0.07 ± 0.18 | 0.09 ± 0.14 |
| P value | †0.052 | 0.225 | 0.841 | 0.709 | 0.539 |
| **Interaction between the GRS and Protein energy (%)** | 0.37 ± 0.50 | 0.77 ± 0.59 | 6.10 ± 19.95 | 0.03 ± 0.75 | 0.20 ± 0.58 |
| P value | †0.451 | 0.196 | 0.760 | 0.968 | 0.728 |

| | | | | | |
|---|---|---|---|---|---|
| **Interaction between the GRS and Fibre (g)** | 0.08 ± 0.05 | 0.14 ± 1.49 | 1.68 ± 1.90 | -0.01 ± 0.07 | 0.04 ± 0.05 |
| P value | †0.081 | 0.925 | 0.376 | 0.898 | 0.503 |
| **Interaction between the GRS and physical activity levels** | 1.14 ± 1.16 | -0.14 ± 1.46 | 23.02 ± 51.29 | 0.99 ± 1.93 | 0.46 ± 1.51 |
| P value | †0.327 | 0.924 | 0.654 | 0.609 | 0.760 |

*Values are beta coefficients ± standard errors.*

*P values were obtained by using a general linear model adjusted for age, BMI, Type 2 diabetes status, Type 2 diabetes medication and sex*

*† P values were obtained by using a general linear model adjusted for age, Type 2 diabetes status, Type 2 diabetes medication and sex*

*Abbreviations: BMI body mass index; WC waist circumference; WHR waist to hip ratio*

**Table 27: Interaction between the FTO-GRS and lifestyle factors on obesity**

| | Interaction between the GRS and dietary factors on Obesity | | | | Interaction between the GRS and physical activity levels on Obesity |
|---|---|---|---|---|---|
| | GRS * Fat energy % | GRS * carbohydrate energy % | GRS * protein energy % | GRS * fibre (g) | |
| **Odds Ratio (95% CI)** | 1.096 (0.980-1.227) | 0.962 (0.889-1.040) | 1.113 (0.729-1.762) | 1.034 (0.984-1.086) | 1.026 (0.263-4.003) |
| **P Value** | 0.109 | 0.326 | 0.578 | 0.182 | 0.970 |

*Values are beta coefficients ± standard errors.*

*P values were obtained by using binary logistic regression adjusted for age, T2D, Type 2 diabetes medication and sex*

### 5.4.5 Discussion

Both obesity and vitamin B12 deficiency, are modifiable risk factors for several chronic diseases. Moreover, both risk factors have been shown previously to be associated with one another. This is the first study to use a genetic approach to establish a relationship between obesity and vitamin B12 levels in an Asian Indian population. Our study confirmed the strength of the association between the GRS generated from the two *FTO* SNPs and BMI and demonstrated the impact of genetically instrumented BMI on serum B12 concentrations. These results suggest that increases in BMI could potentially contribute to the adverse health effects associated with vitamin B12 deficiency. Given that low vitamin B12 concentrations in Asian Indians are common [397, 423], our study highlights the importance of considering obesity as a risk factor for vitamin B12 deficiency with implications on the possible targeting of relevant obesity prevention strategies.

Variants of the *FTO* gene are known to be the strongest genetic predictor of obesity to date [424, 425]. It has been suggested that risk variants at the *FTO* locus trigger the overexpression of ghrelin mRNA, leading to higher levels of the hunger hormone, ghrelin, to be secreted [426], which in turn makes individuals over consume energy-dense foods [427, 428]. In general, the two selected intronic SNPs rs2388405 and rs8050136 could potentially be relevant as intronic enhancers, as they may enhance the expression of the *FTO* gene [416]. In support of this, in a previous study conducted in a South Indian population (CURES), the *FTO* SNP, rs8050136, was associated with an increased risk of obesity [371]. Given the strong role of the *FTO* locus in obesity [11, 371], *FTO* was considered as a suitable candidate to establish the genetic link between obesity-related traits and vitamin B12 concentrations.

Reduced vitamin B12 concentrations in the obese population are thought to result from a nutrient-poor diet, increased nutrient requirements in relation to an increased body size and

the physiological effects of obesity on nutrient absorption/metabolism [84, 85]. Additionally, obesity is a well-known risk factor for T2DM [429] and gastroesophageal reflux disease (GERD) [430]. As a result, obese individuals are more likely to take metformin and proton pump inhibitors (PPIs), which have been shown to reduce serum B12 levels by inhibiting the absorption of the vitamin [93, 431]. In our study, we found a significant association between *FTO* GRS and vitamin B12 concentrations in South Asian adults. Several studies in India, have reported significant associations between vitamin B12 status and obesity related traits [4-7]. A study conducted in North India, reported that there was a negative correlation between waist circumference and reduced levels of vitamin B12 [4]. A study looking at 2,403 school-going adolescents (11–17 years) from Haryana, India reported that more than half (51.2%) of obese adolescents were vitamin B12 deficient [6]. Furthermore, recent findings from the CURES (n = 1500 individuals) demonstrated that the prevalence of vitamin B12 deficiency significantly increased in those with abdominal obesity and the mean levels of vitamin B12 significantly decreased with increasing degrees of glucose tolerance [5]. However, in this study, we were unable to identify a similar trend when considering the GRS, which could be due to the smaller sample size of our study (data not shown). However, our data confirms the association between vitamin B12 concentrations and obesity and suggests that individuals genetically predisposed to obesity are at a higher risk of vitamin B12 deficiency.

Current literature suggests that the genetic profile of an individual can shape the microbiome of the host, and indeed an altered gut flora has been associated with vitamin B12 deficiency [14, 264]. In a study in rodents, it was found that the type of dietary lipids (lard or fish oil) influenced the structure of the microbiome as there was an interaction between gut microbiota and saturated lipids in promoting white adipose tissue inflammation [432]. Chakraborty et al postulated that a higher concentration of inflammatory cytokines could impair vitamin B12 absorption or biosynthesis [6]. Another study reported that low vitamin

B12 status induced excess triacylglycerol biosynthesis and secretion of pro-inflammatory cytokines [433]. Whether the *FTO* genotypes influence the association between obesity and vitamin B12 concentrations by modulating the gut microbiota composition and inducing metabolic inflammation requires further investigation utilizing faecal samples.

The main strength of this study was the use of a validated food frequency questionnaire [372], which has shown high reproducibility and validity for total carbohydrates and dietary fibre, and the use of a GRS. Moreover, the sampling was representative of the overall population of Chennai. Nevertheless, some limitations need to be acknowledged. Although the majority of Indian adults are physically inactive and consume a diet high in carbohydrates [382, 405], no significant interactions were found between the GRS and lifestyle factors on vitamin B12 and metabolic disease outcomes in our study, which could be attributed to the small sample size. The GRS only used two variants from the *FTO* gene, and we cannot fully exclude that other variants of the *FTO* gene may also be important. Another limitation was the use of a cross-sectional design to investigate genetic effects at a single point in time and hence no cause effect inferences can be drawn, for which a longitudinal analysis design over a specific time period would be needed.

## 5.5 Conclusion

In summary, our study suggests that genetic variations at the *FTO* locus appear to influence serum vitamin B12 concentrations. However, we were unable to show an impact of the GRS on lowering B12 concentrations through a dietary influence. Longitudinal studies could help to establish the effect of genetic influences on vitamin B12 concentration.

**Authors' contributions**

**Shelini Surendran performed the statistical analysis, data interpretation and wrote the manuscript**. KSV contributed to drafting the paper; LD assisted with performing the statistical analysis; DB carried out the genotyping analysis; KSV designed the nutrigenetics study; VM, RP and RMA designed the CURES; VR and DB designed the genetic study. RP, NL, VS, CSS and RJ contributed to data collection in India. RP, DB, JAL, NL, VS, VM, RMA and VR critically reviewed the manuscript. All authors read and approved the final version of the manuscript

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# Chapter 6

## A nutrigenetic approach for investigating the relationship between vitamin B12 status and metabolic traits in Indonesian women

For this study, I developed an analysis plan before I undertook the statistical analysis. I screened and validated the dataset to perform statistical analysis. I performed the entire statistical analysis using the SPSS software; I undertook a literature review as part of the introduction to the study and wrote the manuscript. I revised the manuscript based on the comments from all the co-authors before the manuscript was submitted to the journal.

**Published (The published version of the paper is attached as an appendix at the end of the thesis)**

Surendran S, Aji AS, Ariyasra U, Sari SR, Malik SG, Tasrif N, Yani FF, Lovegrove JA,Sudji IR, Lipoeto NI, Vimaleswaran KS (2019). A nutrigenetic approach for investigating the relationship between vitamin B12 status and metabolic traits in Indonesian women. Journal of Diabetes & Metabolic Disorders (**Published**).

### 6.1 Abstract

**Purpose:** Adverse effects of maternal vitamin B12 deficiency have been linked to major clinical outcomes, including increased body mass index and gestational diabetes, however, less is known about vitamin B12 nutrition in non-pregnant women. Hence, the aim of the present study was to explore the relationships between metabolic traits and vitamin B12 status in a cohort of healthy Indonesian women and to investigate whether these relationships were modified by dietary intake using a genetic approach.

**Methods:** A total of 117 Minangkabau women (aged 25-60 years), from the city of Padang, West Sumatra underwent anthropometric, biochemical, dietary intake analysis and genetic

tests. Genetic risk scores (GRS) based on nine vitamin B12 associated single nucleotide polymorphisms (SNPs) (B12-GRS) and nine metabolic SNPs (metabolic-GRS) were constructed.

**Results:** The B12-GRS and metabolic-GRS had no effect on vitamin B12 (P>0.160) and metabolic traits (P>0.085). However, an interaction was observed between the B12-GRS and dietary fibre intake (g) on glycated haemoglobin (HbA1C) levels ($P_{interaction}$=0.042), where among those who consumed a low fibre diet (4.90 ± 1.00 g/day), individuals carrying ≥9 risk alleles for vitamin B12 deficiency had significantly higher HbA1C levels (P=0.025) compared to those carrying ≤8 risk alleles.

**Conclusion:** Our study showed a significant impact of the B12-GRS on HbA1C concentrations through the influence of a dietary factor, however, our study failed to provide evidence for an impact of metabolic-GRS on lowering B12 concentrations. Further replication studies utilizing larger sample sizes are needed to confirm our findings.

## 6.2 Introduction

Vitamin B12 adequacy plays a critical role in a multitude of physiological processes, including DNA synthesis, haematological development and neurological function [2, 50]. Moreover, vitamin B12 is now known to play a much more profound and wide-ranging role in maternal health as well as foetal development [70, 434]. Low maternal plasma concentrations of vitamin B12 have shown negative correlations with body mass index (BMI) levels in healthy women [401] and have been associated with pregnancy complications such as gestational diabetes mellitus [70], recurrent pregnancy loss [435], higher BMI [69] and neural tube defects [436]. Notably, the harmful effects of maternal malnutrition are not just confined to pregnancy complications and birth defects. Findings from the Pune Maternal Nutrition Study (PMNS) in India have shown that low maternal vitamin B12 increases the risk of insulin resistance and

relative adiposity in 6- to 7-y-old children, with the highest levels of insulin resistance occurring when mothers had a combination of a high folate and low vitamin B12 status [69].

Suboptimal vitamin B12 status is common in many parts of the world [59]. Published data on vitamin B12 status of any life-stage group in Indonesian women is lacking with the exception of an earlier report in 2017 showing that the prevalence of a vitamin B12 deficient diet was 34.5% in 606 Indonesian pregnant women (14-49 years) [437]. The Minangkabau culture in Indonesia is of particular interest, as it is the world's largest matrilineal system of kinship, where women hold greater power in both family and society [438]. Food supply is centred around women and compelling evidence suggests that adequate nutrition protects against metabolic disorders related to obesity [439], as a result understanding the dietary patterns of women in relation to their genetic susceptibility is of great importance.

Although vitamin B12 deficiency is associated with a wide range of chronic diseases and conditions, including obesity, and with increasing severity of metabolic dysfunction, such as insulin dysregulation [4-7], the relationship between low vitamin B12 status and obesity related traits has remained inconsistent [93]. It is possible that certain genotypes might jointly contribute to obesity and vitamin B12 deficiency [93] and the implementation of a genetic approach to establish the relationship between vitamin B12 and obesity could be a more desirable option over observational studies, as results are less prone to confounding factors. While genetic studies have implicated several gene loci in the predisposition to vitamin B12 deficiency, no study has yet been carried out in the Indonesian population [14]. Hence, for the first time we used a genetic approach to explore the relationship between metabolic traits and vitamin B12 status and investigated whether these relationships were modified by lifestyle factors in a cohort of Minangkabau women in Padang. Identifying the impact of vitamin B12 status on metabolic traits will help us to reduce the burden of metabolic diseases through implementation of policies for screening of vitamin B12 deficiency.

## 6.3 Methodology

### 6.3.1 Study participants

The Minangkabau Indonesia Study on Nutrition and Genetics (MINANG) study is a cross-sectional pilot study that was conducted in the city of Padang, West Sumatra, Indonesia, from December 2017 to January 2018. This study was conducted as part of the ongoing GeNuIne (Gene-Nutrient Interactions) Collaboration, the main objective of which is to investigate the effect of gene-nutrient interactions (nutrigenetics) on metabolic disease outcomes using population based studies from various ethnic groups [12]. The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Ethical Review Committee of the Medical Faculty, Andalas Univesity (No.311/KEP/FK/2017). All participants provided written informed consent before participating. Participants were allowed to leave the study at will and opt out from any of the procedures. One hundred and thirty-three women were recruited from community health centers in two sub districts in Padang City to represent both urban (50% Padang Timur) and rural areas of Padang (50% Kuranji) population. Inclusion criteria were healthy women (between 25-60 years old) with Minangkabau ethnicity. Among the 133 eligible adults, 10 adults were excluded from the study. Exclusion criteria included the following: having a previous history of type 2 diabetes, cardiovascular disease or hypertension (n=6), having a BMI of more than 40 kg/m$^2$ or being classified as morbidly obese by a physician (n=0), being blood related to other participants in the study (n=0), having any communicable disease (n=4), being pregnant or lactating (n=0) and taking dietary or vitamin supplements (n=0). Among the 123 remaining adults, 5 volunteers did not undergo blood sample collection and were excluded from the study and one participant did not undertake the validated semi-quantitative food frequency questionnaire (FFQ) [440]. The final sample consisted of 117 women who completed an FFQ and underwent blood sample collection for biochemical and genetic analysis (**Figure 14**).

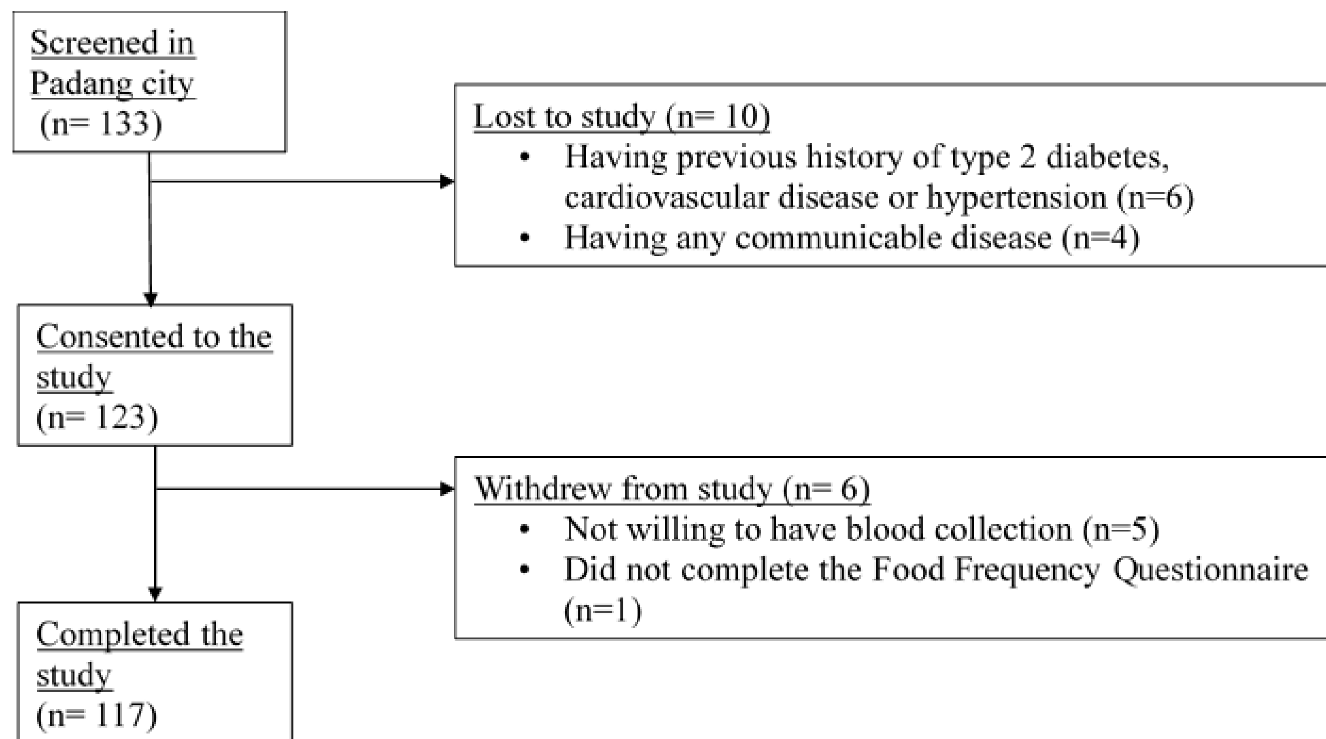


**Figure 14: Flow chart of the subject recruitment process**

### 6.3.2 Anthropometric Measures

Body weight was measured to the nearest 100 g using an electronic scale (Seca 803, Seca GmbH. Co. kg, Hamburg, Germany) and height was measured to the nearest mm using a stadiometer (OneMed Medicom stature meter, YF.05.05.V.A.1022, Indonesia). The BMI was estimated as weight (kg) divided by the height (m) squared. BMI was classified according to the Asia-Pacific classification for BMI/age according to sex [441].The waist (cm) circumference was measured in a standing position with the feet positioned close together. The waist circumference (WC) was measured using a metal tape (Medline-OneMed Medicom, Jakarta, Indonesia) midway between the lower border of the rib cage and the iliac crest, at the end of gentle expiration. Body fat percentage was measured using the Tanita MC780 multi frequency segmental body composition analyser.

### 6.3.3 Biochemical measures

For the determination of biochemical parameters, blood samples (5 ml) were collected by a trained phlebotomist in the morning, after a 12 hour fast. The blood samples were used to measure vitamin B12, glucose, insulin and glycated hemoglobin (HbA1c). All biochemical samples were assayed using the xMark Microplate Spectrophotometer (Bio-Rad Laboratories Inc, Hercules, California, USA). Serum concentrations of vitamin B12, glucose, insulin and HbA1c were assessed using enzyme-linked immunosorbent assay (ELISA) kits from Bioassay Technology Laboratory (Shanghai, China).

### 6.3.4 Assessment of dietary intake and physical activity

Data collection was completed by a qualified nutritionist in the home or in an integrated health service post. Dietary intakes were assessed using a previously validated and published semi-quantitative food frequency questionnaire (SQ-FFQ) containing 223 food items [440]. In brief, participants were asked to estimate the usual frequency (number of times per day, week or month) and the portion sizes of various food items. Portion size photographs of all relevant foods (including some prepared dishes) were used by participants while completing the SQ-FFQ, to aid the estimation of portion size intake [442]. All information provided by the participants was double-checked for accuracy. The recorded data was analyzed with the Indonesian Food Database and Nutrisurvey (EBISpro, Germany) to estimate energy as well as macro- and micronutrient consumption. Wherever appropriate, nutrient intake values were adjusted to energy by the nutrient (energy-adjusted) residual method [345].

"The Global Physical Activity Questionnaire" (GPAQ), developed by the World Health Organization (WHO) was used to measure physical activity [369]. Total time in moderate-to-vigorous physical activity was calculated according to the WHO STEPwise method and was expressed as metabolic equivalent minutes per day (METmins/day). Furthermore, participants

were classified as "active" if they accumulated ≥600 METmins/week or "inactive" if they did <600 METmins/week. Sedentary behaviour (SB, mins/day) was determined from the last question of the GPAQ, based on how long the participants spent sitting while working, in a vehicle, watching television, or lying down, except sleeping [369]

### 6.3.5 SNP selection and genotyping

We selected nine vitamin B12-related SNPs (Methylenetetrahydrofolate reductase [*MTHFR*]- rs1801133, Carbamoyl-phosphate synthase 1 [*CPS1*]- rs1047891, Cubulin [*CUBN*]- rs1801222, CD320 molecule [*CD320*]- rs2336573, Transcobalamin 2 [*TCN2*]- rs1131603, Fucosyltransferase 2 [*FUT2*]- rs602662, Transcobalamin 1 [*TCN1*]- rs34324219, Fucosyltransferase 6 [*FUT6*]- rs778805 and Methylmalonyl-CoA mutase [*MUT*]- rs1141321) based on the recent review article by Surendran et al. [14].

The nine metabolic disease-related SNPs were selected for our analysis based on previously published candidate gene association and genome-wide association (GWA) studies for metabolic disease-related traits [370-378]: Fat mass and obesity-associated [*FTO*]- rs9939609 and rs8050136, Melanocortin 4 Receptor [*MC4R*]- rs17782313 and rs2229616, Transcription factor 7-like 2 [*TCF7L2*]- rs12255372 and rs7903146, Potassium voltage-gated channel subfamily J member 11 [*KCNJ11*]- rs5219, Calpain 10 [*CAPN10*]- rs3792267 and rs5030952)

Genomic DNA was isolated from peripheral blood leukocytes using the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, USA) with spin column methods. The DNA concentration was determined using a NanoDrop spectrophotometer. Genotyping was performed at LGC Genomics (http://www.lgcgroup.com/services/genotyping), which employs the competitive allele-specific PCR-KASP® assay.

### 6.3.6 Statistical analysis

The SPSS statistical package (version 22; SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Results from the descriptive analyses are presented as means and standard deviations (SD) for continuous variables and as percentages for categorical variables. Generalized obesity was defined according to the Asia-Pacific classification of BMI for Asians as non-obese (BMI $< 23$ kg/m$^2$) and obese (BMI $\geq 23$ kg/m$^2$) [441]. We performed an independent t-test to compare the means of the quantitative variables between non-obese individuals vs obese individuals. Comparison of the proportion of individuals engaging in different types of physical activity levels (vigorously active, moderately active and sedentary) between non-obese vs obese individuals was analyzed by the Chi Square test. The normality of variable distribution was verified by the Shapiro-Wilk test; WC, body fat percentage, glucose, insulin, HbAC1 and vitamin B12 levels were not normally distributed in our study population; therefore, the data were natural log-transformed prior to analysis.

Allele frequencies were estimated by gene counting. The Chi-square test was used to compare the proportions of genotypes or alleles. Fifteen of the SNPs were in Hardy Weinberg Equilibrium (HWE) ($P > 0.05$) (**Table 28**). HWE was not calculated for the SNPs *TCN2* rs1131603 and *TCN1* rs34324219 as no minor alleles were present. The SNP *FUT2* rs602662 deviated from HWE; however, this SNP was not excluded from analysis. The KASP$^{TM}$ genotyping technology used in this study, has been independently assessed to be over 99.8% accurate [443]. Validation of the KASP$^{TM}$ genotyping was conducted at LGC genomics and the quality of the genotyping results were independently assessed and confirmed by the project manager. This ruled out genotyping artefacts as possible reasons for deviation from HWE. Hence, it is possible that the SNP *FUT2* rs602662 could have deviated from HWE due to population or racial grouping substructure (Sub-grouping), non-random mating, linkage disequilibrium (incomplete mixing of different ancestral population) or chance findings [379].

Interestingly, the SNP *FUT2* rs602662 deviated from HWE within the Sri Lankan (GOOD) population and  in a GWA study conducted in India; the authors ruled out that the deviation was not due to a genotyping error and still used this SNP for analysis in their study [233]. It is possible, that *FUT2* rs602662 does not reach HWE in South / South East Asian populations. An alternative reason could be that the HWE is not reached in this population, due to the small sample size and the possibility of interbreeding (especially as consanguineous marriages are common in these populations).

**Table 28: Genotype distribution of vitamin B12 related SNPs and metabolic disease-related SNPs**

| Gene | rs number | Major allele | Minor allele | Common Homozygotes (%) | Heterozygotes (%) | Rare Homozygotes (%) | Minor allele frequency | HWE P value |
|---|---|---|---|---|---|---|---|---|
| *MTHFR* | *rs1801133* | C | T | 92 (79.30) | 24 (20.70) | 0 (0.00) | 0.10 | 0.214 |
| *CPS1* | *rs1047891* | C | A | 48 (41.00) | 56 (47.90) | 13 (11.10) | 0.35 | 0.579 |
| *CUBN* | *rs1801222* | C | T | 84 (74.30) | 27 (23.90) | 2 (1.80) | 0.14 | 0.920 |
| *CD320* | *rs2336573* | C | T | 86 (74.10) | 29 (25.00) | 1 (0.90) | 0.13 | 0.390 |
| *TCN2* | *rs1131603* | T | C | 117 (100) | 0 (0.00) | 0 (0.00) | 0 | N/A |
| *FUT2* | *rs602662* | G | A | 111 (94.90) | 4 (3.40) | 2 (1.70) | 0.03 | **0.000** |
| *TCN1* | *rs34324219* | C | A | 117 (100) | 0 (0.00) | 0 (0.00) | 0 | N/A |
| *FUT6* | *rs778805* | T | C | 33 (28.20) | 61 (52.10) | 23 (19.70) | 0.46 | 0.586 |
| *MUT* | *rs1141321* | G | A | 67 (59.30) | 40 (35.40) | 6 (5.30) | 0.23 | 0.993 |
| *CAP10* | *rs3792267* | G | A | 108 (91.50) | 9 (7.60) | 1 (0.80) | 0.05 | 0.123 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *CAP10* | *rs5030952* | C | T | 77 (66.40) | 31 (26.70) | 8 (6.90) | 0.20 | 0.063 |
| *KCNJ11* | *rs5219* | C | T | 55 (47.00) | 47 (40.20) | 15 (12.80) | 0.33 | 0.329 |
| *TCF7L2* | *rs12255372* | G | T | 97 (82.90) | 20 (17.10) | 0 (0.00) | 0.09 | 0.312 |
| *TCF7L2* | *rs7903146* | C | T | 95 (81.90) | 21 (18.10) | 0 (0.00) | 0.09 | 0.284 |
| *FTO* | *rs9939609* | T | A | 70 (60.30) | 39 (33.60) | 7 (6.00) | 0.23 | 0.618 |
| *MC4R* | *rs17782313* | T | C | 89 (76.10) | 26 (22.20) | 2 (1.70) | 0.13 | 0.929 |
| *FTO* | *rs8050136* | C | A | 69 (60.00) | 39 (33.90) | 7 (6.10) | 0.23 | 0.638 |
| *MC4R* | *rs2229616* | G | A | 116 (99.10) | 1 (0.90) | 0 (0.00) | 0.00 | 0.963 |

*MAF; minor allele frequency, HWE; Hardy Weinberg Equilibrium, $X^2$; Chi-Squared value*

A schematic representation of the study design is presented in **Figure 15**. The unweighted, risk-allele GRS method was calculated for each participant as the sum of risk allele counts across each SNP which predicted vitamin B12 status. The B12-GRS was generated from the vitamin B12-related SNPs in the *MTHFR, CPS1, CUBN, CD320, TCN2, FUT2, TCN1, FUT6, MUT* genes. Furthermore, another unweighted GRS was created using allele markers previously reported to be associated with metabolic disease traits. The Metabolic-GRS was generated from the SNPs in the *CAP10*, *KCNJ11*, *TCF7L2*, *FTO* and *MC4R* genes. A value of 0, 1 or 2 was assigned to each SNP, which denotes the number of risk alleles on that SNP. These values were then calculated by adding the number of risk alleles across each SNP. The average number of risk alleles per person for the B12-GRS was 8.18 (SD = 1.36), which ranged from 5 to 12. The sample was stratified, by the median, into a "low genetic risk group," for those with a GRS ≤ 8 risk alleles ($n$ = 73), and into a "high genetic risk group," for those with a GRS ≥ 9 risk alleles ($n$ = 44). For the metabolic-GRS, the average number of risk alleles per person was 4.66 (SD = 1.76), which ranged from 2 to 9. The sample was stratified, by the median, into a "low genetic risk group," for those with a GRS ≤ 4 risk alleles ($n$ = 61), and into a "high genetic risk group," for those with a GRS ≥ 5 risk alleles ($n$ = 56). Linear regression was used to examine the association of the two GRS scores with the biochemical and anthropometric outcomes (vitamin $B_{12}$, glucose, insulin, HbA1c, BMI, WC and body fat percentage). The interaction between the two GRS scores and dietary factors on biochemical and anthropometric outcomes was determined by including interaction term (GRS*lifestyle factor) in the regression model. Models were adjusted for age, BMI, and total energy intake, wherever appropriate. Correction for multiple testing was applied using Bonferroni correction [2 GRS * 7 biochemical and anthropometric measurements (vitamin B12, glucose, insulin, HbA1c, BMI, WC, body fat percentage) * 5 lifestyle factors (dietary carbohydrate (energy %), dietary protein (energy %), dietary fat (energy %), dietary fibre (g)

and physical activity levels) = 70 tests; 0.05/70 = 0.000714; P<0.000714]. All data are expressed as mean ± SD. Given that there are no studies on GRS in relation to B12 status and metabolic outcomes and no previously reported effect sizes for the South-East Asians, we were unable to perform a power calculation.

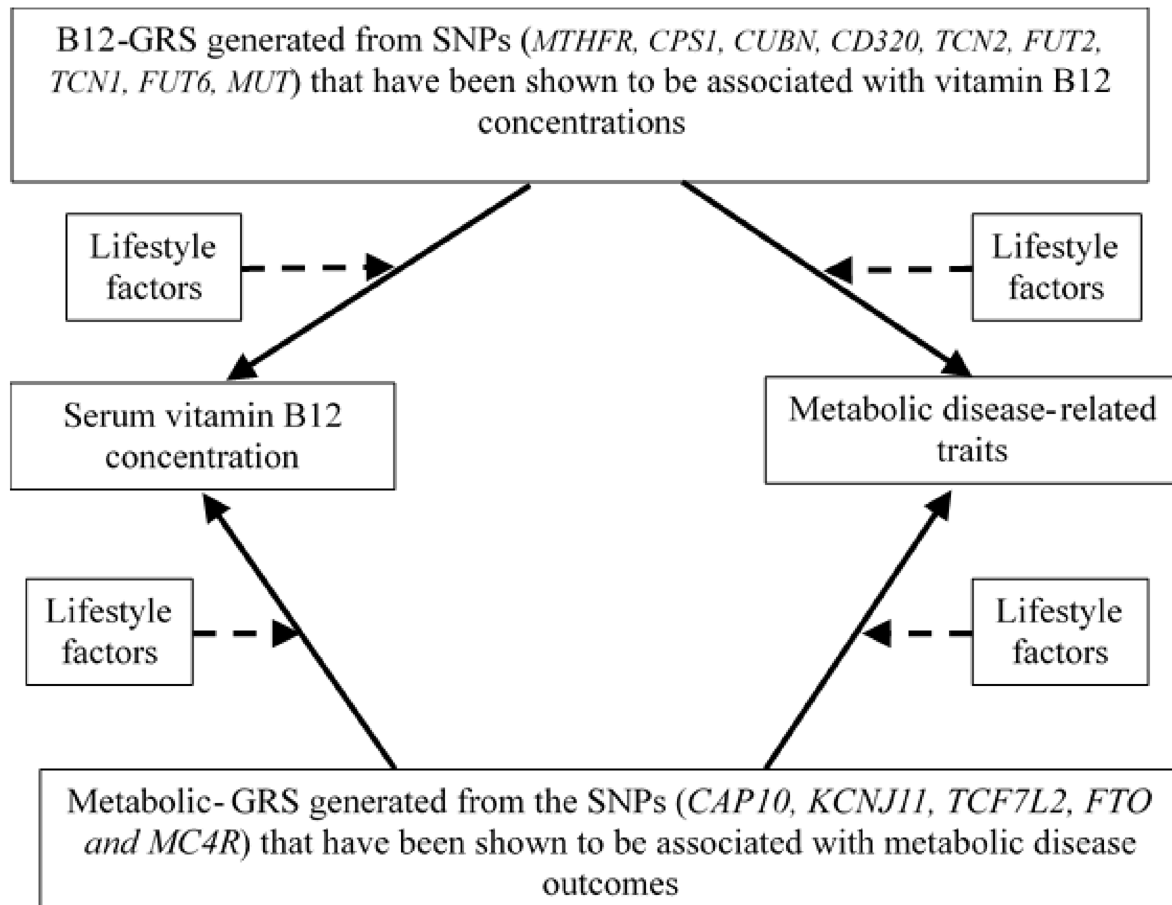


**Figure 15: Diagram representing the study design.**

*Four possible associations and four possible interactions were examined. One-sided arrows with unbroken lines represent genetic associations and one-sided arrows with broken lines represent interactions between a GRS and a lifestyle factor on serum vitamin B12/ metabolic traits. The association of the metabolic-GRS with vitamin B12 and metabolic disease-related traits and the association of B12–GRS with vitamin B12 and metabolic disease related traits were tested. Lastly, the impact of lifestyle factors (macronutrient intakes and physical activity levels) on these genetic associations was investigated.*

## 6.4 Results

### 6.4.1 Characteristics of the participants

In this study, 117 women (mean age, 40.40 ± 10.20 years; BMI, 25.10 ± 4.20 kg/m$^2$) were included. **Table 29** illustrates the main characteristics of the study participants.

**Table 29: Anthropometric and biochemical characteristics of women participants**

| | All women (N=117) | Non-obese* (N=32) | Obese** (N=85) | P value*** |
|---|---|---|---|---|
| Age (yrs) | 40.40 ± 10.20 | 35.70 ± 11.30 | 42.10 ± 9.20 | **0.006** |
| Height (cm) | 152.90 ± 5.20 | 154.90 ± 4.70 | 152.20 ± 5.20 | **0.012** |
| BMI (kg/m$^2$) | 25.10 ± 4.20 | 20.10 ± 2.10 | 27.00 ± 3.10 | **<0.001** |
| WC (cm) | 83.10 ± 12.50 | 72.80 ± 13.30 | 87.00 ± 9.70 | **<0.001** |
| Body fat (%) | 35.70 ± 7.00 | 27.00 ± 5.20 | 39.00 ± 4.30 | **<0.001** |
| Fasting serum Glucose (mg/dl) | 92.20 ± 20.20 | 85.70 ± 9.00 | 94.70 ± 22.70 | **0.033** |
| Fasting serum Insulin (mIU/L) | 32959 ± 26327 | 30372 ± 26179 | 33933 ± 26470 | 0.517 |
| HbA1C (ng/ml) | 662 ± 624 | 638 ± 606 | 672 ± 633 | 0.794 |

| | | | | |
|---|---|---|---|---|
| Fasting vitamin B12 (pg/mL) | 591 ± 579 | 426 ± 137 | 433 ± 193 | 0.795 |
| Physical Activity Levels | Sedentary (39.30%)<br>Moderate (49.60%)<br>Vigorous (11.10%) | Sedentary (46.90%)<br>Moderate (40.60%)<br>Vigorous (12.50 %) | Sedentary (36.50%)<br>Moderate (52.90 %)<br>Vigorous (10.60 %) | 0.490[a] |
| Total energy (kcal/d) | 1774 ± 609 | 1849 ± 585 | 1746 ± 619 | 0.416 |
| Protein (g) | 76.90 ± 36.50 | 80.50 ± 29.00 | 75.50 ± 39.00 | 0.514 |
| Fat (g) | 59.00 ± 33.10 | 67.30 ± 27.70 | 55.80 ± 34.60 | 0.096 |
| Carbohydrate (g) | 233 ± 71 | 230 ± 70 | 235 ± 72 | 0.714 |
| Dietary fibre (g) | 8.80 ± 4.50 | 9.70 ± 4.80 | 8.50 ± 4.40 | 0.222 |
| Saturated Fat (g) | 20.90 ± 11.10 | 23.70 ± 11.10 | 19.80 ± 10.90 | 0.085 |
| MUFA (g) | 8.20 ± 4.50 | 9.80 ± 5.20 | 7.50 ± 4.20 | **0.015** |

| PUFA (g) | 6.30 ± 3.50 | 6.80 ± 3.20 | 6.10 ± 3.60 | 0.332 |
|---|---|---|---|---|

*Data shown are represented as means ± SD*

*P values were calculated by using the Independent t test*

**Non-Obese individuals refers to the percentage of individuals with a BMI of under 23 according to the Asia-Pacific classification of BMI.*

***Obesity cases refers to the percentage of individuals with a BMI of equal to or over 23 according to the Asia-Pacific classification of BMI.*

****P values for the differences in the means/ proportions between non-obese and obese individuals*

*[a]P values were calculated by using the Chi Squared test*

*Abbreviations: BMI Body mass index; WC Waist circumference; MUFA Monounsaturated fatty acids; PUFA Polyunsaturated fatty acids.*

### 6.4.2 Association between B12-GRS and metabolic-GRS with biochemical and anthropometric measurements

After correction for multiple testing, none of the associations of the B12-GRS with vitamin B12 and metabolic traits (P> 0.160) were statistically significant (**Table 30**). Furthermore, no associations between the metabolic-GRS and vitamin B12 or metabolic traits (P>0.085) were observed (**Table 31**).

**Table 30: Association of the B12-GRS with obesity traits, biochemical traits and anthropometric measurements**

| GRS | BMI ($kg/m^2$) | WC (cm) | Body Fat (%) | Fasting serum glucose (mg/dl) | Fasting serum insulin (mIU/L) | HbAC1 (ng/ml) | Vitamin B12 (pg/ml) |
|---|---|---|---|---|---|---|---|
| **≤ 8 risk alleles** | 24.90 ± 4.10 | 82.10 ± 13.20 | 35.40 ± 7.10 | 89.90 ± 9.30 | 31331 ± 24636 | 625 ± 579 | 452 ± 187 |
| **≥ 9 risk alleles** | 25.50 ± 4.30 | 84.60 ± 11.00 | 36.30 ± 6.90 | 96.10 ± 30.60 | 35659 ± 29008 | 726 ± 693 | 392 ± 156 |
| **P value** | 0.468† | 0.456* | 0.898* | 0.193* | 0.328* | 0.444* | 0.160* |

*Values are given as mean ± standard deviation.*

*P values for differences between ≤8 and ≥9 risk alleles were obtained using linear regression model adjusted for age, sex and BMI.*

*† P values were obtained by using a general linear model adjusted for age and sex*

**P values were based on the log transformed values*

*Abbreviations: BMI body mass index; WC waist circumference; HbAC1 glycated haemoglobin*

**Table 31: Association of the metabolic-GRS with obesity traits and biochemical and anthropometric measurements**

| GRS | BMI (kg/$m^2$) | WC (cm) | Body Fat (%) | Fasting serum glucose (mg/dl) | Fasting serum insulin (mIU/L) | Glycated Haemoglobin (ng/ml) | Vitamin B12 (pg/ml) |
|---|---|---|---|---|---|---|---|
| **≤ 4 risk alleles** | 24.50 ±4.10 | 82.30 ± 14.40 | 35.00 ± 6.80 | 95.00 ± 26.00 | 33764 ± 27805 | 670 ± 651 | 436 ± 174 |
| **≥ 5 risk alleles** | 25.80 ± 4.20 | 83.90 ± 10.20 | 36.50 ± 7.20 | 89.20 ± 10.30 | 32082 ± 24837 | 654 ± 598 | 426 ± 184 |
| **P value** | 0.085† | 0.570* | 0.383* | 0.361* | 0.785* | 0.653* | 0.778* |

*Values are given as mean ± standard deviation*

*P values for differences between ≤4 and ≥5 risk alleles were obtained using linear regression model adjusted for age, sex and BMI.*

*† P values were obtained by using a general linear model adjusted for age and sex*

**P values were based on the log transformed values*

*Abbreviations: BMI body mass index; WC waist circumference; HbAC1 glycated haemoglobin*

### 6.4.3 Interaction between the B12-GRS and dietary factors on biochemical and anthropometric measurements

We observed an interaction between the B12-GRS and dietary fibre intake (g) on log transformed HbA1C ($P_{interaction}$= 0.042) (**Figure 16 and Table 32**). Individuals who carried 9 or more risk alleles for vitamin B12 deficiency had 8.10 % higher HbAC1 concentrations (ng/ml) in the lowest tertile of fibre intake (g) (Mean ± S.D: 4.90 ± 1.00 g) compared to those with 8 or less risk alleles for vitamin B12 deficiency.

**Table 32: Interaction between the B12-GRS and metabolic-GRS and lifestyle factors on biochemical outcomes and anthropometric measurements**

| *Interaction between the GRS * lifestyle factors on BMI* | | | | |
|---|---|---|---|---|
| B12-GRS * fat energy % | B12-GRS * carbohydrate energy % | B12-GRS * protein energy % | B12-GRS * fibre (g) | B12-GRS * Physical activity levels |
| 0.933† | 0.685† | 0.993† | 0.155† | 0.682† |
| metabolic-GRS * fat energy % | metabolic-GRS * carbohydrate energy % | metabolic-GRS * protein energy % | metabolic-GRS * fibre (g) | metabolic-GRS * Physical activity levels |
| 0.422† | 0.230† | 0.110† | 0.273† | 0.757† |
| *Interaction between the GRS * lifestyle factors on Log waist circumference (cm)* | | | | |
| B12-GRS * fat energy % | B12-GRS * carbohydrate energy % | B12-GRS * protein energy % | B12-GRS * fibre (g) | B12-GRS * Physical activity levels |
| 0.444 | 0.875 | 0.395 | 0.547 | 0.706 |
| metabolic-GRS * fat energy % | metabolic-GRS * carbohydrate energy % | metabolic-GRS * protein energy % | metabolic-GRS * fibre (g) | metabolic-GRS * Physical activity levels |

| 0.812 | 0.072 | **0.032** | 0.648 | 0.796 |
|---|---|---|---|---|

| ***Interaction between the GRS * lifestyle factors on Log Body fat (%)*** | | | | |
|---|---|---|---|---|
| B12-GRS * fat energy % | B12-GRS * carbohydrate energy % | B12-GRS * protein energy % | B12-GRS * fibre (g) | B12-GRS * Physical activity levels |
| 0.275 | 0.064 | **0.034** | 0.697 | 0.419 |
| metabolic-GRS * fat energy % | metabolic-GRS * carbohydrate energy % | metabolic-GRS * protein energy % | metabolic-GRS * fibre (g) | metabolic-GRS * Physical activity levels |
| 0.775 | 0.844 | 0.568 | 0.423 | 0.253 |

| ***Interaction between the GRS * lifestyle factors on Log fasting serum glucose (mg/dl)*** | | | | |
|---|---|---|---|---|
| B12-GRS * fat energy % | B12-GRS * carbohydrate energy % | B12-GRS * protein energy % | B12-GRS * fibre (g) | B12-GRS * Physical activity levels |
| 0.347 | 0.260 | 0.368 | 0.380 | 0.315 |
| metabolic-GRS * fat energy % | metabolic-GRS * carbohydrate energy % | metabolic-GRS * protein energy % | metabolic-GRS * fibre (g) | metabolic-GRS * Physical activity levels |
| 0.634 | 0.771 | 0.929 | 0.537 | 0.056 |

| ***Interaction between the GRS * lifestyle factors on Log fasting serum insulin (mIU/L)*** | | | | |
|---|---|---|---|---|
| B12-GRS * fat energy % | B12-GRS * carbohydrate energy % | B12-GRS * protein energy % | B12-GRS * fibre (g) | B12-GRS * Physical activity levels |
| 0.757 | 0.341 | 0.073 | 0.215 | 0.629 |
| metabolic-GRS * fat energy % | metabolic-GRS * carbohydrate energy % | metabolic-GRS * protein energy % | metabolic-GRS * fibre (g) | metabolic-GRS * |

| | | | | Physical activity levels |
|---|---|---|---|---|
| 0.108 | 0.104 | 0.890 | 0.947 | 0.723 |
| ***Interaction between the GRS * lifestyle factors on Log HbAC1 (ng/ml)*** | | | | |
| B12-GRS * fat energy % | B12-GRS * carbohydrate energy % | B12-GRS * protein energy % | B12-GRS * fibre (g) | B12-GRS * Physical activity levels |
| 0.175 | 0.091 | 0.150 | **0.042** | 0.475 |
| metabolic-GRS * fat energy % | metabolic-GRS * carbohydrate energy % | metabolic-GRS * protein energy % | metabolic-GRS * fibre (g) | metabolic-GRS * Physical activity levels |
| 0.298 | 0.166 | 0.155 | 0.851 | 0.969 |
| ***Interaction between the GRS * lifestyle factors on Log Vitamin B12 (pg/ml)*** | | | | |
| B12-GRS * fat energy % | B12-GRS * carbohydrate energy % | B12-GRS * protein energy % | B12-GRS * fibre (g) | B12-GRS * Physical activity levels |
| 0.772 | 0.936 | 0.270 | 0.157 | 0.078 |
| metabolic-GRS * fat energy % | metabolic-GRS * carbohydrate energy % | metabolic-GRS * protein energy % | metabolic-GRS * fibre (g) | metabolic-GRS * Physical activity levels |
| 0.983 | 0.682 | 0.298 | 0.171 | 0.242 |

*P values were obtained by using a general linear model adjusted for age, sex, and BMI*

*† P values were obtained by using a general linear model adjusted for age and sex*

*Abbreviations: BMI body mass index*

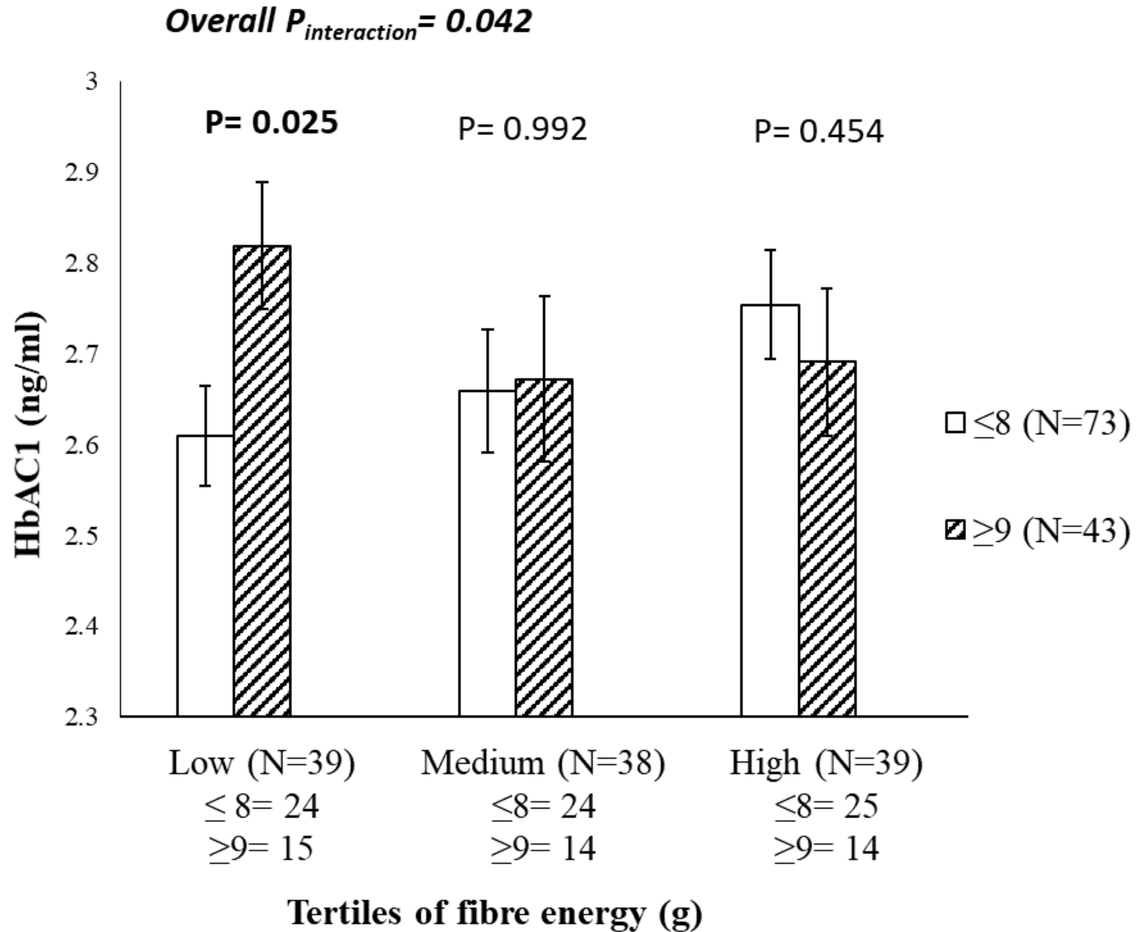


**Figure 16: Interaction between the B12-GRS and dietary fibre intake (g) on log HbAC1 (ng/ml) ($P_{interaction}$ = 0.042)**

*Among those who consumed a low fibre diet, individuals who carried 9 or more risk alleles had significantly higher levels of log HbAC1 compared to individuals carrying 8 or less risk alleles (P = 0.025). Error bars indicate Standard error.*

Interactions were also seen between the B12-GRS and protein (energy %) on log transformed body fat percentage (P = 0.034). However, further stratification of participants based on their consumption of low-, medium- and high-dietary protein (energy %) did not show a statistically significant association between the GRS and the outcomes in any of the tertiles.

### 6.4.4 Interaction between the metabolic-GRS and dietary factors on biochemical and anthropometric measurements

An interaction was found between the metabolic-GRS and protein (energy %) on log transformed WC (P=0.032) (**Table 32 and Figure 17**). Individuals who carried 5 or more risk alleles for metabolic disease had 2.15% lower WC measurements (cm) in the lowest tertile of protein energy intake (%) (Mean ± S.D: 1.91 ± 0.06 %) compared to those with 4 or less risk alleles (P= 0.027) (**Figure 17**).

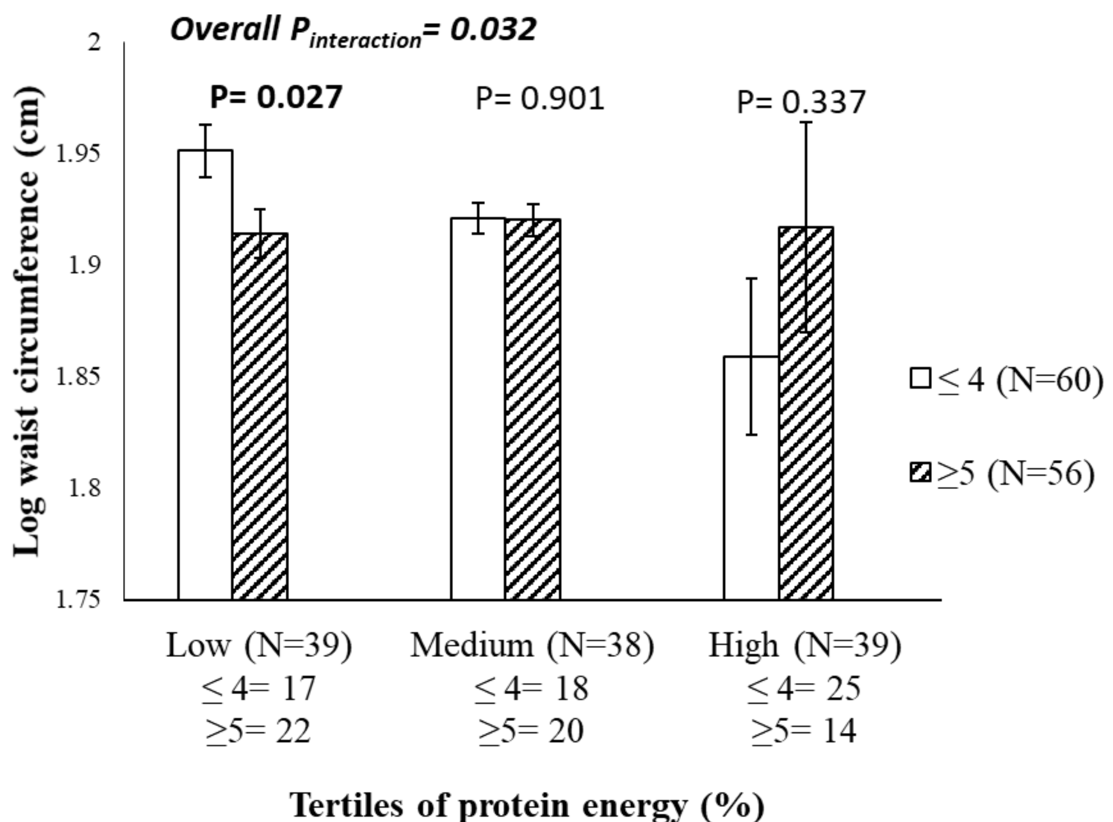


**Figure 17: Interaction between the metabolic-GRS and protein energy (%) on log waist circumference ($P_{interaction}$ = 0.032)**

*Among those who consumed a low protein diet, individuals who carried 5 or more risk alleles had significantly lower waist circumference measurements compared to individuals carrying 4 or less risk alleles (P = 0.027). Error bars indicate Standard error.*

### 6.4.5 Interaction between the B12-GRS and physical activity on biochemical and anthropometric measurements

No statistically significant interactions were observed between the two GRSs (vitamin B12 and metabolic traits) and physical activity on biochemical and anthropometric measurements (P>0.056) **(Table 32).**

After correction for multiple testing (Bonferroni corrected <0.000714), none of these GRS-lifestyle interactions were considered statistically significant.

## 6.5 Discussion

To our knowledge, this is the first study to use a nutrigenetic approach to explore the relationship between vitamin B12 status and metabolic traits in Indonesian women. Our study demonstrated the impact of genetically instrumented B12 concentrations on HbA1C levels, a marker of glycaemic control [444], through the influence of dietary fibre intake. Given that previous studies have shown that the consumption of dietary fibre is inadequate in Indonesian adults [445-447], our findings, if replicated in future studies, may have significant public health implications in terms of encouraging a consumer education campaign targeted around increasing fibre intake, in order to reduce HbA1C levels, which may be associated with improved glycaemic control.

In the present study, we constructed a GRS consisting of nine vitamin B12 decreasing SNPs in genes involved in vitamin B12 metabolism [14]. Our study showed that individuals carrying less than 8 risk alleles for vitamin B12 deficiency had higher vitamin B12 concentrations, compared to those carrying more than 9 risk alleles. However, there was no statistically significant difference between individuals carrying 8 or less risk alleles vs 9 or more risk alleles for the B12-GRS, which could be attributed to the small sample size. Furthermore, we were unable to identify any association between the B12-GRS and metabolic disease traits in our study, implying that linear decreases in vitamin B12 may not have a role

in the development of metabolic disease traits. Our finding goes in line with a Mendelian Randomization study investigating the effect of genetically instrumented vitamin B12 concentrations on BMI, where there was no evidence to suggest the causal role of decreased serum vitamin B12 levels in obesity [16].

Interestingly, in our study, we found a significant interaction between the B12-GRS and dietary fibre intake (g) on log HbA1C levels, where, among those who consumed a low fibre diet (Mean ± S.D: 13.60 ± 4.30), individuals carrying more than 9 risk alleles had significantly higher HbAC1 levels compared to those carrying 8 or less risk alleles. The average fibre intake in Indonesia is 10.5 g/day [448], which is lower compared to the mean fibre intake in the UK ( ~18g/day) and USA (~16g/day) [373]. In comparison to the mean fibre intake in Indonesia, the results in our study reported a lower mean fibre intake (8.80 ± 4.50 g). It is important that dietary intakes of fibre are increased in this population, as it may help maintain lower levels of HbAC1 levels in individuals carrying more than 9 risk alleles of the B12-GRS. Even though our study is the first to report this gene-diet interaction, a meta-analysis conducted from 15 randomized studies have shown that high fibre intake can reduce HbAC1 levels in type 2 diabetic subjects [449]. High fibre intake is generally recommended to reduce the risk of gestational diabetes (GDM) in pregnant women [450]. It has been shown that each 10g/day increment in total fibre intake, corresponds to a 26% reduced risk of GDM [450]. It is possible that high dietary fibre may increase satiety and consequently reduce total energy intake [451, 452]. Increased dietary fibre intake may also affect glucose homeostasis, by delaying gastric emptying, resulting in a slower absorption of glucose into the blood stream [450]. Additionally, low vitamin B12 status prevents erythropoiesis and prolongs the lifespan of erythrocytes, resulting in increased HbA1c levels [453]. This is the first study to provide evidence for an interaction between B12-GRS and HbA1c; hence, we do not have any previous studies to compare our findings with.

Accurately determining obesity has become an exceedingly important step in preventing the onset of metabolic syndrome or cardiometabolic diseases, which are brought about through excess adiposity. The underestimation of obesity, particularly in young women who appear to have a healthy BMI measure, could falsely lead to incorrect conclusions about body composition and future risk of diseases associated with increased adiposity, such as breast cancer [454]. The ability to measure body fat percentage is currently the preferred method of determining body composition over BMI, as it distinguishes between fat and lean body mass [455]. To date, little is known about the average body fat percentage in healthy Indonesian women. Although, a recent study conducted in 308 Indonesian women of Javanese ethnicity living in Yogyakarta Special Region Province (aged between 18-65), reported lower body fat % values (33.30 ± 7.70%) compared to our present study (35.70 ± 7.00 %) [456]. Within our study, an interaction between the B12-GRS and protein intake (energy %) on log transformed body fat percentage was observed. The exact mechanism of how dietary protein results in a more favourable body composition profile in individuals genetically predisposed to vitamin B12 deficiency is not known. A previous study conducted in 1,834 participants in Canada reported that high protein diets could reduce overall body fat percentage, even in the absence of energy restriction [457]. Further to this, it has been hypothesised that high protein diets increase the release of the anorectic gut hormone peptide YY (PYY), thus enhancing greater inter-meal satiety and reducing weight gain [458]. This suggestion for a novel interaction of protein (energy intake) in relation to body fat distribution in individuals genetically predisposed to vitamin B12 deficiency warrants further replication.

In a review analysing the nutrient intakes of pregnant women in Indonesia, it was reported that pregnant Indonesian women generally have protein intakes below the estimated average requirements [459]. The association between low protein intake and obesity outcomes has attracted interest amongst health care professionals. Observational studies in the USA have

reported that body weight and WC were reduced when protein was consumed above the recommended daily allowance [0.8 g/kg body weight (BW)] [460]. It has been noted in animal models, that pregnant rats consuming a low protein diet were more prone to GDM and to having offspring with a low birthweight [461, 462]. In our study, we found a significant interaction between the metabolic-GRS and protein energy (%) on log WC, where individuals consuming a low protein diet, despite carrying 5 or more risk alleles, had a lower waist to hip ratio compared to individuals carrying 4 or less risk alleles. There are no previous reports of the risk variants used in our GRS, but Goni et al [36] found that total protein intake interacted with a GRS of 16 obesity/lipid metabolism polymorphisms to modify the effect on body fat mass in 711 individuals of Caucasian ancestry. In our study, we only observed interaction of the metabolic-GRS with WC but not BMI, which suggests that effects of the GRS are likely to be on central obesity as opposed to common obesity in Indonesian women.

Significant interactions between genetic variants and physical activity on obesity traits have been reported in several studies from Europe and Asia [463, 464]. However, this is the first study to investigate interactions between the two GRSs and physical activity on metabolic traits and B12 concentrations in Indonesian women. In our study, as much as 39% of the women had low physical activity levels. These findings were much higher than the findings reported from another cross sectional study conducted across five major cities in Indonesia, who reported that 20% of women had a low physical activity status [465]. Although the majority of women in our study were physically inactive, no significant interactions were found between the GRSs and physical activity on metabolic traits/B12 status, which could be due to the small sample size of our study.

Major strengths of our study are that this is the first study of its kind to evaluate vitamin B12 status among Indonesian women. Furthermore, this study used a comprehensive, validated, interviewer administered food frequency questionnaire [440] to measure the long-

term macronutrient intake of the population. Nevertheless, several limitations of this study need to be considered. One of the main limitations of the study is the small sample size (N=117); however, we were still able to identify significant associations and gene-lifestyle interactions. Measurement errors in dietary assessment are inevitable since self-reported data on dietary intake are all subject to bias. We only included dietary data on total energy and macronutrient intake, but no data on specific foods or more specific types of micronutrients, which may potentially interact with GRS. Circulating concentrations of other vitamin B12 biomarkers, such as Holo-transcobalamin (holoTC) or Methylmalonic Acid (MMA) were not measured. Furthermore, all the women included in our analysis were of Minangkabau descent, and thus it is unknown whether our results can be generalized to other communities in Indonesia.

## 6.6 Conclusion

In conclusion, our study showed a significant effect of the B12-GRS on HbA1C concentrations, through the influence of a low dietary fibre intake. Additionally, our study failed to provide evidence for an impact of metabolic-GRS on lowering B12 concentrations. After correction for multiple testing, none of the interactions were statistically significant; hence, further replication studies utilizing larger sample sizes are needed to confirm our findings, before public health recommendations and personalised nutrition advice can be developed for Minangkabau Indonesian women.

**Author contributions:**

**Shelini Surendran performed the statistical analysis, data interpretation and wrote the manuscript.** ASA assisted with statistical analyses and data interpretation; KSV was involved in drafting the manuscript; KSV and ASA carried out data collection; KSV designed the nutrigenetics study; NIL, FFY and KSV conceived, supervised and designed the study; UA and SRS helped data collection, monitoring and evaluation of participants, and with project administration; NT was involved in data collection and with dietary data analysis; IRS was

involved with laboratory analysis. NIL SGM and JAL critically reviewed the manuscript. All authors read and approved the final manuscript.

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# Chapter 7

## A genetic approach to investigate the relationship between vitamin B12 status and cardio-metabolic traits in response to changes in dietary fat composition in adults with moderate cardiovascular disease risk

For this study, I collected and transferred the blood serum samples from the freezer in the Department of Food and Nutritional Sciences at the University of Reading to Royal Berkshire Hospital (Reading, UK) for vitamin B12 analysis. In addition, I organised the genotyping of the selected genetic variants at the LGC Genomics, UK. I was involved in generating and cleaning the dataset to perform statistical analysis. I developed the analysis plan, ran the entire statistical analysis using the SPSS software and wrote the first draft of the manuscript. I revised the manuscript based on the comments from all the co-authors. This paper has been submitted to the journal 'Lipids in Health and Disease'.

**Under review**

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### 7.1 Abstract

**Background:** Low vitamin B12 status has been reported to be a risk factor for several cardiometabolic traits such as obesity, diabetes and cardiovascular disease (CVD). Animal models have shown that the modification of dietary fat intake can affect vitamin B12 status. Hence, we investigated whether vitamin B12- and metabolic disease-related genetic variants

modify vitamin B12 concentrations and cardiometabolic traits in response to replacement of saturated fatty acids (SFA) with monounsaturated (MUFA) or n-6 polyunsaturated (PUFA) fatty acids.

**Methods:** This describes a secondary analysis of the Dietary Intervention and VAScular function (DIVAS) study. The DIVAS study was a randomized, single-blind, parallel-group dietary intervention, in which 119 men and women aged 21-60 y from the United Kingdom with moderate CVD risk followed one of three isoenergetic diets rich in SFA, MUFA or n-6 PUFA for 16 weeks. Genetic risk scores (GRS) based on three vitamin B12 associated single nucleotide polymorphisms (SNPs) (B12-GRS) and six metabolic SNPs (metabolic-GRS) were constructed.

**Results:** After the 16-week intervention, post-hoc tests indicated no significant interactions between changes in vitamin B12 concentrations and the three dietary groups and the B12- and metabolic-GRSs . For the metabolic-GRS, individuals with 6 or more risk alleles showed a significant reduction in 24-hour ambulatory systolic blood pressure after the MUFA diet (n = 13; -7± 8 mm Hg) compared to the SFA (n = 15; 2 ± 7 mm Hg /or n-6 PUFA dietary groups (n = 16; 2 ± 9 mm Hg) ($P_{interaction}$ = 0.012). However, this interaction was not considered statistically significant after correction for multiple testing.

**Conclusions:** In summary, this post-hoc analysis demonstrated a greater sensitivity of the metabolic-GRS to dietary fat composition with a 9 mmHg lower 24-hour ambulatory systolic blood pressure observed following substitution of SFA with MUFA, but not n-6 PUFA. However, our findings failed to provide evidence for an impact of the B12-GRS on cardiometabolic traits. Further large intervention studies incorporating prospective genotyping are required to confirm or refute our findings.

## 7.2 Introduction

Vitamin B12 is an essential micronutrient associated with the one carbon metabolic pathway, a cycle related to the synthesis of DNA, protein and lipids [72, 466]. In recent years, new functional roles of vitamin B12 have emerged, linking the water-soluble vitamin to various non-communicable diseases. Observational studies have provided evidence for an association of low serum B12 concentrations with unfavourable metabolic phenotypes, including future type 2 diabetes (T2D) [70], insulin resistance [67], cardiovascular disease (CVD) [10, 115], obesity[84] and dyslipidaemia [9]. The basis of low vitamin B12 concentrations and its association with these metabolic phenotypes is still under debate and could be the result of several mechanisms. It has been hypothesised that low vitamin B12 leads to adipocyte dysfunction by modulating lipid metabolism and enhancing cellular inflammation [467]. Furthermore, vitamin B12 insufficiency has been shown to disturb the methylation of genes involved in the regulation of cholesterol biosynthesis, such as sterol regulatory element binding transcription factor 1 (*SREBF1*) and LDL receptor, consequently increasing the expression of cholesterol biosynthesis in adipose tissue [468]. On the other hand, it has been suggested that vitamin B12 deficiency is more prevalent in obese subjects due to increased vitamin B12 demands secondary to an increased surface area of the body [84, 91] or as a result of individuals consuming a diet low in micronutrient density [92].

At present the true prevalence of vitamin B12 deficiency in the United Kingdom is currently unknown. A recent nationwide survey reported the estimated prevalence of vitamin B12 deficiency (≤150 pmol/L) in 299 women of childbearing age (19-36 yrs) to be 12.4% [469]. In addition, a cross sectional analysis involving 689 men in the UK (226 omnivores, 231 vegetarians and 232 vegans) reported that 52% of vegans, 7% of vegetarians and 0.44% of omnivores were vitamin B12 deficient (<118 pmol/L) [470]. Vitamin B12 concentrations of individuals are highly heterogenous, with interindividual variability persisting as a result of

environmental and genetic factors [14]. Several single nucleotide polymorphisms (SNPs) in the fucosyltransferase 2 (*FUT2*) gene have shown associations with circulating vitamin B12 and, to date, rs492602 and rs602662 have been the most extensively studied [14]. Furthermore, recent studies have shown suggestive associations between variants of *FUT2* with diabetes (Type 1 and Type 2) and BMI [16, 255-257]. Given that obesity and being overweight may modulate the status of vitamin B12 [7, 84], it is important to examine the role of genes involved in obesity-related traits with vitamin B12 status.

In the Dietary Intervention and VAScular function (DIVAS) study, the isoenergetic replacement of 9.5–9.6% total energy (TE) dietary saturated (SFAs) with cis-monounsaturated (MUFAs) fatty acids for 16 weeks in 195 UK adults at moderate CVD risk resulted in beneficial effects on lipid biomarkers and ambulatory night-time blood pressure [471]. Given that the modification of dietary fat intake has been shown to affect vitamin B12 status in rats [472], this post-hoc analysis presents additional outcome measures from the DIVAS study exploring the effect of dietary fat composition on vitamin B12 status in individuals with moderate CVD risk. To investigate whether genetic polymorphisms contribute to any observable changes in vitamin B12 status and cardiometabolic disease risk markers, a retrospective post hoc analysis of the DIVAS study was conducted. We examined whether nine SNPs (6 metabolic SNPs and 3 vitamin B12 SNPs) modified the response of vitamin B12 concentrations and cardiometabolic traits, after substitution of SFA with MUFA or polyunsaturated fatty acids (n-6 PUFA) in 119 participants at moderate CVD risk.

## 7.3 Methodology

### 7.3.1 Study participants

The DIVAS study was a single-blinded, parallel, randomised controlled trial. A detailed description of the study criteria and methods has been previously published elsewhere [471, 473]. In brief, non-smoking men and women, aged between 21 and 60 years

with moderate risk of CVD, were recruited from Reading, UK and the surrounding area in three cohorts between November 2009 and July 2012. A scoring tool [473] was used to determine the presence of single or multiple CVD risk factors, including elevated fasted measures of serum total cholesterol (TC) and glucose, low high-density lipoprotein (HDL) cholesterol, raised blood pressure, increased BMI or waist circumference (WC), and a family history of premature myocardial infarction or T2D. Participants who were eligible had a risk score of ≥2 combined points, reflecting a moderate CVD risk (≥50% above the population mean). Other criteria for exclusion were the presence of abnormal fasting blood biochemistry, and taking dietary/vitamin supplements or medication for hypertension, raised lipids, or inflammatory disorders [473]. **Table 33** shows the characteristics of the study participants at baseline.

**Table 33: Baseline characteristics of study participants in the whole group and stratified by sex**

| Characteristics | n | Total participants | n | Men | n | Women | P value * |
|---|---|---|---|---|---|---|---|
| Age (years) | 119 | 47 ± 9 | 53 | 48 ± 9 | 66 | 46 ± 9 | 0.257 |
| BMI (kg/m$^2$) | 119 | 26.4 ± 4.0 | 53 | 26.7 ± 3.5 | 66 | 26.2 ± 4.3 | 0.494 |
| WC (cm) | 111 | 90.7 ± 11.9 | 49 | 96.2 ± 11.1 | 62 | 86.4 ± 10.8 | **<0.0001** |
| WHR | 100 | 0.87 ± 0.09 | 39 | 0.94 ± 0.07 | 61 | 0.82 ± 0.06 | **<0.0001** |
| 24 h Ambulatory Systolic BP (mm Hg) | 97 | 122 ± 11 | 39 | 126 ± 9 | 58 | 120 ± 11 | **0.007** |
| 24 h Ambulatory Diastolic BP (mm Hg) | 97 | 75 ± 8 | 39 | 78 ± 7 | 58 | 74 ± 7 | **0.019** |
| Total Cholesterol (mmol/L) | 109 | 5.61 ± 1.09 | 47 | 5.81 ± 1.09 | 62 | 5.45 ± 1.07 | 0.090 |
| HDL Cholesterol (mmol/L) | 109 | 1.54 ± 0.36 | 47 | 1.37 ± 0.32 | 62 | 1.68 ± 0.33 | **<0.0001** |
| LDL Cholesterol (mmol/L) | 109 | 3.80 ± 0.98 | 47 | 4.11 ± 0.97 | 62 | 3.57 ± 0.93 | **0.004** |
| TAG (mmol/L) | 109 | 1.29 ± 0.60 | 47 | 1.64 ± 0.60 | 62 | 1.02 ± 0.45 | **<0.0001** |
| Glucose (mmol/L) | 109 | 5.12 ± 0.43 | 47 | 5.24 ± 0.44 | 62 | 5.03 ± 0.40 | **0.008** |
| Insulin (pmol/L) | 119 | 1.40 ± 0.24 | 53 | 1.43 ± 0.25 | 66 | 1.37 ± 0.22 | 0.097 |
| HOMA-IR | 109 | 1.09 ± 0.71 | 47 | 1.25 ± 0.89 | 62 | 0.97 ± 0.49 | 0.054 |
| Vitamin B12 (ng/L) | 96 | 413.5 ± 161.6 | 42 | 413.1 ± 171.7 | 57 | 413.8 ± 155.3 | 0.982 |

*Data shown are represented as means ± SD, wherever appropriate. P values for the differences in the means between men and women. P values were calculated by using an independent t-test*

*BMI body mass index, BP blood pressure, HDL high- density lipoprotein cholesterol, HOMA-IR homeostasis model assessment—insulin resistance, LDL low- density lipoprotein cholesterol, TAG triacylglycerol, WC waist circumference, WHR waist to hip ratio*

The West Berkshire Local Research ethics committee (09/ H0505/56) and the University of Reading Research Ethics Committee (09/40) gave a favourable ethical opinion for conduct, and each volunteer gave written informed consent before participating. The trial was registered at www.clinicaltrials.gov as NCT01478958. All protocols and procedures were performed according to the Declaration of Helsinki. In our retrospective analysis, 119 of the 195 participants who completed the DIVAS study consented to genetic analysis and were included in the present study. Only 96 participants had samples available for vitamin B12 analysis.

### 7.3.2 Study design and diets

A detailed description of the DIVAS study design and methods has been reported elsewhere [471, 473]. Clinical visits took place at the Hugh Sinclair Unit of Human Nutrition, University of Reading, during weeks 0 (baseline; V1) and 16 (after intervention; V2) [471]. The participants were randomly assigned to one of three 16 weeks of dietary intervention diets, based on a minimization program that matched for age, sex, BMI, and total CVD risk score. The three isoenergetic intervention diets (%TE target compositions, SFA:MUFA:n–6 PUFA) were rich in SFAs (17:11:4), MUFAs (9:19:4), or n–6 PUFAs (9:13:10). All three intervention diets provided 36% TE from total fat, and intakes of other macronutrients were unchanged. After the intervention, a greater SFA exchange than the target 8% TE was achieved: SFA vs MUFA was 9.5% TE and SFA vs n-6 PUFA was 9.6% TE [473]. Dietary intakes were determined from 4-d weighed diet diaries completed at baseline (week 0) and during the intervention (weeks 8 and 16), which were analysed by using Dietplan 6.6 (Forestfield software).

### 7.3.3 Anthropometric measurements and biochemical parameters

In brief, volunteers attended the Hugh Sinclair Unit of Human Nutrition (University of Reading) at baseline (visit 1) and week 16 (visit 2) after an overnight fast. Study participants were asked to refrain from alcohol or organised exercise regimens 24 h before visits. Participants were provided with a low fat (<10 g fat) evening meal to standardise short-term fat intake and only drank low-nitrate water during this time. After a 12 h overnight fast, a blood sample was taken at each visit.

Serum vitamin B12 concentrations were measured using an electrochemiluminescence immunoassay (ECLIA) kit on a Roche 6000 series e601 immunoassay analyser (Roche Diagnostics GmbH, Hoffmann-La Roche ltd, Mannheim, Germany) at Royal Berkshire Hospital. Fasting serum glucose and lipids (TC, triacylglycerol (TAG), and HDL cholesterol) were measured using an ILAB600 clinical chemistry analyser (reagents and analyser: Werfen UK Ltd). The LDL cholesterol concentration was calculated using the Friedewald formula [343]. Fasting plasma insulin levels were determined by commercial ELISA kits (Dako Ltd, High Wycombe, UK). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the fasting glucose and insulin data [fasting insulin (pmol/L) x fasting glucose (mmol/l)]/135] [474].

At weeks 0 and 16, BMI was calculated as weight (in kg) divided by the square of body height (m). Height was recorded to the nearest 0.5 cm using a wall-mounted stadiometer and weight was measured using a digital scale (Tanita Europe) using standard settings (standard body type and 1 kg for clothing). Twenty-four hour ambulatory blood pressure (ABP) was measured every 30 min from 07:00 to 21:59 and every 60 min from 22:00 to 06:59, approximately 48 h before the clinical visits, using A/A grade automated oscillometric ABP monitors (A&D Instruments Ltd.). Participant activity forms which contained the recordings

of participants' sleep times, were used to calculate the mean 24-h day and night blood pressure measurements [471].

### 7.3.4 SNP selection and genotyping

In total, nine SNPs were examined in the present study. SNPs for vitamin B12-GRS were selected using the tagSNP approach. TagSNPs in the gene fucosyltransferase 2 (*FUT2*) were selected using genotype data from the International HapMap collected in individuals of Northern and Western European ancestry (CEU) (HapMap Data Rel 27 Phase 2+3, Feb 09, NCBI B36 assembly, dbSNP b126). The Haploview software V3.3 (http://www.broadinstitute.org/haploview/haploview-downloads) was used to assess the linkage disequilibrium (LD) structure between SNPs. Tagger software was used to select tagSNPs with the 'pairwise tagging only' option and an r2 threshold of >0.8 (±10kb upstream and downstream of the genes). In the tagSNP selection, after excluding SNPs with low minor allele frequency (<5%), there were 3 tagSNPs (rs602662, rs492602 and rs16982241) representing the common genetic variations across the *FUT2* gene. The six metabolic disease-related SNPs were selected for our analysis based on previously published candidate gene association and genome-wide association (GWA) studies for cardiometabolic disease-related traits and associations of the SNPs with dietary intake of macronutrients [370-372, 475-481]: Fat mass and obesity-associated [*FTO*]- rs8050136 and rs9939609, Melanocortin 4 Receptor [*MC4R*]- rs17782313 and rs2229616 and Transcription factor 7-like 2 [*TCF7L2*]- rs7903146 and rs12255372.

DNA was extracted from the buffy coat using a QIAamp DNA blood kit (QIAGEN) and stored at −20 °C from 119 of the participants who consented for genotyping. The genotyping of the SNPs was outsourced to LGC Genomics

(http://www.lgcgroup.com/services/ genotyping), which employs the competitive allele-specific PCR-KASP® assay.

### 7.3.5 Statistical analysis

Statistical analysis was carried out using the SPSS software (version 21; SPSS Inc, Chicago, IL, USA). All biochemical outcomes were expressed as means ± standard deviation (SD) in the tables and text, and as standard error in the figure. All the tested variables were checked for normality prior to statistical analysis; BMI, HOMA-IR and insulin levels were not normally distributed in our study population; therefore, the data were natural log-transformed prior to analysis. The minor allele frequency was calculated by gene counting. Nine of the SNPs were in Hardy Weinberg Equilibrium (HWE) ($P > 0.05$) (**Table 34**). HWE was not calculated for the SNP *MC4R* rs2229616 as no minor alleles were present. Independent t-tests were used to compare means between men and women at baseline.

**Table 34: Genotype distribution of vitamin B12-related SNPs and metabolic disease-related SNPs**

| Gene | rs number | Major allele | Minor allele | Common Homozygotes n (%) | Heterozygotes n (%) | Rare Homozygotes n (%) | Minor allele frequency | HWE P value |
|---|---|---|---|---|---|---|---|---|
| *FUT2* | rs602662 | G | A | 34 (28.6) | 60 (50.4) | 25 (21.0) | 0.46 | 0.877 |
| *FUT2* | rs492602 | A | G | 38 (31.9) | 58 (48.7) | 23 (19.3) | 0.44 | 0.918 |
| *FUT2* | rs16982241 | G | A | 91 (76.5) | 26 (21.8) | 2 (1.7) | 0.13 | 0.928 |
| *TCF7L2* | rs7903146 | C | T | 62 (52.1) | 46 (38.7) | 11 (9.2) | 0.29 | 0.564 |
| *TCF7L2* | rs12255372 | G | T | 66 (55.9) | 42 (35.6) | 10 (8.5) | 0.26 | 0.378 |
| *MC4R* | rs17782313 | T | C | 70 (59.8) | 38 (32.5) | 9 (7.7) | 0.24 | 0.243 |
| *MC4R* | rs2229616 | G | A | 113 (95.0) | 6 (5.0) | 0 (0) | 0.03 | -* |
| *FTO* | rs8050136 | C | A | 40 (33.9) | 57 (48.0) | 21 (17.8) | 0.42 | 0.929 |
| *FTO* | rs9939609 | T | A | 41 (34.5) | 57 (47.9) | 21 (17.6) | 0.42 | 0.877 |

*Abbreviations: HWE; Hardy Weinberg Equilibrium*

** HWE was not calculated for the SNP MC4R rs2229616 as no minor alleles were present.*

### 7.3.6 Genetic Risk Score

The unweighted, risk-allele GRS method was calculated for each participant as the sum of risk allele counts across each SNP which predicted vitamin B12 status or metabolic disease risk. The B12-GRS was generated from the SNPs in the gene *FUT2 (*rs602662, rs492602 and rs16982241), which have been shown to be associated with vitamin B12 concentrations. Furthermore, another unweighted GRS was created using allele markers previously reported to be associated with metabolic disease traits. The metabolic-GRS was generated from the SNPs in the genes *FTO* (rs8050136 and rs9939609)*, TCF7L2 (*rs7903146 and rs12255372) and *MC4R* (rs17782313 and rs2229616). A value of 0, 1 or 2 was assigned to each SNP, which denotes the number of risk alleles on that SNP. These values were then calculated by adding the number of risk alleles across each SNP. The average number of risk alleles per person for the B12-GRS was 4 (SD = 1), which ranged from 2 to 6. The sample was stratified, by the median into a "low genetic risk group" for those with a GRS ≤ 3 risk alleles ($n$ = 41), and into a "high genetic risk group" for those with a GRS ≥ 4 risk alleles ($n$ = 78). For the metabolic-GRS, the average number of risk alleles per person was 5 (SD = 2), which ranged from 2 to 11. The sample was stratified, by the median, into a "low genetic risk group" for those with a GRS ≤ 5 risk alleles ($n$ = 68), and into a "high genetic risk group" for those with a GRS ≥ 6 risk alleles ($n$ = 51). The baseline and week 16 associations for the two GRSs with continuous phenotypes were evaluated by the general linear model (GLM). Moreover, potential interactions between the two GRSs and dietary intervention on changes in vitamin B12 concentrations and cardiometabolic traits over the 16-week intervention were analysed by using GLM, where an interaction term was included in the model. Potential confounders associated with the outcomes were adjusted in all GLM analyses (i.e. age, sex, BMI, and ethnicity). When a significant diet x genotype interaction was found, data were split by genotype group and analysed further by using GLM. A Bonferroni correction was applied and

the significant P value was 0.0019 [0.05/2 GRSs * 13 phenotypic outcomes (vitamin B12, 24 h ambulatory systolic blood pressure, 24 h ambulatory diastolic blood pressure, total cholesterol, HDL cholesterol, LDL cholesterol, TAG, glucose, insulin, HOMA-IR, BMI, WC and WHR)].

## 7.4 Results

Baseline clinical and biochemical characteristics of the individuals from the retrospective analysis from the DIVAS study are presented in Table 1.Men had higher WC, WHR, 24 h ambulatory systolic and diastolic blood pressures, fasting LDL cholesterol, TAG and glucose concentrations (P<0.019) and lower levels of fasting HDL cholesterol (P<0.0001) compared to women (**Table 33**). No significant differences in fasting serum vitamin B12 concentrations (P = 0.982) between men (413 ± 171 (ng/L) and women (414 ± 155 (ng/L) were observed in this study (**Table 33**). Additionally, there was no overall significant difference (P>0.539) in dietary vitamin B12 intake (**Table 35**) and serum vitamin B12 concentrations (**Table 35**), between the dietary intervention groups after the 16-week intervention.

**Table 35: Reported daily composition of vitamin B12 and serum vitamin B12 at baseline (week 0) and after diets rich in SFAs, MUFAs, and n–6 PUFAs (week 16) in adults with moderate risk of cardiovascular disease**

| | **SFA** | | | **MUFA** | | | **n-6 PUFA** | | | ****P value** |
|---|---|---|---|---|---|---|---|---|---|---|
| | Baseline (n=40) | Post (n=39) | Δ (n=37) | Baseline (n=35) | Post (n=34) | Δ (n=33) | Baseline (n=42) | Post (n=40) | Δ (n=40) | |
| **Dietary vitamin B12 (μg/day)** | 5.29 ± 2.20 | 5.25 ± 1.93 | -0.02 ± 1.65 | 4.74 ± 1.55 | 5.22 ± 2.28 | 0.46 ± 2.27 | 6.89 ± 6.47 | 6.11 ± 3.32 | -0.86 ± 7.56 | 0.539 |
| | | *P=0.939 | | | *P=0.254 | | | *P=0.474 | | |
| **Vitamin B12 (ng/L)** | Baseline (n=27) | Post (n=27) | **Δ** (n=26) | Baseline (n=27) | Post (n=27) | **Δ** (n=26) | Baseline (n=36) | Post (n=34) | **Δ** (n=34) | |
| | 384 ± 164 | 384 ± 151 | -0.08 ± 71.66 | 395 ± 116 | 379 ± 112 | -20.0 ± 66.4 | 457 ± 182 | 436 ± 160 | -26.4 ± 56.5 | 0.188 |
| | | *P=0.995 | | | *P=0.138 | | | ***P=0.010** | | |

*Values are given as means ± standard deviations. Dietary intakes estimated from 4-d weighed diet diaries at baseline (week 0) and after intervention (mean of weeks 8 and 16).*

**The difference between baseline and Post-intervention intakes were identified by using paired-samples T test.*

*** The overall effect of diet based on change from baseline after 16-week intervention was derived by univariate analysis (general linear model) for between-group comparisons adjusting for BMI, age, sex and ethnicity.*

The genotype distributions of the polymorphisms involved in the B12-GRS and metabolic-GRS are shown in **Table 34**. The participants' characteristics at the beginning of the dietary interventions (week 0) are presented in **Table 36** according to the B12-GRS and metabolic-GRS scores. There was an association between the B12-GRS and fasting HDL cholesterol concentration ($P_{association}$ = 0.035), where individuals who carried 4 or more risk alleles for vitamin B12 deficiency had higher concentrations ( 1.58 ± 0.37 mmol/L) compared to those with 3 or less risk alleles (1.47 ± 0.33 mmol/L). There was a significant association between the B12-GRS and fasting TAG concentration ($P_{association}$ = 0.016), where individuals carrying 4 or more risk alleles for vitamin B12 deficiency had 19.6 % lower TAG levels (1.19 ± 0.56 mmol/L) compared to individuals carrying 3 or less risk alleles (1.48 ± 0.65 mmol/L). However, these findings were not significant after correction for multiple testing (Bonferroni corrected P>0.0019). None of the variables (including vitamin B12, 24 h ambulatory systolic and diastolic blood pressure, total cholesterol, HDL cholesterol, LDL cholesterol, TAG, glucose, insulin, HOMA-IR, BMI, WC and WHR) were associated with the metabolic-GRS at baseline (**Table 36**). After 16 weeks of dietary intervention, there was no significant association of the B12-GRS and metabolic GRSs with changes in the vitamin B12 concentrations after Bonferroni correction (**Table 37 and Table 38**).

**Table 36: Association of the B12-GRS and metabolic-GRS with obesity traits and fasting biochemical traits**

| Number of risk alleles | BMI ($kg/m^2$) | WC (cm) | WHR | 24 h Ambulatory SBP (mm Hg) | 24 h Ambulatory DBP (mm Hg) | Total cholesterol (mmol/L) | HDL-C (mmol/L) | LDL-C (mmol/L) | TAG (mmol/L) | Glucose (mmol/L) | Insulin (pmol/L) | HOMA-IR | Vitamin B12 (ng/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | **Vitamin B12-GRS** | | | | | | | |
| **≤3 risk alleles** | 26.3 ± 3.4 | 91.4 ± 10.4 | 0.87 ± 0.07 | 124 ± 11 | 76.9 ± 7.3 | 5.79 ± 1.14 | 1.47 ± 0.33 | 4.02 ± 1.03 | 1.48 ± 0.65 | 5.18 ± 0.40 | 28.0 ± 14.05 | 1.04 ± 0.49 | 435 ± 185 |
| **≥4 risk alleles** | 26.5 ± 4.2 | 90.3 ± 12.8 | 0.87 ± 0.10 | 121 ± 10 | 74.7 ± 7.6 | 5.51 ± 1.06 | 1.58 ± 0.37 | 3.69 ± 0.94 | 1.19 ± 0.56 | 5.06 ± 0.44 | 29.5 ± 19.2 | 1.11 ± 0.80 | 403 ± 149 |
| **P value** | 0.657†* | 0.207 | 0.625 | 0.197 | 0.219 | 0.685 | **0.035** | 0.367 | **0.016** | 0.075 | 0.729* | 0.529* | 0.311 |
| | | | | | | **Metabolic-GRS** | | | | | | | |
| **≤5 risk alleles** | 26.1 ± 3.38 | 89.4 ± 10.8 | 0.86 ± 0.08 | 121 ± 10 | 75.1 ± 7.4 | 5.66 ± 1.04 | 1.56 ± 0.37 | 3.84 ± 0.95 | 1.31 ± 0.58 | 5.13 ± 0.48 | 27.7 ± 12.4 | 1.04 ± 0.50 | 411 ± 144 |
| **≥6 risk alleles** | 26.9 ± 4.6 | 92.4 ± 13.1 | 0.88 ± 0.10 | 124 ± 11 | 75.8 ± 7.7 | 5.54 ± 1.15 | 1.53 ± 0.34 | 3.76 ± 1.02 | 1.27 ± 0.64 | 5.10 ± 0.34 | 30.8 ± 22.7 | 1.16 ± 0.92 | 417 ± 185 |
| **P value** | 0.335†* | 0.913 | 0.947 | 0.435 | 0.870 | 0.309 | 0.928 | 0.350 | 0.142 | 0.714 | 0.634* | 0.711* | 0.784 |

*Values are given as mean ± standard deviation*

*P values for differences between ≤3 and ≥4 risk alleles were obtained using linear regression model adjusted for age, BMI, ethnicity and sex*

*† P values were obtained by using a general linear model adjusted for age, ethnicity and sex*

**P values were based on the log transformed values*

**Table 37: Changes in anthropometric traits and fasting biochemical traits after dietary intervention over 16 weeks according to the B12-GRS**

| B12-GRS | SFA diet | | P association | MUFA diet | | P association | n-6 PUFA diet | | $P_{association}$ | $P_{interaction}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | ≤3 risk alleles | ≥ 4 risk alleles | | ≤ 3 risk alleles | ≥ 4 risk alleles | | ≤ 3 risk alleles | ≥ 4 risk alleles | | |
| Log BMI (kg/m$^2$) | -0.00 ± 0.01 | -0.00 ± 0.01 | 0.479 † | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.530† | 0.00 ± 0.01 | 0.00 ± 0.01 | 0.135† | 0.900†† |
| WC (cm) | -0.66 ± 4.99 | -1.11 ± 3.44 | 0.770 | -1.91± 2.88 | -1.51 ± 3.18 | 0.545 | -0.43 ± 2.32 | -0.08 ± 3.79 | 0.881 | 0.798 |
| WHR | 1.26 ± 4.29 | -1.57 ± 3.97 | 0.115 | -1.87 ± 4.36 | -0.66 ± 4.03 | 0.354 | 0.03 ± 3.78 | 0.11 ± 4.48 | 0.978 | 0.166 |
| 24 h Ambulatory systolic blood pressure (mm Hg) | -1.80 ± 8.32 | 1.74 ± 7.37 | 0.162 | -4.91 ± 5.59 | -0.29 ± 9.57 | 0.087 | -0.73 ± 8.84 | 1.41 ± 8.64 | 0.424 | 0.822 |
| 24 h Ambulatory diastolic blood pressure (mm Hg) | -0.10 ± 6.08 | 1.48 ± 4.32 | 0.156 | -1.82 ± 4.69 | 0.18 ± 6.48 | 0.203 | -0.46 ± 5.87 | -0.28 ± 6.17 | 0.869 | 0.788 |
| Total cholesterol (mmol/L) | 0.41 ± 0.64 | 0.30 ± 0.48 | 0.256 | -0.55 ± 0.89 | -0.36 ± 2.73 | 0.923 | -0.05 ± 0.71 | -0.33 ± 1.57 | 0.704 | 0.849 |
| HDL Cholesterol (mmol/L) | 0.64 ± 2.01 | -0.22 ± 1.39 | 0.085 | -0.02 ± 0.15 | 0.01 ± 0.18 | 0.987 | 0.50 ± 1.89 | 0.05 ±2.04 | 0.524 | 0.566 |
| LDL Cholesterol (mmol/L) | -0.29 ± 2.48 | 0.54 ± 1.58 | 0.392 | -1.28 ± 3.30 | 0.12 ± 1.92 | 0.105 | 0.14 ± 3.61 | 0.02 ± 1.81 | 0.955 | 0.393 |
| TAG (mmol/L) | -0.67 ± 2.10 | 0.34 ± 1.41 | 0.123 | -0.01 ± 0.42 | 0.03 ± 0.33 | 0.990 | -0.10 ± 0.29 | -0.85 ± 2.41 | 0.344 | 0.078 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Glucose (mmol/L) | -0.46 ± 2.08 | -0.01 ± 0.32 | 0.579 | 0.05 ± 0.33 | 0.62 ± 2.13 | 0.278 | -0.12 ± 0.46 | -0.20 ± 1.45 | 0.763 | 0.563 |
| Log Insulin (pmol/L) | -0.09 ± 0.28 | -0.01 ± 0.21 | 0.098 | -0.01 ± 0.09 | 0.01 ± 0.19 | 0.944 | 0.02 ± 0.21 | -0.03 ± 0.16 | 0.605 | 0.331 |
| Log HOMA-IR | -0.09 ± 0.30 | -0.01 ± 0.22 | 0.121 | -0.01 ± 0.10 | 0.02 ± 0.21 | 0.966 | 0.02 ± 0.24 | -0.02 ± 0.18 | 0.612 | 0.397 |
| vitamin B12 (ng/L) | 20.52 ± 75.53 | -9.14 ± 69.51 | 0.403 | -37.60 ± 90.80 | -12.16 ± 53.71 | 0.255 | -38.45 ± 59.12 | -20.57 ± 55.66 | 0.210 | 0.214 |

*P values for association between GRS and changes of means over 16 weeks with one of three diets were obtained by using general linear model adjusted for age, sex, BMI, and ethnicity. P values for interaction between genotypes and changes of means over 16 weeks of intervention with one of three diets were obtained by using general linear model adjusted for age, sex, BMI, and ethnicity. Values are mean ± SD*

*† P values for association between GRS and changes of means over 16 weeks with one of three diets were obtained by using general linear model adjusted for age, sex and ethnicity.*

*†† P values for interaction between GRS and changes of means over 16 weeks of intervention with one of three diets were obtained by using general linear model adjusted for age, sex and ethnicity.*

*Abbreviations: BMI body mass index, HDL high- density lipoprotein, HOMA-IR homeostasis model assessment—insulin resistance, LDL- low-density lipoprotein, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids, SFA saturated fatty acids, TAG triacylglycerol, WC waist circumference, WHR waist to hip ratio*

**Table 38: Changes in anthropometric traits and fasting biochemical traits after dietary intervention over 16 weeks according to the metabolic-GRS**

| Metabolic-GRS | SFA diet | | P association | MUFA diet | | P association | n-6 PUFA diet | | P association | P interaction |
|---|---|---|---|---|---|---|---|---|---|---|
| | ≤ 5 risk alleles | ≥ 6risk alleles | | ≤ 5 risk alleles | ≥ 6 risk alleles | | ≤ 5 risk alleles | ≥ 6 risk alleles | | |
| Log BMI ($kg/m^2$) | -0.00 ± 0.01 | 0.00 ± 0.01 | 0.843 † | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.780 † | 0.00 ± 0.01 | 0.00 ± 0.01 | 0.241 † | 0.569 † † |
| WC (cm) | -0.95 ± 3.77 | -0.94 ± 4.34 | 0.404 | -1.60 ± 3.21 | -1.74 ± 2.89 | 0.812 | -0.15 ± 2.71 | -0.29 ± 4.09 | 0.656 | 0.937 |
| WHR | -0.55 ± 4.79 | -0.79 ± 3.58 | 0.311 | 0.15 ± 4.55 | -2.56 ± 2.91 | 0.251 | -0.52 ± 3.62 | 0.95 ± 4.88 | 0.094 | 0.168 |
| 24 h Ambulatory systolic blood pressure (mm Hg) | -0.11 ± 8.03 | 1.60 ± 7.49 | 0.409 | 1.63 ± 7.33 | -7.00 ± 7.95 | **0.007** | -0.56 ± 8.61 | 1.92 ± 8.74 | 0.475 | **0.012** |
| 24 h Ambulatory diastolic blood pressure (mm Hg) | -0.06 ± 4.70 | 2.27 ± 4.94 | 0.348 | 1.05 ± 6.01 | -2.79 ± 5.19 | 0.115 | -1.31 ± 5.69 | 0.64 ± 6.27 | 0.415 | 0.069 |
| Total cholesterol (mmol/L) | 0.29 ± 0.45 | 0.38 ± 0.61 | 0.102 | -0.48 ± 2.75 | -0.33 ± 0.71 | 0.947 | -0.43 ± 1.61 | 0.09 ± 0.55 | 0.081 | 0.781 |
| HDL Cholesterol (mmol/L) | 0.44 ± 1.65 | -0.297 ± 1.59 | 0.206 | 0.03 ± 0.13 | -0.07 ± 0.21 | 0.079 | 0.29 ± 2.52 | 0.09 ± 0.26 | 0.874 | 0.541 |
| LDL Cholesterol (mmol/L) | 0.20 ± 2.77 | 0.34 ± 0.54 | 0.400 | 0.30 ± 2.16 | -1.63 ± 2.76 | 0.056 | -0.01 ± 2.47 | 0.17 ± 2.78 | 0.960 | 0.153 |
| TAG (mmol/L) | 0.53 ± 1.67 | -0.44 ± 1.62 | 0.071 | -0.04 ± 0.41 | 0.12 ± 0.21 | 0.354 | -0.31 ± 1.57 | -1.01 ± 2.45 | 0.209 | 0.201 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Fasting blood glucose (mmol/L) | -0.04 ± 0.31 | -0.25 ± 1.61 | 0.862 | -0.08 ± 0.20 | 1.39 ± 2.79 | **0.024** | 0.04 ± 0.42 | -0.51 ± 1.82 | 0.347 | **0.006** |
| Log fasting Insulin (pmol/L) | -0.05 ± 0.24 | -0.01 ± 0.22 | 0.967 | 0.02 ± 0.19 | -0.02 ± 0.12 | 0.532 | -0.00 ± 0.20 | -0.02 ± 0.16 | 0.950 | 0.785 |
| Log HOMA-IR | -0.06 ± 0.26 | -0.02 ± 0.23 | 0.926 | 0.03 ± 0.20 | -0.02 ± 0.13 | 0.441 | 0.00 ± 0.22 | -0.02 ± 0.18 | 0.822 | 0.833 |
| Vitamin B12 (ng/L) | -14.17 ± 52.66 | 0.08 ± 38.51 | 0.437 | -21.29 ± 88.03 | -54.61 ± 59.53 | 0.072 | -17.84 ± 69.06 | -2.40 ± 91.26 | 0.544 | 0.533 |

*P values for association between GRS and changes of means over 16 weeks with one of three diets were obtained by using general linear model adjusted for age, sex, BMI, and ethnicity. P values for interaction between genotypes and changes of means over 16 weeks of intervention with one of three diets were obtained by using general linear model adjusted for age, sex, BMI, and ethnicity. Values are mean ± SD*

*[†] P values for association between GRS and changes of means over 16 weeks with one of three diets were obtained by using general linear model adjusted for age, sex and ethnicity.*

*[††] P values for interaction between GRS and changes of means over 16 weeks of intervention with one of three diets were obtained by using general linear model adjusted for age, sex and ethnicity.*

*Abbreviations: BMI body mass index, HDL high- density lipoprotein, HOMA-IR homeostasis model assessment—insulin resistance, LDL low-density lipoprotein, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids, SFA saturated fatty acids, TAG triacylglycerol, WC waist circumference, WHR waist to hip ratio*

At 16 weeks, after adjustment for age, sex, ethnicity and baseline BMI, a significant interaction between the metabolic-GRS and dietary intervention (SFA vs MUFA vs n-6 PUFA) on changes in 24 h ambulatory systolic blood pressure ($P_{interaction} = 0.012$) was observed (**Table 38 and Figure 18**). Individuals with 6 or more risk alleles for the metabolic-GRS had significantly lower 24 h ambulatory systolic blood pressure levels after the MUFA (*n* = 13; -7 ± 8 mm Hg) compared with the SFA (*n* = 15; 2 ± 7 mm Hg; *P* = 0.033) and n-6 PUFA-rich diets (*n* = 16; 2 ± 9 mm Hg; P = 0.001) ($P_{association}$= 0.009) (**Table 38 and Figure 18**).

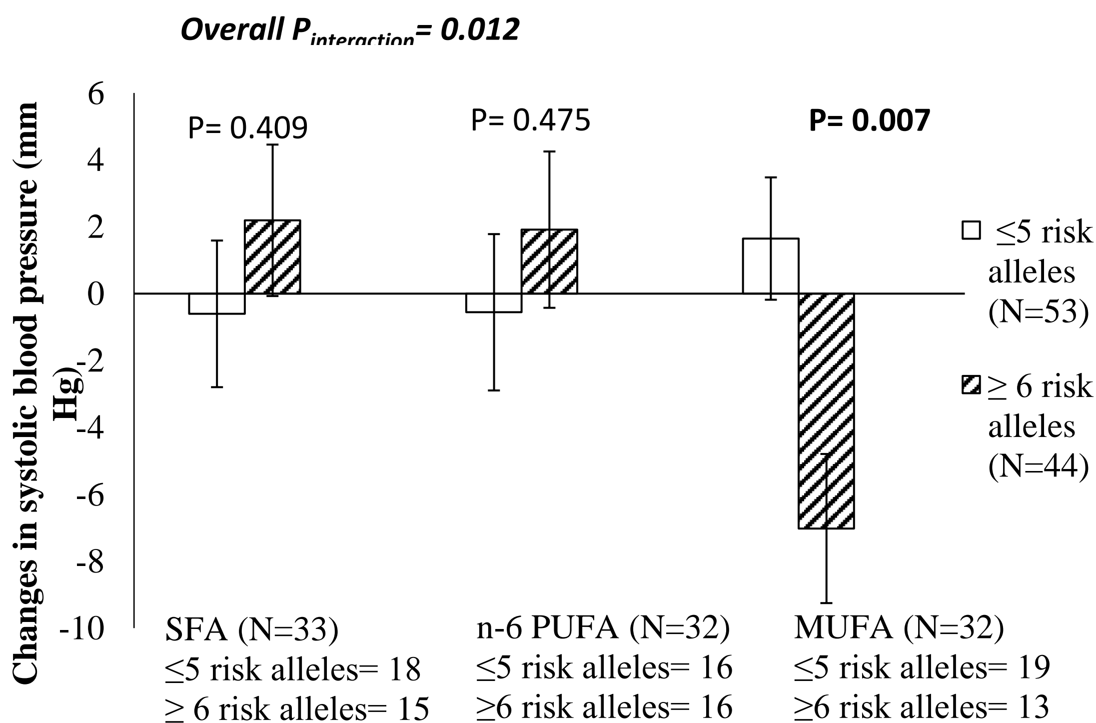


**Figure 18: Mean (±SE) of changes in 24 h ambulatory systolic blood pressure following three intervention diets [rich in either saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and n-6 polyunsaturated fatty acids (PUFA)] according to the metabolic-GRS ($P_{interaction}$ = 0.012).**

*A general linear model analysis was performed with adjustments for age, sex, body mass index, and ethnicity. Individuals carrying ≥ 6 risk alleles had lower 24 h ambulatory systolic blood*

*pressure after consuming the MUFA diet compared to the SFA or n-6 PUFA diets ($P_{association}$ = 0.007). Error bars indicate Standard error.*

In addition, we also observed an interaction between the metabolic-GRS and the dietary fat intervention (SFA vs MUFA vs n-6 PUFA) on changes in fasting glucose concentrations ($P_{interaction}$ = 0.006) (**Table 38**), where individuals with 6 or more risk alleles had significantly higher fasting glucose concentrations after the MUFA (*n* = 21; 1.39 ± 2.79 mmol/L) compared with the SFA (*n* = 26; -0.25 ± 1.61 mmol/L; *P* = 0.045) and n-6 PUFA-rich diets (*n* = 14; -0.51 ± 1.82 mmol/L; *P* = 0.045) ($P_{association}$ = 0.020). However, these interactions were not statistically significant after correction for multiple testing (**Table 38**).

## 7.5 Discussion

To our knowledge, this is the first report to investigate the relationship between vitamin B12 concentrations and cardiometabolic traits, after replacing dietary SFA with MUFA or n-6 PUFA in adults at moderate risk of CVD by using a genetic approach. Our findings from this retrospective analysis of the DIVAS study failed to show an impact of the B12-GRS on cardiometabolic traits in the presence of a dietary intervention manipulating fatty acid intake for 16 weeks. However, our analysis showed that individuals carrying 6 or more risk alleles (42.9% of the study population) of the metabolic-GRS had significantly lower 24 h ambulatory systolic blood pressure after the 16-week replacement of SFA with MUFA. Our findings indicate that the metabolic-GRS influences inter-individual variation in 24 h ambulatory systolic blood pressure and a 9.5% percentage replacement of SFA with MUFA could potentially be implemented as a dietary approach to reduce CVD risk in individuals at increased genetic risk.

The quantity and quality of fats consumed in the diet are important features that influence the risk of hypertension [482]. In a meta-analysis of 9 randomized controlled trials which examined the long-term effects (≥ 6 months) of a high-MUFA (>12%TE MUFA) vs

low-MUFA (≤12%TE MUFA) diet on markers of CVD risk, it was shown that high-MUFA diets significantly reduced systolic and diastolic blood pressures, compared with individuals consuming low-MUFA diets [483]. It is important to note that the studies included in the meta-analysis had inconsistent total energy intakes, furthermore some of the studies compared high carbohydrate diets or high protein diets as the comparator, whereas others used high PUFA diets [483]. To date, twin studies have demonstrated the heritability of blood pressure to range between 39-63% [484], with lower estimates of heritability from the general population (20-40%) [485]. Our retrospective data analysis demonstrated a significant interaction between metabolic-GRS and dietary fat composition on 24 h ambulatory systolic blood pressure in adults at moderate CVD risk, where the substitution of SFA with MUFA reduced 24 h ambulatory systolic blood pressure despite individuals carrying 6 or more risk alleles compared to the SFA and n-6 PUFA-rich diets. Our findings are also in line with previous epidemiological studies which have shown that blood pressure is positively correlated with high intakes of SFA [486-488] and that the replacement of SFA with n-6 PUFA may not be beneficial in reducing blood pressure [489, 490]. Hence, from interpreting our study findings it is possible that the replacement of 9.5% of SFA with MUFA might overcome the genetic risk of increased blood pressure. [491] MUFA-rich foods, such as oleic acid (a major component of olive oil), have been implicated in lowering blood pressure by modifying membrane phospholipid composition and inducing hypotensive effects through the $\alpha_2$-adrenergic receptor system in rats [492]. Animal studies have reported impaired endothelial vasodilator function [493] and an activation of sympathetic nervous system activities [494] in rats fed with SFA. It has been suggested that the detrimental effect of SFA on blood pressure might be secondary to a decrease in insulin sensitivity, which is likely to have contributed to the activation of the sympathetic nervous system and the resulting blood pressure increase of these rats[494, 495]. However, the association between fasting plasma insulin and hypertension

have been inconsistent among human studies [496-498]. Linoleic acid, the primary form of dietary n-6 PUFA, has previously been shown to induce hypotensive effects and hyperpolarization of pig coronary artery vascular smooth muscle cells (VSMC) through a mechanism which activates the Na+/K+-ATPase pump. The activation of the Na+/K+-ATPase pump alters the membrane potential of VSMC, leading to the relaxation of the coronary arteries [499]; but our analysis in humans at moderate risk of CVD was unable to confirm any beneficial effect of replacing 9.6% of SFA with n-6 PUFA on blood pressure. Our finding is in accordance with a recent systematic review based on five previous human epidemiological (n = 33,834), and four intervention studies (n = 483) which also reported conflicting evidence of the impact of n-6 PUFA-rich diets on blood pressure [490]. Hence, given the findings from our data analysis, it can be assumed that an increase in MUFA in the diet is likely to regulate systolic blood pressure among individuals carrying 6 or more risk alleles of the metabolic-GRS, however, further analysis is required to confirm these findings.

Furthermore, the metabolic-GRS was found to modify the association between the MUFA rich diet and changes in fasting glucose concentrations. Among individuals consuming the MUFA rich diet, those carrying ≥ 6 risk alleles of the metabolic-GRS had a tendency for a greater increase in glucose concentrations after 16 weeks compared to individuals consuming an SFA or n-6 PUFA rich diet. Even though this contradicts the impact of MUFA on metabolic disease outcomes [491, 500], a recent meta-analysis of nine randomized controlled intervention trials (n = 1,547) also failed to identify any significant difference in fasting glucose concentrations when comparing high- (>12% of Total Energy Consumption) with low-MUFA diets (≤12% of Total Energy Consumption) [483]. Hence, MUFA-rich diets may not be beneficial in reducing glucose concentrations in individuals at genetic risk of developing metabolic disease-related outcomes. Unexpectedly, our data analysis indicated a significant reduction in fasting glucose concentrations after 16 weeks amongst those consuming a n-6

PUFA-rich or SFA-rich diet when they carried more than 6 risk alleles of the metabolic-GRS. However, these findings should be interpreted with caution, given the potentially negative effects associated with consuming a high SFA diet [501] and the complex role of factors influencing metabolic disease outcomes [502]. This finding was not considered statistically significant after correction for multiple testing; hence, further large studies are warranted to explore this gene-diet interaction.

The present study has some limitations. Our data analysis showed an effect of the plasma vitamin B12-GRS in the opposite direction to what we expected, where an increased genetic risk of vitamin B12 deficiency was positively associated with increased fasting HDL cholesterol and decreased TAG at baseline. In addition, our analysis indicated a significant fasting glucose increasing effect amongst those replacing 9.5% of SFA with MUFA when they carried more than 6 risk alleles of the metabolic-GRS. In attempting to explain these discrepancies it is important to note that this study included six individuals who had vitamin B12 deficiency (< 200 ng/L), which could likely have influenced these outcomes. Furthermore, we were unable to see any associations of the B12-GRS with any of the other outcomes, which could be attributed to the small sample size of the study. In comparison to cross-sectional studies, randomized clinical trials are often limited by the sample size; in the DIVAS study, only 119 participants out of 195 consented for analysis of genetic data and thus the sample size for the analysis was limited. Importantly, our data analysis did not measure circulating concentrations of other vitamin B12 biomarkers, such as holo-transcobalamin (holoTC) or methylmalonic acid. Furthermore, the DIVAS study included an ethnically diverse population (Asian-7% and Black- 7%); however, to avoid population stratification, ethnicity was adjusted for in the analyses. Even after excluding other ethnic groups and only analysing Caucasian individuals, our findings still remained the same (data not shown). One of the main strengths of the DIVAS study was that it examined the effects of dietary fatty acid manipulation for a

long duration (16 wk) on vitamin B12 concentrations and cardiometabolic traits in a robust randomised controlled intervention study. Secondly, the dietary fat manipulation in this intervention study had minimal impact on other dietary components and total energy intake, effectively negating the confounding effect of these variables on the effects of the GRS on dietary response.

In conclusion, our data analysis was able to show an interaction between the metabolic-GRS and dietary fat intake on 24 h ambulatory systolic blood pressure. The results suggest a greater sensitivity of individuals carrying 6 or more risk alleles (42.9% of the population) to dietary fat composition, with an 24 h ambulatory systolic blood pressure lowering effect observed following substitution of SFA with MUFA but not with n-6 PUFA. However, our analysis was unable to provide evidence for an impact of the B12-GRS on vitamin B12 concentrations and cardiometabolic traits in response to the 16-week dietary fat intervention. Future studies with a larger sample size examining B12-GRS and metabolic-GRS, particularly prospectively genotyped dietary intervention studies, are required to confirm the gene-diet interactions identified in our study.

**Author contributions:**

**Shelini Surendran performed the statistical analysis, data interpretation and wrote the manuscript**. KSV assisted in drafting the manuscript. MW was a member of the original research team that conducted the DIVAS study and analysed the original outcome measures; KGJ and JAL designed the intervention study; KSV conceived the genetic aspects of the study; MW, KGJ and JAL critically reviewed the manuscript; All authors approved the final draft of this article prior to submission.

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# Chapter 8

## Discussion and conclusion

### 8.1 Discussion

The field of Nutrigenetics has been important in providing evidence for the interaction between genes and nutrients in the development of chronic diseases, such as obesity, insulin dysregulation and adverse cardiometabolic outcomes. The findings from this thesis has contributed to the field of nutrigenetics by demonstrating that genetic heterogeneity in gene-diet interactions related to vitamin B12 concentrations and metabolic traits exist across different ethnic groups. The results in this thesis allow nutritionists to better understand how genetic variants related with B12 absorption and metabolism interact with dietary factors in the development of metabolic disorders. The field of nutrigenetics will one day allow nutritionists to provide personalised dietary recommendations to their patients based on their genotype in order to delay or prevent the development of diseases related to low vitamin B12 status.

Although, several nutrigenetic studies have examined the interaction between genes and nutrition on chronic disease outcomes, the findings have yielded inconsistent results due to two main factors (i) genetic heterogeneity between individuals and (ii) small sample sizes with limited replication, hence, it has not been possible to develop a personalised diet for each ethnic group [12]. Up unto now it has been difficult to conduct nutrigenetic studies in less economically developed countries, due to the limited infrastructure, funding and expertise [12]. This thesis has set out to use a genetic approach to examine the association between selected common single nucleotide polymorphisms (SNPs) associated with vitamin B12 concentrations and SNPs associated with metabolic traits on vitamin B12 concentrations and metabolic outcomes in different ethnic groups. Also, the interaction between these SNPs and

dietary factors (protein, carbohydrate and fat) on vitamin B12 concentrations and cardio-metabolic traits was investigated.

One of the reasons for examining different ethnic groups was to replicate the findings from one study into another independent cohort from another population. A total of three different study designs from five different populations [Brazilian adolescents, Sri Lankan adults, South Asian Indian adults, Indonesian adult women and British adults] were used to test our objectives. The studies included in this PhD were: one case-control study [Chennai Urban Rural Study (CURES; Asian Indian, n=900)], three cross-sectional cohort studies [Genetics of obesity and Diabetes study (GOOD study; Sinhalese Sri Lankan adults, n=109), The Minangkabau Indonesia Study on Nutrition and Genetics (MINANG study; Indonesian women; n=118) and Brazilian adolescents (n=113)] and a 16 week-dietary randomized, single-blind, parallel-group dietary intervention [Dietary Intervention and VAScular function (DIVAS study; British adults, n=119)]. The general linear regression model was used for statistical analysis using the SPSS version 25, to conduct association and interaction analyses. Potential confounders such as age, sex and body mass index (BMI) were adjusted wherever necessary. A summary of the findings from this thesis is presented below.

### 8.1.1 Impact of genes and diet on homocysteine, vitamin B12, folate and lipids in a Brazilian adolescent Population

Cardiovascular diseases (CVD) have remained the leading cause of mortality in Brazil and are major causes of disability affecting the quality of life [328, 329]. Genes involved in the one carbon metabolism are of particular interest because of their role in CVD [355]. However, the interaction between SNPs involved in the one-carbon metabolism pathway and macronutrient intake on cardiovascular risk factors in the Brazilian population has not yet been investigated. The aim of this study was to examine the association of ten SNPs involved in the

one-carbon metabolism pathway [methylenetetrahydrofolate reductase (*MTHFR*)- rs1801133 and rs1801131; 5-methyltetrahydrofolate-homocysteine methyltransferase or methionine synthase (*MTR*)- rs1805087 ; 5-methyltetrahydrofolatehomocysteine methyltransferase reductase or methionine synthase reductase (*MTRR*)- rs1801394; transcobalamin 2 (*TCN2*)- rs1801198, catechol-O-methyltransferase (*COMT*)- rs4680 and rs4633; betaine-homocysteine S-methyltransferase (*BHMT*)- rs3797546 and rs492842; and fucosyltransferase 2 (*FUT2*)- rs602662] with vitamin B12, folic acid, homocysteine and blood lipids [high- (HDL), low- (LDL) density lipoproteins, triacylglycerol (TAG) and oxidized-LDL (ox-LDL)], and to investigate whether lifestyle factors (dietary factors/physical activity levels) modified the association of the SNPs in 113 Brazilian adolescents (aged 10-19 years) with cardiovascular risk.

The results of this study confirm the association of the fucosyltransferase 2 (*FUT2*) SNP rs602662 with vitamin B12. Additionally, associations were observed between the SNP rs4633 at the catechol-O-methyltransferase (*COMT*) gene with folic acid concentrations and finally the SNP rs1801394 at the 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR*) gene with ox-LDL concentrations. No gene-lifestyle interactions were observed on vitamin B12 concentrations. Additionally, none of the vitamin B12 associated SNPs interacted with lifestyle factors to influence cardio-metabolic outcomes.

This study provided evidence for the interactions between *COMT* SNP rs4680 and carbohydrate intake on ox-LDL levels and the *FUT2* SNP rs602662 and protein intake on homocysteine concentrations. However, upon stratification of participants based on their consumption of dietary carbohydrate/protein (low, medium and high intake), no statistically significant associations between the SNPs and the outcomes in any of the tertiles were detected, which could potentially be due to the small sample size. This is the first study to provide novel gene-diet interactions at the *COMT* and *FUT2* gene loci, on ox-LDL and homocysteine

concentrations; hence, we have no other studies to compare our findings with. Given that ox-LDL and homocysteine are well-known independent risk factors for cardiovascular disease [24, 354], our findings may have significant public health implications. However, it is important to note that after correction for multiple testing, none of these associations and interactions were statistically significant.

The main strength of this study is that SNPs were examined on vitamin B12, folic acid, homocysteine and lipid traits during early stages of life when lifestyle and behavioral factors have had less time to substantially modify the phenotype and hence, this study on Brazilian adolescents has significant importance. Furthermore, this study population is characterized by obesity and/or dyslipidemia which make it suitable for a study on lipids. Additionally, little is known about gene–diet interactions which influence ox-LDL concentrations, and thus our study adds to the limited body of research. After correction for multiple testing, none of the SNP–environment interactions were detected. Hence, our findings warrant confirmation in larger and well-powered prospective studies, before any public health recommendations can be developed for the adolescent Brazilian population.

### 8.1.2 Impact of genes and diet on vitamin B12 concentrations and metabolic diseases in an Asian Sri Lankan population

The prevalence of obesity in Sri Lanka has increased markedly in recent years [361], with approximately 34.4% of the Sri Lankan adult population being diagnosed as overweight or obese [361, 362]. Although, vitamin B12 deficiency has been linked to obesity [7, 84, 85] and diabetes [67, 69, 70], no study to date has tested the genetic link between metabolic traits and vitamin B12 status in a Sinhalese cohort. Furthermore, no study has investigated the status of vitamin B12 within the Sinhalese population. Hence, the objective of this study was to use a gene-based approach to explore the relationship between metabolic traits and vitamin B12

status in 109 Sinhalese adults (61 men and 48 women, aged 25-50 years), and to investigate whether these relationships were modified by lifestyle factors (dietary factors/physical activity levels).

Genetic risk scores (GRS) based on ten metabolic disease-related genetic variants [Fat mass and obesity-associated (*FTO)*- rs9939609 and rs8050136, Melanocortin 4 Receptor (*MC4R)*- rs17782313 and rs2229616, Transcription factor 7-like 2 (*TCF7L2)*- rs12255372 and rs7903146, Potassium voltage-gated channel subfamily J member 11 (*KCNJ11)*- rs5219, Calpain 10 (*CAPN10)*- rs3792267, rs2975760 and rs5030952] and ten vitamin B12-related genetic variants [Methylenetetrahydrofolate reductase (*MTHFR)*- rs1801133, Carbamoyl-phosphate synthase 1 (*CPS1)*- rs1047891, Cubulin (*CUBN)*- rs1801222, CD320 molecule (*CD320)*- rs2336573, Transcobalamin 2 (*TCN2)*- rs1131603, Citrate lyase beta like (*CLYBL)*- rs41281112, Fucosyltransferase 2 (*FUT2)*- rs602662, Transcobalamin 1 (*TCN1)*- rs34324219, Fucosyltransferase 6 (*FUT6)*- rs778805 and Methylmalonyl-CoA mutase (*MUT)*- rs1141321] were constructed.

This study confirmed the association between the B12-GRS and serum vitamin B12 concentrations. However, no association between the B12-GRS and metabolic traits were observed. Furthermore, the metabolic-GRS did not show any association with vitamin B12 concentrations and metabolic traits. The findings in this study have suggested that a genetically lowered vitamin B12 concentration may have an impact on central obesity in the presence of a dietary influence (protein energy intake %). However, further stratification of participants based on their consumption (low, medium and high dietary intake) of dietary protein (energy %) did not show statistically significant associations between the GRS and the outcome in any of the tertiles, which could account for the small sample size. Additionally, the metabolic GRS interacted with carbohydrate energy intake (%) to influence waist to hip ratio levels, where individuals carrying more than 9 risk alleles had a higher waist-to-hip ratio among those in the

highest tertile of carbohydrate energy percentage. Given that the daily consumption of protein is low and carbohydrate intake is high in Sri Lankan adults [380], my findings may have significant public health implications in terms of revising dietary guidelines, which could prevent central obesity and its related CVD-related outcomes.

### 8.1.3 Impact of genes and diet on vitamin B12 concentrations and metabolic diseases in an Indonesian women population (Minangkabau community)

Optimal vitamin B12 status is essential for women to maintain adequate maternal health and to avoid foetal developmental complications [70, 434]. Additionally, low vitamin B12 concentrations have shown negative correlations with body mass index (BMI) in healthy women [401]. Currently, the data on vitamin B12 status in healthy Indonesian women is unknown and the relationship between low vitamin B12 status and obesity related traits has yielded conflicting results [93]. Thus, I used a genetic approach to explore the relationship between metabolic traits and vitamin B12 status and investigated whether these relationships were modified by lifestyle factors (dietary intake or physical activity levels) in an Indonesian women population (117 adults, aged 25-60 years). The Minangkabau population from Indonesia was selected in this study on the basis that women hold a higher authoritarian role in society and that all food choices in the family are dictated by women [438].

Genetic risk scores (GRS) based on nine B12-related SNPs [Methylenetetrahydrofolate reductase (*MTHFR*)- rs1801133, Carbamoyl-phosphate synthase 1 (*CPS1)*- rs1047891, Cubulin (*CUBN)*- rs1801222, CD320 molecule (*CD320*)- rs2336573, Transcobalamin 2 (*TCN2)*- rs1131603, Fucosyltransferase 2 (*FUT2)*- rs602662, Transcobalamin 1 (*TCN1)*- rs34324219, Fucosyltransferase 6 (*FUT6)*- rs778805 and Methylmalonyl-CoA mutase (*MUT)*- rs1141321] and nine metabolic disease-related SNPs [Fat mass and obesity-associated (*FTO)*-

rs9939609 and rs8050136, Melanocortin 4 Receptor (*MC4R)*- rs17782313 and rs2229616, Transcription factor 7-like 2 (*TCF7L2)*- rs12255372 and rs7903146, Potassium voltage-gated channel subfamily J member 11 (*KCNJ11)*- rs5219, Calpain 10 (*CAPN10*])- rs3792267 and rs5030952] were constructed.

This study replicated the non-significant findings observed in the Sri Lankan study and confirmed the lack of association between the B12-GRS and metabolic traits, and the metabolic-GRS with vitamin B12 and metabolic traits. However, unlike in the Sri Lankan study, the B12-GRS in the Indonesian population did not show any associations with B12 concentrations, which could potentially be accounted to the higher mean vitamin B12 status observed in the Indonesian study (436 ± 427.18 pmol/L) compared to the Sri Lankan study (380.65 ± 132.83 pmol/L). In the Indonesian study, we observed a novel interaction between the B12-GRS and dietary fibre intake (g) on glycated haemoglobin. Individuals who consumed a low fibre diet (4.90 ± 1.00 g/day) and those who carried ≥9 risk alleles for vitamin B12 deficiency had significantly higher HbA1C levels compared to those carrying ≤8 risk alleles. Previous studies have shown that dietary fibre consumption is low in the Indonesian population, and thus our findings are important as it could encourage a consumer education campaign centred around encouraging fibre intake to reduce HbA1C levels, which could improve glycaemic control in the Indonesian population.

### 8.1.4 Impact of genes and diet on vitamin B12 concentrations and metabolic diseases in an Asian Indian population

Asian Indians exhibit a unique phenotype collectively known as the 'South Asian Phenotype' which consists of higher levels of total and visceral fat, higher waist circumference and an increased susceptibility to Type 2 diabetes [364]. Approximately >12% of the Indian population is either overweight or obese [390]. Although there is a strong genetic component

to developing the 'South Asian Phenotype', consuming an unhealthy diet and leading a sedentary lifestyle can further contribute to this phenotype [405, 408]. Several studies have implicated that obesity is related to many micronutrient deficiencies including vitamin B12 [6, 395, 396].

Our study examined whether dietary intake and physical activity levels modified associations between a GRS using two previously studied *FTO* SNPs (rs8050136 and rs2388405) and vitamin B12 concentrations and metabolic disease-related outcomes in 900 Asian Indians (300 normal glucose-tolerant individuals, 300 prediabetic and 300 type 2 diabetes individuals). In this study, participants were randomly recruited from the Chennai Urban Rural Epidemiology Study (CURES), a cross-sectional case-control epidemiological study conducted on a representative population of Chennai in Southern India [410].

To date, the *FTO* (Fat mass and obesity associated) gene, has been the strongest obesity risk loci in several populations [11]. Our study confirmed the association between *FTO* gene variants and obesity traits. A novel finding of this study was the potential association between the *FTO*-GRS and vitamin B12 concentrations, after adjustment for age, sex, BMI and type 2 diabetes. Carriers of more than one risk allele for the *FTO*-GRS had lower vitamin B12 concentrations, compared to individuals carrying zero risk alleles. The mechanism explaining the differences in vitamin B12 concentrations in the *FTO*-GRS could potentially be due to the *FTO* genotypes modulating gut microbiota and inducing metabolic inflammation, consequently impairing B12 absorption [6, 432]. Interestingly, associations between obesity-related SNPs and vitamin B12 concentrations in other populations investigated in this thesis (Brazil, Sri Lanka, UK and Indonesia) were not observed. It is possible that these associations were not observed due to the following reasons [322]: (1) difference in effect allele frequencies, (2) genetic heterogeneity across different ethnic groups, (3) variance in linkage disequilibrium structure and (4) gene-gene and gene-environment interactions (5) Variations in sample sizes

of the study (6) number of metabolic-SNPs assessed. Additionally, no significant interactions were observed between the *FTO*-GRS and lifestyle factors (diet and physical activity) in the Indian population.

Given that low vitamin B12 concentrations in Asian Indians are common [397, 423] and that 28-44% of Asians carry at least one copy of the *FTO* risk allele [415], our study highlights the importance of considering obesity as a risk factor for vitamin B12 deficiency with implications on the possible targeting of relevant obesity prevention strategies. Following such advice could substantially reduce vitamin B12 deficiency among Asian Indians.

#### 8.1.5 Impact of genes and diet on vitamin B12 concentrations and cardio-metabolic diseases in a British population

Given that the modification of dietary fat intake has been shown to affect vitamin B12 status in a previous animal study [472], a post-hoc analysis of the Dietary Intervention and VAScular function (DIVAS) study was carried out with the aim of replicating similar findings in humans. In this study, I investigated whether vitamin B12- and metabolic disease-related genetic variants modified vitamin B12 concentrations and cardiometabolic traits in response to replacement of saturated fatty acids (SFA) with monounsaturated (MUFA) or n-6 polyunsaturated (PUFA) fatty acids in British adults at 1.5-fold higher risk of CVD. Genetic risk scores (GRS) based on three vitamin B12 related tagSNPs [(*FUT2)*- rs602662, rs492602 and rs16982241] representing the entire common genetic variations across the *FUT2* gene were selected. Furthermore, seven metabolic disease-related genetic variants [(*FTO*)- rs8050136, rs9939609 and rs10163409, (*MC4R*)- rs17782313 and rs2229616 and (*TCF7L2*)- rs7903146 and rs12255372] were constructed.

No significant associations of the B12-GRS and metabolic-GRS with vitamin B12 concentrations were observed within the participants. However, it should be noted that the

mean vitamin B12 concentrations were lower in participants who carried ≥4 risk alleles of the B12-GRS and ≥6 risk alleles of the metabolic-GRS. Our data analysis showed an effect of the B12-GRS in the reverse direction to what was expected, where an increased genetic risk of vitamin B12 deficiency was positively associated with increased fasting HDL cholesterol and decreased TAG at baseline, these findings were not replicated in other study populations which had used a different study design (cross-sectional) in my thesis (Brazil, Sri Lanka, India and Indonesia). Our findings from this retrospective analysis of the DIVAS study failed to show an impact of the B12-GRS on vitamin B12 concentrations and cardiometabolic traits in the presence of a dietary intervention manipulating fatty acid intake for 16 weeks. Furthermore, the post-hoc tests indicated no significant interactions between changes in vitamin B12 concentrations and the three dietary groups with the metabolic-GRSs.

Interestingly, the DIVAS study showed two novel interactions between the metabolic-GRS and the dietary fat intervention on metabolic traits. My study demonstrated that isoenergetically substituting a high SFA diet with a 9.5% replacement of MUFA had a significant effect on lowering ambulatory systolic blood pressure in individuals carrying 6 or more risk alleles (43.8% of the study population) of the metabolic-GRS. Additionally, our study indicated the metabolic-GRS modified the association between the MUFA-rich diet and changes in fasting glucose concentrations. Among individuals consuming the MUFA-rich diet, those carrying ≥ 6 risk alleles of the metabolic-GRS had a tendency for a greater increase in glucose concentrations after 16 weeks compared to individuals consuming an SFA or n-6 PUFA rich diet. Further studies are warranted to confirm these results and investigate the mechanisms underlying the effect of these genes on blood pressure levels in response to replacing SFA with MUFA.

#### 8.1.6 General trends observed across the study population

Differences in macronutrients existed among the five groups studied in this thesis. In general, Sri Lankans and Indians had similar macronutrient intakes, whereas Minangkabau women from Indonesia had intakes like those of Brazilian adolescents. Carbohydrate intakes as a percentage of calories were higher among South Asians (Sri Lankan: 69.6 ± 8.8 % and Indian: 64.3 ± 6.3 %) than the other groups (Brazil: 47.7 ± 20.6 %, Indonesian: 54.1 ± 9.4 % and British: 49.1 ± 7.1%) (**Table 39**). The acceptable macronutrient distribution range (AMDR) for carbohydrate in adolescents (aged 14 and over) and adults is 45-65 percent of total energy [503]. The Sri Lankan population consumed carbohydrates above the AMDR. Furthermore, the Indian population was very close to the upper limit of the acceptable intake of carbohydrates. The British, Indonesian and Brazilian population were well within the AMDR for carbohydrate intake. The percent of calories from protein was highest among the Brazilian adolescent population (17.0 ± 8.4 %) and lowest among the South Asian populations (Sri Lankan: 11.3 ± 2.3 % and Indian: 11.3 ± 1.1 %). British adults with moderate cardiovascular risk had the highest total fat intakes as percentage of calories (34.1 ± 5.4 %), compared to the other populations studied (Brazil: 25.4 ± 13.2 %, Sri Lanka: 21.9 ± 5.3%, Indian: 23.8 ± 4.7 and Indonesian: 28.9 ± 8.0%) (**Table 38**). All of the populations in this thesis had macronutrient intakes within the AMDR for protein (10-35%) and fat (20-35%) intake [503].

Aspects of the sampling strategy could have affected our comparisons between the five groups. Firstly, we did not examine age differences between macronutrient intakes. The Brazilian population included only adolescents, whereas in the Sri Lankan population adults up to the age of fifty years were included. The Indian, Indonesian and British populations all included adults over the age of fifty. It is important to note, that the younger populations are more likely to adopt new dietary patterns, as opposed to older populations, making it easier to propose dietary advice. Indian adults were sampled in both urban and rural areas, whereas the

MINANG study was conducted in a rural area. Further to this, the DIVAS, GOOD and Brazilian population were conducted in urban areas. More future studies, which look at both rural and urban populations are needed, as well as controlling for potential confounders such as socio-economic factors [504]. Furthermore, the studies did not report what days of the week sampling took place. It is important to note, that the nutrient intake in the weekend may vary in comparison to the weekdays [505].

The highest levels of insufficient physical activity (<600 MET mins/week using the GPAQ) were reported in the CURES study (82.0%), whereas the lowest levels of insufficient physical activity levels were reported in the Brazilian adolescent population (31%) (**Table 39**). There are many limitations, which act to limit the generalisability of the physical activity levels including: the sample size of the cohort and the inclusion of cohorts with high-risk (DIVAS, CURES and the Brazilian population). Additionally, there are inter-cultural differences that exist between how 'moderate' and 'vigorous' physical activity is reported [506]. In the future, physical activity should also be measured with the combined use of a piezoelectric pedometer and accelerometer, instead of relying on subjective measures.

The observations described in this analysis both confirm previous findings and offer new-light on population-based differences in vitamin B12 levels. Compared with the Indonesian population, South Asians demonstrated lower vitamin B12 concentrations. Although the Brazilian adolescent population had indicators of cardiovascular risk, higher vitamin B12 concentrations were observed in this population compared with healthy participants from the Indian and Sri Lankan population. Furthermore, the lowest levels of vitamin B12 across the five populations in this study was from the DIVAS study (**Table 39**), which could be accounted by the populations older age group. It is difficult to generalise these findings, as some of the populations included cohorts at risk of disease and furthermore the sample size of the population was limited.

**Table 39: Macronutrient Intakes, Biochemical and physical activity levels: A Comparison of the Brazilian, GOOD, CURES, MINANG and DIVAS studies**

| Macronutrient | Brazil (N=113)[a] | Sri Lanka GOOD study (n=109) | India CURES study (n=548) | Indonesia MINANG study (n=117)[b] | DIVAS (n=119)[c] |
|---|---|---|---|---|---|
| Protein (%) | 17.0 ± 8.4 | 11.3 ± 2.3 | 11.3 ± 1.1 | 16.9 ± 3.3 | 15.6 ± 2.9 |
| Carbohydrate (%) | 47.7 ± 20.6 | 69.6 ± 8.8 | 64.3 ± 6.3 | 54.1 ± 9.4 | 49.1 ± 7.1 |
| Fat (%) | 25.4 ± 13.2 | 21.9 ± 5.3 | 23.8 ± 4.7 | 28.9 ± 8.0 | 34.1 ± 5.4 |
| Total Fibre (g) | N/A | 16.8 ± 8.2 | 32.2 ± 11.3 | 8.8 ± 4.5 | N/A |
| Total energy (Kcal/day) | 2522 ± 586 | 2098 ± 456 | 2597 ± 773 | 1774 ± 609 | 2148 ± 572 |
| Anthropometric | | | | | |
| Age (yrs) | 14 ± 2 | 38 ± 7 | 49.39 ± 11.45 | 40 ± 10 | 47 ± 9 |
| BMI ($kg/m^2$) | 24.0 ± 4.9 | 24.6 ± 4.1 | 26.75 ± 5.04 | 25.1 ± 4.2 | 26.4 ± 4.0 |
| WHR | N/A | 0.92 ± 0.11 | 0.90 ± 0.09 | N/A | 0.87 ± 0.09 |
| Fat (%) | N/A | 27.25 ± 7.37 | N/A | 35.70 ± 7.00 | N/A |
| 24 h Ambulatory Systolic BP (mm Hg) | N/A | 120 ± 15 | 129 ± 20 | 113 ± 9 | 122 ± 11 |
| 24 h Ambulatory diastolic BP (mm Hg) | N/A | 75 ± 16 | 80 ± 12 | 77 ± 6 | 75 ± 8 |
| Biochemical | | | | | |
| Vitamin B12 (pg/ml) | 520 ± 232 | 516 ± 180 | 417 ± 255 | 591 ± 579 | 414 ± 162 |
| Homocysteine (µmol/l) | 7.04 ± 2.99 | N/A | 13.67 ± 8.09 | N/A | N/A |
| Folic acid (ng/ml) | 11.02 ± 3.27 | N/A | 8.59 ± 5.81 | N/A | N/A |

| | | | | | |
|---|---|---|---|---|---|
| Triacylglycerol (mg/dl) | 94.05 ± 54.16 | 144.19 ± 86.81 | 146.54 ± 116.88 | 97.67 ± 42.80 | 114.16 ± 53.10 |
| HDL (mg/dl) | 46.29 ± 11.79 | 42.56 ± 8.24 | 40.89 ± 8.85 | 58.99 ± 10.20 | 59.46 ± 13.90 |
| LDL (mg/dl) | 90.28 ± 21.00 | 134.03 ± 28.60 | 114.37 ± 35.56 | 127.77 ± 39.17 | 146.72 ± 37.84 |
| VLDL (mg/dl) | 18.85 ± 10.82 | 28.84 ± 17.36 | N/A | N/A | N/A |
| Oxidized-LDL (U/L) | 6.42 ± 13.69 | N/A | N/A | N/A | N/A |
| Fasting plasma glucose (mg/dl) | N/A | 86 ± 13 | 116 ± 49 | 92 ± 20 | 92 ± 8 |
| Fasting serum insulin (μIU/ml) | N/A | 9.9 ± 7.2 | 9.23 ± 6.25 | 32959 ± 26327 | 4.17 ± 2.52 |
| Glycated Haemoglobin (%) | N/A | 5.4 ± 0.5 | 6.5 ± 1.7 | N/A | N/A |
| Physical activity levels (%) | | | | | |
| Low | 31 | 72.5 | 82.0 | 39.3 | N/A |
| Medium | 69 | 19.3 | 16.2 | 49.6 | N/A |
| High | | 8.3 | 1.8 | 11.1 | N/A |

[a]Children with risk factors for cardiovascular disease were included

[b]Women were included for analysis

[c]Adults with moderate risk factors for cardiovascular disease were included

Several genetic loci have supported the presence of ethnic differences for metabolic traits and vitamin B12 status within the populations studied in this thesis. The *FTO* rs8050136 genotype is an example of genetic heterogeneity according to race. The minor allele ‘A’ of the SNP rs8050136 was present in 13% of Indian participants vs 23% Indonesian participants (**Table 40**). Although India and Sri Lanka are geographically close, the minor allele was more frequent in the Sinhalese population (34%). Furthermore, the prevalence of the rs8050136 minor allele in the British DIVAS population (42%) was the most prevalent in the population, and was in agreement with previous reported values for the Caucasian population (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?do_not_redirect&rs=rs8050136).

The *FUT2* SNP rs602662 is one of the most commonly studied variant related to vitamin B12 status. The rs602662 SNP is an example of a B12 SNP that has demonstrated ethnic specificity, for example, within the Indonesian population the minor allele frequency was extremely low (0.03%) in comparison to the DIVAS (46%), GOOD (31%) and Brazilian population (41%). Although no genotyping errors were identified, the SNP rs602662 did not reach HWE within the Indonesian and Sri Lankan populations (**Table 40**). It is possible that the Hardy-Weinberg equilibrium is not met in South Asian / South East Asian populations. An alternative reason could be that the HWE was not reached in these populations, due to the small sample size and the possibility of interbreeding (especially as consanguineous marriages are common in these populations).

**Table 40: Genotype frequencies: A Comparison of the Brazilian, GOOD, CURES, MINANG and DIVAS studies**

| Gene | rs number | Major allele | Minor allele | Common Homozygotes (%) | Heterozygotes (%) | Rare Homozygotes (%) | Minor allele frequency | HWE | ethnicity |
|---|---|---|---|---|---|---|---|---|---|
| *FTO* | rs8050136 | C | A | 291 (75.2) | 90 (23.5) | 6 (1.6) | 0.13 | 0.749 | India |
| | rs8050136 | C | A | 69 (60.0) | 39 (33.9) | 7 (6.1) | 0.23 | 0.638 | Indonesia |
| | rs8050136 | C | A | 40 (33.9) | 57 (48.0) | 21 (17.8) | 0.42 | 0.929 | British |
| | rs8050136 | C | A | 48 (44.0) | 47 (43.1) | 14 (12.8) | 0.34 | 0.641 | Sri Lanka |
| | rs2388405 | T | C | 342 (83.6) | 62 (15.2) | 5 (1.2) | 0.09 | 0.259 | India |
| | rs9939609 | T | A | 70 (60.3) | 39 (33.6) | 7 (6.0) | 0.23 | 0.618 | Indonesia |
| | rs9939609 | T | A | 41 (34.5) | 57 (47.9) | 21 (17.6) | 0.42 | 0.877 | British |
| | rs9939609 | T | A | 48 (44.0) | 47 (43.1) | 14 (12.8) | 0.34 | 0.641 | Sri Lanka |
| *MC4R* | rs17782313 | T | C | 89 (76.1) | 26 (22.2) | 2 (1.7) | 0.13 | 0.949 | Indonesia |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | rs17782313 | T | C | 70 (59.8) | 38 (32.5) | 9 (7.7) | 0.24 | 0.243 | British |
| | rs17782313 | T | C | 48 (44.0) | 50 (45.9) | 11 (10.1) | 0.33 | 0.700 | Sri Lanka |
| | rs2229616 | G | A | 116 (99.1) | 1 (0.9) | 0 (0.0) | 0.00 | 0.963 | Indonesia |
| | rs2229616 | G | A | 113 (95.0) | 6 (5.0) | 0 (0) | 0.03 | 0.778 | British |
| | rs2229616 | G | A | 99 (91.7) | 9 (8.3) | 0 (0) | 0.04 | 0.651 | Sri Lanka |
| *TCF7L2* | rs12255372 | G | T | 97 (82.9) | 20 (17.1) | 0 (0.0) | 0.09 | 0.312 | Indonesia |
| | rs12255372 | G | T | 66 (55.9) | 42 (35.6) | 10 (8.5) | 0.26 | 0.378 | British |
| | rs12255372 | G | T | 57 (52.3) | 45 (41.3) | 7 (6.4) | 0.27 | 0.633 | Sri Lanka |
| | rs7903146 | C | T | 95 (81.9) | 21 (18.1) | 0 (0.0) | 0.09 | 0.284 | Indonesia |
| | rs7903146 | C | T | 62 (52.1) | 46 (38.7) | 11 (9.2) | 0.29 | 0.564 | British |
| | rs7903146 | C | T | 45 (41.3) | 54 (49.5) | 10 (9.2) | 0.34 | 0.274 | Sri Lanka |
| *KCNJ11* | rs5219 | C | T | 55 (47.0) | 47 (40.2) | 15 (12.8) | 0.33 | 0.329 | Indonesia |
| | rs5219 | C | T | 49 (45.0) | 45 (41.3) | 15 (13.8) | 0.34 | 0.373 | Sri Lanka |
| *CAP10* | rs3792267 | G | A | 108 (91.5) | 9 (7.6) | 1 (0.8) | 0.05 | 0.123 | Indonesia |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | rs3792267 | G | A | 79 (72.5) | 24 (22.0) | 6 (5.5) | 0.17 | 0.035 | Sri Lanka |
| | rs5030952 | C | T | 77 (66.4) | 31 (26.7) | 8 (6.9) | 0.20 | 0.063 | Indonesia |
| | rs5030952 | C | T | 101 (92.7) | 8 (7.3) | 0 (0) | 0.04 | 0.691 | Sri Lanka |
| | rs2975760 | T | C | 66 (60.6) | 38 (34.9) | 5 (4.6) | 0.22 | 0.874 | Sri Lanka |
| *MUT* | rs1141321 | G | A | 67 (59.3) | 40 (35.4) | 6 (5.3) | 0.23 | 0.993 | Indonesia |
| | rs1141321 | G | A | 28 (25.7) | 60 (55.0) | 21 (19.3) | 0.47 | 0.271 | Sri Lanka |
| *FUT6* | rs778805 | T | C | 33 (28.2) | 61 (52.1) | 23 (19.7) | 0.46 | 0.586 | Indonesia |
| | rs778805 | C | T | 29 (26.6) | 53 (48.6) | 27 (24.8) | 0.49 | 0.776 | Sri Lanka |
| *TCN1* | rs34324219 | C | A | 117 (100) | 0 (0.0) | 0 (0.0) | 0.00 | N/A | Indonesia |
| | rs34324219 | C | A | 107 (98.2) | 2 (1.8) | 0 (0) | 0.01 | 0.923 | Sri Lanka |
| *FUT2* | rs602662 | G | A | 111 (94.9) | 4 (3.4) | 2 (1.7) | 0.03 | 0.000 | Indonesia |
| | rs602662 | G | A | 34 (28.6) | 60 (50.4) | 25 (21.0) | 0.46 | 0.877 | British |
| | rs602662 | G | A | 34 (30.1) | 52 (46.0) | 24 (21.2) | 0.45 | 0.625 | Brazil |
| | rs602662 | G | A | 60 (55.6) | 30 (27.8) | 18 (16.7) | 0.31 | 0.000 | Sri Lanka |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | rs492602 | A | G | 38 (31.9) | 58 (48.7) | 23 (19.3) | 0.44 | 0.918 | British |
| | rs16982241 | G | A | 91 (76.5) | 26 (21.8) | 2 (1.7) | 0.13 | 0.928 | British |
| *TCN2* | rs1131603 | T | C | 117 (100) | 0 (0.0) | 0 (0.0) | 0.00 | N/A | Indonesia |
| | rs1131603 | T | C | 107 (98.2) | 2 (1.8) | 0 (0) | 0.01 | 0.923 | Sri Lanka |
| | rs1801198 | G | C | 60 (53.1) | 33 (29.2) | 10 (8.8) | 0.26 | 0.100 | Brazil |
| *CD320* | rs2336573 | C | T | 86 (74.1) | 29 (25.0) | 1 (0.9) | 0.13 | 0.390 | Indonesia |
| | rs2336573 | C | T | 99 (90.8) | 10 (9.2) | 0 (0) | 0.05 | 0.616 | Sri Lanka |
| *CUBN* | rs1801222 | C | T | 84 (74.3) | 27 (23.9) | 2 (1.8) | 0.14 | 0.920 | Indonesia |
| | rs1801222 | C | T | 78 (72.2) | 29 (26.9) | 1 (0.9) | 0.14 | 0.338 | Sri Lanka |
| *CPS1* | rs1047891 | C | A | 48 (41.0) | 56 (47.9) | 13 (11.1) | 0.35 | 0.579 | Indonesia |
| | rs1047891 | C | A | 56 (51.9) | 44 (40.7) | 8 (7.4) | 0.28 | 0.873 | Sri Lanka |
| *MTHFR* | rs1801133 | C | T | 92 (79.3) | 24 (20.7) | 0 (0.0) | 0.10 | 0.214 | Indonesia |
| | rs1801133 | C | T | 89 (81.7) | 19 (17.4) | 1 (0.9) | 0.10 | 0.990 | Sri Lanka |
| | rs1801133 | C | T | 55 (48.7) | 41 (36.3) | 12 (10.6) | 0.30 | 0.310 | Brazil |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | rs1801131 | A | C | 56 (49.9) | 43 (38.1) | 13 (11.5) | 0.31 | 0.293 | Brazil |
| *CLYBL* | rs41281112 | C | T | 105 (96.3) | 4 (3.7) | 0 (0) | 0.02 | 0.845 | Sri Lanka |
| *BHMT* | rs3797546 | T | C | 67 (59.3) | 27 (23.9) | 7 (6.2) | 0.20 | 0.081 | Brazil |
| | rs492842 | T | C | 35 (31) | 43 (38.1) | 27 (23.9) | 0.46 | 0.071 | Brazil |
| *COMT* | rs4680 | G | A | 35 (31) | 48 (42.5) | 24 (21.2) | 0.45 | 0.335 | Brazil |
| | rs4633 | C | T | 44 (38.9) | 51 (45.1) | 16 (14.2) | 0.37 | 0.844 | Brazil |
| *MTR* | rs1805087 | A | G | 77 (68.1) | 32 (28.3) | 2 (1.8) | 0.16 | 0.521 | Brazil |
| *MTRR* | rs1801394 | A | G | 45 (39.8) | 49 (43.4) | 16 (14.2) | 0.37 | 0.655 | Brazil |

Abbreviations: *HWE* Hardy-Weinberg equilibrium

### 8.1.7 Limitations and strengths

A major limitation to this thesis was that some of the studies had relatively small sample sizes; this may have affected our results by having a wider confidence interval. However, despite this limitation, we were still able to observe gene-diet interactions on vitamin B12 and cardio-metabolic disease outcomes. In this PhD project, most of the studies employed a cross-sectional study design (Brazilian adolescents, CURES, GOOD, MINANG) which investigated the genetic effects at a single point in time, thus we were unable to examine the causal relationship between the SNP–diet interactions on vitamin B12 concentrations and metabolic traits. Furthermore, some of the studies might have introduced biases, including the inclusion of type 2 diabetes participants in the CURES study (selection bias); however, there was adjustment for diabetes status in the statistical analysis. The cross-sectional studies investigated gene-diet interactions; where the dietary component was based on macronutrient intake. However, no data on specific types of micronutrient was available, and this could have limited further potential gene-diet interactions from being observed. Furthermore, the data collected from FFQs and physical activity questionnaires, were based on self-reported data and this could have led to measurement bias. The data was limited in some of the studies, for example the GOOD, MINANG and DIVAS studies did not investigate folate or homocysteine concentrations in their participants. Additionally, none of the studies investigated other vitamin B12 biomarkers, such as Holo-transcobalamin (holoTC) or Methylmalonic Acid (MMA), which have been shown to have greater sensitivity for vitamin B12 status.

The main strengths of this thesis were that different study designs were employed (cross-sectional and intervention) to confirm the observed findings. Three of the cross-sectional studies used FFQs (CURES, GOOD, MINANG) [368, 411, 440] that were validated to measure long-term macronutrient intake of the population. Vitamin B12 status has not been previously

reported in healthy Indonesian women and Sri Lankan adults, thus this thesis was the first to investigate vitamin B12 status in Sri Lanka and Indonesia (women only). Further to this, four of the studies (CURES, GOOD, MINANG and DIVAS) calculated genetic risk scores (GRS) for the risk of vitamin B12 deficiency and cardio-metabolic related traits. The benefits of using a GRS as opposed to using a single gene, is that GRS can increase the statistical power of the study [387] and can provide better information for disease association compared to the single SNPs. The use of the tagging approach allowed me to select three tagSNPs representing the entire common genetic variations across the *FUT2* gene in the DIVAS study. Furthermore, my thesis was the first to report gene-diet interactions on vitamin B12 concentrations and metabolic traits in Sri Lanka and Indonesia.

## 8.2 Conclusion

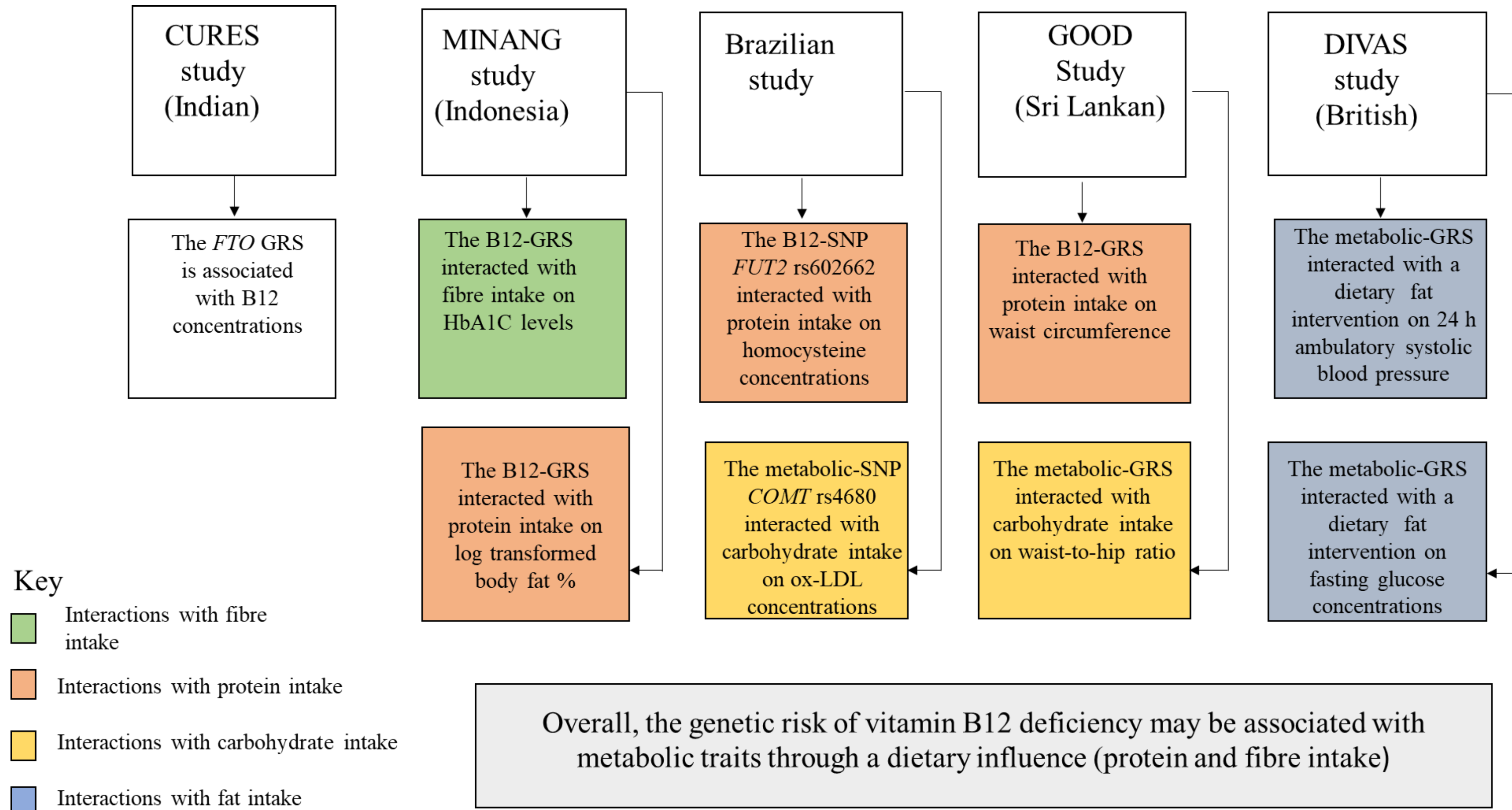
In conclusion, my research identified novel interactions between the B12-GRS and dietary factors (protein intake and fibre intake) on central obesity indicators within the Sri Lankan and Indonesian populations. Furthermore, the B12-related SNP *FUT2* rs602662 showed a significant interaction with protein intake on homocysteine concentrations (a marker of cardiovascular disease) in the Brazilian adolescent population. However, no gene-lifestyle interactions on cardio-metabolic outcomes were observed in the British population. Given that high fibre and protein diets are recommended for preventing metabolic disease outcomes [460] [449], the gene-diet interaction findings observed in my study will have significant public health implications, where people carrying risk alleles for vitamin B12 deficiency could be advised to alter their diet according to their ethnic background. Interestingly, in the Indian population, I found the relationship between vitamin B12 and obesity in the other direction to what was observed in the Indonesian, Brazilian and Sri Lankan populations. In the CURES Indian study, I found that being genetically predisposed to obesity could in fact lower vitamin B12 concentrations, without influence from dietary factors. Ultimately, the findings in this

thesis contribute to a better understanding of how genetic variants related to B12 absorption and metabolism interacts with lifestyle factors in the progression of metabolic traits (**Figure 19**).

Replications of the findings from this thesis require confirmation from studies with a larger population size and must include individuals from different ethnic groups. It is important that more randomized controlled trials are carried out in the future, to identify the cause and effect of a dietary intervention on a metabolic outcome (based on an individual's genotype). Furthermore, it is important that prospective genotyping is carried out to prevent any issues related to having an unbalance in genotype groups, which could confound the results. This thesis only addressed vitamin B12 concentrations, and only two of the studies in this thesis (Indian and Brazilian) studied folate and homocysteine levels, therefore there is a need to take these risk factors into consideration in order to implement dietary strategies to prevent the metabolic disease-related outcomes. One of the benefits of this thesis was the use of genetic risk scores to provide evidence for the genetic effects of the phenotypes observed and this increased the statistical power of our study. It is important that future nutrigenetic studies utilize a comprehensive panel of SNPs associated with vitamin B12 or metabolic disease related traits to calculate a genetic risk score.

In conclusion, my study has demonstrated significant interactions between the B12-GRS and dietary intake on metabolic traits. However, these gene-diet interactions require replication in an independent cohort utilizing a larger number of samples and functional studies to understand the role of these interactions at the molecular level are highly warranted before implementing personalised nutrition strategies to overcome the burden of metabolic diseases.

**Figure 19: The main study findings of this thesis**



## 8.3 Future prospects

In this thesis, I found that the relationship between B12 deficiency and metabolic outcomes may be influenced by dietary factors such as protein and fibre intake. It is important that the gene-diet interactions observed in this thesis are replicated, before public health recommendations can be enforced. Also, it is important to further investigate whether people with increased weight require more B12 containing foods, for the possibility of implementing B12 deficiency screening programmes in the population. If low vitamin B12 concentrations stimulate metabolic diseases through a dietary influence, it is important that mechanistic studies are carried out to determine how vitamin B12 interacts with adipose tissue metabolism or how epigenetic mechanisms contribute to the epidemic of metabolic diseases. Current literature suggests that the genetic profile of an individual can shape the microbiome of the host, and an altered gut flora has been associated with vitamin B12 deficiency [14, 264]. This possibly requires investigation, given the known link between an altered distribution in gut microbiota and obesity.

In the future, validation of the findings of this thesis will be carried out by replicating the study in a cohort with an increased sample size and power. Moreover, it is difficult to truly isolate the macronutrient accountable for any nutrigenetic effects, especially for fat and carbohydrates, as one macronutrient usually compensates the other [507]. Therefore, dietary intervention studies are more favourable as they place strong emphasis on compliance to the dietary exposure. Dietary intervention studies have the potential to avoid measurement bias found within FFQs and to uncover relationships between SNPs, diet and metabolic traits. Additionally, longitudinal research studies are required to examine the causal relationship between the SNP–diet interactions on vitamin B12 concentrations and metabolic traits. Future studies should also measure specific dietary micronutrients for more in-depth gene-diet interactions to be carried out.

Future research should include more long-term, randomised controlled studies that robustly measure both body composition (e.g. dual-energy X-ray absorptiometry, magnetic resonance imaging and/or computed tomography scans) and body size (e.g. body weight or BMI) [93]. Additionally, investigating how different clinical outcomes associated with vitamin B12 deficiency is affected by B12 supplements at different doses is another area that requires attention. It is also necessary to study the effects of adiposity on vitamin B12 status across all body sizes and to measure vitamin B12 status using markers such as homocysteine, methylmalonic acid (MMA) and holotranscobalamin. Looking into groups that are more at risk of vitamin B12 deficiency, such as elderly individuals, pregnant women, vegetarians, other patient groups (e.g. Type 2 diabetes patients taking metformin medication), ethnic minorities and elite athletes must also be considered.

It is yet to be elucidated whether the relationship between vitamin B12 and cardio-metabolic traits, can be modified in response to other dietary sources (e.g. fortified foods); such evidence will have important implications on setting dietary requirements in a clinical setting. Insights from this PhD thesis show promise for the use of personalised nutrition in the area of vitamin B12 and obesity, whereby certain vitamin B12-related SNPs may be used to predict an individual's risk of developing metabolic traits and may be modified according to an individual's lifestyle (dietary pattern/physical activity levels). Nutrigenetic research is urgently required, given the importance of developing public health strategies to decrease the prevalence and impact of overweight and obesity.

Although extraordinary improvements have been accomplished in identifying several gene-diet interactions, little is known about the underlying cardio-metabolic pathways of these gene-diet interactions [508, 509]. Nutrigenomics is an approach which explores the effect of specific nutrients on gene expression and their metabolic consequences [510]. The definition of nutrigenomics has further extended to 'nutritional factors which protect the genome from

any damage'. Nutrigenomics focuses on the impact of dietary factors on the genome, proteome (the complete set of proteins expressed by an organism) and the metabolome (the total number of metabolites). Researchers are now able to demonstrate that certain nutritional interventions are positive in a select proportion of the population, whilst others show no effect, and some may act unfavourably [511]. This field of research, will ultimately help in understanding how the diet interacts with metabolic pathways and their role in diet-related diseases [512]. On the other hand, nutrigenetics explores the effect of genetic variation on the interaction between a particular diet and disease [513]. The role of nutrigenomics and nutrigenetics will help revolutionise our ability to design optimal dietary advice for health maintenance and for metabolic disease prevention [221]. Currently, a large gap exists between nutrition recommendations and an individual's eating behaviour [514]. Population-based dietary advice, where a 'one size fits all' approach is given, has been relatively unsuccessful in benefitting the patient, due to a myriad of factors including: lack of motivation and failure of patient compliance [514]. Evidence now suggests that the use of genetic testing or personalised advice as a catalyst, can demonstrate behavioural changes in nutrition [515, 516].

'Foodomics' approaches are becoming increasingly essential tools in preventative health care. This approach combine 'omics' (i.e. transcriptomics, proteomics, metabolomics) technologies to prevent and treat non-communicable diseases [508]. The 'Foodomics' approach is used to help understand the gene-based differences among individuals in response to specific dietary patterns, and to identify the interactions of bioactive compounds from dietary components at a biochemical, molecular and cellular level [517]. An example of the foodomics approach was shown in the work of Valdes et al., who studied the effect of rosemary extracts on colon cancer cells, using multi-platform 'omics' analyses for clarifying the signalling and metabolic pathways involved in cancer progression [518].

Precision nutrition is another technique that aims to develop more comprehensive nutritional recommendations based on an individual's internal and external environment [519]. This approach suggests that it is possible to understand the complex relationship between an individual and their food consumption, individuals physical activity level, their food behaviour, microbiota, metabolome and their phenotype (health status), in order to offer nutritional interventions/ advice which can benefit the individual [510, 519]. As a result, precision nutrition requires a greater degree of scientific certainty and replication studies, compared to the other methods [510].

The majority of studies assessing the link between genetic variation and disease risk have been studied in populations with European ancestry (78%). At present, there is a need to study more diverse ethnic groups, in order to translate our understanding of genetic disease architecture into clinical practice. At present, approximately 85% of the Genotype-Tissue Expression project study (examines the genes in tissues obtained from different people and studies how inherited changes in genes lead to common diseases) contains samples from European descent [207], again highlighting the need for more studies in minority populations. At present, the ability to study minority populations have been hindered, and this may be due to previous experiences of these populations being exploited/mistrusted in biomedical research [207]. In order to generate reliable phenotype data from diverse ethnic groups, it is important that less economically developed regions contain adequate funding, equipment for biochemical and genetic testing and experienced personnel [207].

In summary, knowledge in the field of nutrigenetics holds a promising future in preventing the development of dietary-related chronic diseases. Although, there are many studies which show interactions between genes and lifestyle factors, there are inconsistences in the evidence given which may limit the application of nutrigenetics to the general public at present [514]. There is a need to utilize larger studies that are well-powered and that examine

lifestyle/dietary behaviours across various ethnic groups in order to implement personalised nutrition.

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# Chapter 9

## Appendices

### 9.1 Research plan: The Influence of One-carbon Metabolism Gene Polymorphisms and Gene–environment Interactions on Homocysteine, Vitamin B12, Folate and Lipids in a Brazilian Adolescent Population

**Main study objective:**

To determine whether 10 SNPs from seven selected candidate genes related to the one-carbon metabolism cycle were associated with vitamin B12, homocysteine, folic acid and lipid-related outcomes and whether these associations were modified by environmental factors (diet and physical activity).

1)To work out which genetic model to use for each SNP: additive, dominant or recessive

2) To test the association between the 10 SNPs and folic acid levels

3) To test the association between the 10 SNPs and Hcy levels

4) To test the association between the 10 SNPs and vitamin B12 levels

5) To test the interaction between the 10 SNPs and dietary factors on folate levels

6) To test the interaction between the 10 SNPs and dietary factors on Hcy

7) To test the interaction between the 10 SNPs and dietary factor on vitamin B12 levels

**Previous Studies looking at the association of the 10 SNPs with biochemical traits:**

| Gene symbol | SNP rs number | Vitamin B12 | Folate | Hcy |
|---|---|---|---|---|
| *MTHFR* | rs1801133 | | [520] | [254, 520-522] |
| *MTHFR* | rs1801131 | | [523, 524] | [523] |
| *MTR* | rs1805087 | | [234] | |
| *MTRR* | rs1801394 | | [234] | |
| *TCN2* | rs1801198 | [285] | | |
| *COMT* | rs4680 | | | [525] |
| *COMT* | rs4633 | | | [338] |
| *BHMT* | rs492842 | | | It is unknown whether this SNP is associated with Hcy, although previous studies have implicated associations between the *BHMT* gene and homocysteine. |

| | | | | |
|---|---|---|---|---|
| *BHMT* | rs3797546 | | | It is unknown whether this SNP is associated with Hcy, although previous studies have implicated associations between the *BHMT* gene and homocysteine. |
| *FUT20* | rs602662 | [234, 254] | | |

**Co-variates in this study:**

- Age will be coded according the two age ranges[341] :

- (10-14 years) = Recoded as 0

-(15-19 years) = Recoded as 1

- Gender will be coded 0 (Male) or 1 (Female) [341]
- BMI was estimated and classified according to the WHO (2007) for BMI/age according to gender and was coded into 4 groups:[341]

- below 15$^{th}$ percentile=below normal weight -> Recoded as 1

- between 15$^{th}$ and 85$^{th}$ percentile= normal-weight -> Recoded as 2

-between 85$^{th}$ and 97$^{th}$ percentile= overweight -> Recoded as 3

- above 95$^{th}$ percentile=obese. -> Recoded as 4

**Plan of action:**

| **Objective 1: To work out which genetic model to use for each SNP: additive, dominant or recessive.** | | |
|---|---|---|
| **Aims:** | **Statistical test used:** | **1) Reason for statistical test used**<br>**2) Outcome of statistical test used**<br>**3) covariates** (when appropriate) |
| 1a) To determine the frequencies of each of the genotypes, so that an appropriate genetic model can be selected. | Descriptive statistics- Frequencies | 1) Reason for test: exposure variables (*SNPs*) are categorical variables<br>2) To be given the frequencies of the common homozygous, heterozygous and rare homozygous genotypes. Therefore, an appropriate model can be given to the SNP: dominant, recessive or additive. As well as this, the minor allele frequency can be calculated. |

| | | |
|---|---|---|
| **Objective 2: To test the association between the 10 SNPs and Folic acid levels** | | |
| 2a) Testing the association between each SNP and folic acid levels | One-Way Anova; Post hoc Tukey | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and the outcome variable (folic acid) is a continuous variable.<br>2) Outcome of test: Identifying the difference of folic acid levels between different genotypes (common, heterozygotes and rare). |
| 2b) Adjusting for covariates to check if the covariates influence the association between each SNP and folic acid levels | Univariate linear regression | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and the outcome variable (folic acid levels) is a continuous variable.<br>2) Outcome of test: identifying the impact of the variants on folic acid levels<br>3) Covariates to be adjusted: Age, gender, BMI [341] |
| **Objective 3: To test the association between 10 SNPs and Homocysteine levels (Hcy)** | | |
| 3a) Testing the association between each SNP and Hcy levels | One-Way Anova; Post hoc Tukey | 1) Reason for test: The exposure variable (*SNP*) is a categorical variable and the outcome variable (Hcy levels) is a continuous variable.<br>2) Outcome of test: Identifying the difference of Hcy levels between different genotypes. |
| 3b) Adjusting for covariates to check if the covariates influence the association between each SNP and Hcy levels. | Univariate linear regression | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and the outcome variable (Hcy levels) is a continuous variable.<br>2) Outcome of test: identifying the impact of the variants on Hcy levels<br>3) Covariates to be adjusted: Age, gender, BMI [341] |
| **Objective 4:To test the association between the 10 SNPs and vitamin B12 (cbl)** | | |
| 4a) Testing the association between each SNP and cbl levels | One-Way Anova; Post hoc Tukey | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and the outcome variable (cbl levels) is a continuous variable.<br>2) Outcome of test: Identifying the difference of cbl levels between different genotypes. |
| 4b) Adjusting for covariates to check if the covariates influence the association between each SNP and cbl levels | Univariate linear regression | 1) Reason for test: exposure variable (*SNP*) is a category variable and outcome variable (cbl levels) is a continuous variable<br>2) Outcome of test: identifying the impact of the variants on cbl levels<br>3) Covariates to be adjusted: Age, gender, BMI [341] |
| **Objective 5: To test the association between the 10 SNPs and lipid concentrations (TAG, HDL-cholesterol, LDL-cholesterol, and ox-LDL)** | | |
| 5a) Testing the association between | One-Way Anova; Post hoc Tukey | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and the outcome |

| | | |
|---|---|---|
| each SNP and each lipid concentration | | variable (each lipid concentration) is a continuous variable.<br>2) Outcome of test: Identifying the difference of lipid concentrations between different genotypes. |
| 5b) Adjusting for covariates to check if the covariates influence the association between each SNP and each lipid concentration | Univariate linear regression | 1) Reason for test: exposure variable (*SNP*) is a category variable and outcome variable (each lipid concentration) is a continuous variable<br>2) Outcome of test: identifying the impact of the variants on lipid concentration.<br>3) Covariates to be adjusted: Age, gender, BMI [341] |
| **Objective 6: To test the interaction between the 10 SNPs and dietary factors on Folic acid levels** | | |
| 6a) Testing the interaction between the macronutrient and SNPs on folic acid levels | Univariate linear regression | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and the outcome variable (folic acid levels) is a continuous variable.<br>2) Outcome of test: identifying the impact of the gene variants and the macronutrients: carbohydrate, protein and fat on folic acid levels.<br>3) Covariates to be adjusted: Age, gender, BMI [341]. |
| 6b) Testing to find out if high or low macronutrients are causing the interaction | Univariate linear regression | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and outcome variable (Folic acid levels) is a continuous variable.<br>2) Outcome of test: identifying the impact of gene variants and the consumption of different quantities of the macronutrient on folic acid levels<br>3) Covariates to be adjusted: Age, gender, BMI.[341]<br>(Data split based on tertiles of carbohydrate, protein and fat) [253] |
| **Objective 7: To test the interaction between the 10 SNPs and dietary factors on Hcy levels** | | |
| 7a) Testing the interaction between the macronutrient and SNPs on Hcy levels | Univariate linear regression | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and the outcome variable (Hcy levels) is a continuous variable.<br>2) Outcome of test: identifying the impact of the gene variants and the macronutrients: carbohydrate, protein, fat on folic acid levels.<br>3) Covariates to be adjusted: Age, gender, BMI [341]. |

| 7b) Testing to find out if high or low macronutrients are causing the interaction | Univariate linear regression | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and outcome variable (Hcy levels) is a continuous variable.<br>2) Outcome of test: identifying the impact of gene variants and the consumption of different quantities of the macronutrient on Hcy levels<br>3) Covariates to be adjusted: Age, gender, BMI [341].<br>(Data split based on: Tertiles of carbohydrate, protein and fat) [253] |
|---|---|---|
| **Objective 8: To test the interaction between the 10 SNPs and dietary factors on cbl levels** | | |
| 8a) Testing the interaction between the macronutrient and SNPs on cbl levels | Univariate linear regression | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and the outcome variable (cbl levels) is a continuous variable.<br>2) Outcome of test: identifying the impact of the gene variants and the macronutrients: carbohydrate, protein, fat on cbl levels.<br>3) Covariates to be adjusted: age, gender, BMI [341] |
| 8b) Testing to find out if high or low macronutrients are causing the interaction | Univariate linear regression | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and outcome variable (cbl levels) is a continuous variable.<br>2) Outcome of test: identifying the impact of gene variants and the consumption of different quantities of the macronutrient on cbl levels<br>3) Covariates to be adjusted: Age, gender, BMI [341]<br>(Data split based on tertiles of carbohydrate, protein and fat)[253] |
| **Objective 9: To test the interaction between the 10 SNPs and dietary factors on lipid concentrations (TAG, HDL-cholesterol, LDL-cholesterol, and ox-LDL)** | | |
| 9a) Testing the interaction between the macronutrient and SNPs on lipid concentrations | Univariate linear regression | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and the outcome variable (lipid concentrations) is a continuous variable.<br>2) Outcome of test: identifying the impact of the gene variants and the macronutrients: carbohydrate, protein, fat on lipid concentrations.<br>3) Covariates to be adjusted: age, gender, BMI [341] |
| 9b) Testing to find out if high or low | Univariate linear regression | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and outcome |

| | | |
|---|---|---|
| macronutrients are causing the interaction | | variable (lipid concentrations) is a continuous variable.<br>2) Outcome of test: identifying the impact of gene variants and the consumption of different quantities of the macronutrient on lipid concentrations<br>3) Covariates to be adjusted: Age, gender, BMI [341]<br>(Data split based on: Tertiles of carbohydrate, protein and fat)[253] |
| **SPECIAL NOTES** | | |
| When looking at carbohydrates, proteins and fat in grams, you will need to adjust for Kcal. If you are using energy intake of the macronutrients you do not need to adjust for Kcal. | Compute variables | • For Carbohydrate interactions:<br>1g of Carbohydrates = 4kcal<br>• For Fat interactions:<br>1g=9 kcal<br>• For Protein interactions:<br>1g= 4kcal [526] |
| When significant interactions with macronutrients are detected, further investigation will take place to underline the specific type of macronutrient responsible for the interaction. | Univariate linear regression | No subgroups of protein and carbohydrates were recorded in this data set.<br><br>For fat interactions:<br>• Test for interaction between SNPs with saturated fatty acid intake, SNPs with monounsaturated fatty acid intake and SNPs with polyunsaturated fatty acid intake. Tertiles will be made for each of these sub-groups. |
| After carrying out the interaction analysis using Univariate liner regression and obtaining P values, make sure any problems due to multiple comparisons are counteracted | Bonferroni Correction | 1) This method adjusts for multiple comparison and reduces experiment-wise error rate in genetic association studies[527] |

## 9.2 Research plan – A genetic approach to examine the relationship between vitamin B12 status and metabolic traits in a South Asian population

**Main objectives:**

The purpose of this study is to use a genetic approach to explore the relationship between metabolic traits and vitamin B12 status in a Sri Lankan population and to investigate whether these relationships were modified by dietary intake.

**Hypothesis:**

I will test the hypotheses that low plasma vitamin B12 concentrations caused by genetic variants are associated with an increased risk of obesity and type 2 diabetes, and that the effect of genetic variants associated with high BMI on obesity traits is partly mediated through the reduction of serum vitamin B12 concentration. The latter hypothesis requires that genetically increased obesity traits are associated with low vitamin B12 concentration and with increased risk of diabetes/obesity traits.

**Specific aims:**

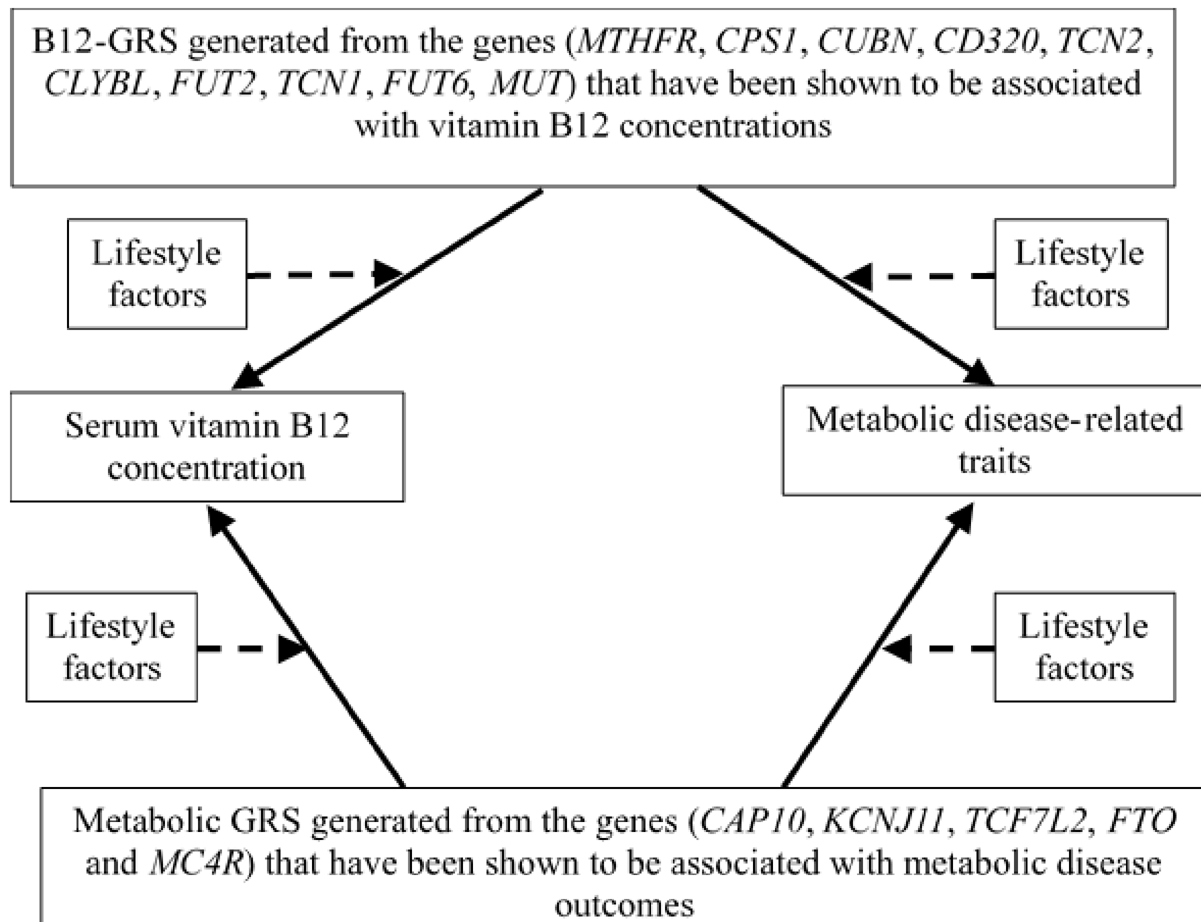


**Diagram representing the study design.** The diagram shows four possible associations, and four possible interactions. One-sided arrows with unbroken lines represent genetic associations and one-sided arrows with broken lines represent interactions between a lifestyle factor and GRS on serum vitamin B12/ metabolic traits.

1. I will test the association between the metabolic-GRS and vitamin B12 concentrations and metabolic disease-related traits.
2. I will then test the associations between the B12–GRS and vitamin B12 status and metabolic disease related traits.
3. Lastly, I will test whether these genetic associations are modified by lifestyle factors (macronutrient intake and physical activity levels).

**Previous Studies looking at the association of the 10 B12-related SNPs with vitamin B12 concentrations:**

| Gene | rs number | Studies which show an association with vitamin B12 |
|---|---|---|
| *Methylenetetrahydrofolate reductase (MTHFR)* | rs1801133 | [288] |
| *Carbamoyl-phosphate synthase 1 (CPS1)* | rs1047891 | [205] |
| *Cubulin (CUBN)* | rs1801222 | [205, 234, 253] |
| *CD320 molecule (CD320)* | rs2336573 | [205, 237, 253] |
| *Transcobalamin 2 (TCN2)* | rs1131603 | [205, 233] |
| *Citrate lyase beta like (CLYBL)* | rs41281112 | [205, 206] |
| *Fucosyltransferase 2 (FUT2)* | rs602662 | [205, 233, 234, 253, 254, 258, 260] |
| *Transcobalamin 1 (TCN1)* | rs34324219 | [205, 233] |
| *Fucosyltransferase 6 (FUT6)* | rs778805 | [205] |
| *Methylmalonyl-CoA mutase (MUT)* | rs1141321 | [205, 206, 234] |

**Previous Studies looking at the association of the 10 metabolic disease-related SNPs with diabetes and obesity traits:**

| **Gene** | **rs number** | **Studies which show its association with Type 2 diabetes in South Asian populations** | **Studies which show its association with obesity in South Asian populations** |
|---|---|---|---|
| *Calpain 10 (CAPN10)* | rs3792267 | [374, 528] | |
| *Calpain 10 (CAPN10)* | rs2975760 | [528] | |
| *Calpain 10 (CAPN10)* | rs5030952 | [528] | |
| *Potassium voltage-gated channel subfamily J member 11 (KCNJ11)* | rs5219 | [529] | |
| *Transcription factor 7-like 2 (TCF7L2)* | rs12255372 | [372, 373, 530-532] | |

| *Transcription factor 7-like 2 (TCF7L2)* | rs7903146 | [372, 373, 530, 532-536] | [479, 537] |
|---|---|---|---|
| *Fat mass and obesity associated (FTO)* | rs9939609 | [538, 539] | [370, 479, 539-546] |
| *Melanocortin 4 Receptor (MC4R)* | rs17782313 | | [370, 545, 547-550] |
| *Fat mass and obesity associated (FTO)* | rs8050136 | | [371, 542, 544, 546] |
| *MC4R* | rs2229616 | | [551] (South East Asian population) |

**Disease cut off-values**:

1) Only normal glucose tolerant (NGT) individuals were included in this study. According to the World Health Organization (WHO) NGT individuals are those with 2-h plasma glucose value < 7.8 mmol/l (140 mg/dl) [552]
2) Generalized obesity was defined according to the World Health Organization Asia Pacific Guidelines for Asians as non-obese (BMI < 25 kg/m2 ) and obese (BMI ≥ 25 kg/m2 )[422].
3) Vitamin B12 status is defined as:
   <148 pmol/L (%) – deficient individuals
   148-221 pmol/L- suboptimal individuals
   >221 pmol/L – normal individuals [2]

**Plan of action:**

| **Objective 1: To work out whether each SNP was in Hardy-Weinberg equilibrium** | | |
|---|---|---|
| **Aims:** | **Statistical test used:** | **1) Reason for statistical test used**<br>**2) Outcome of statistical test used**<br>**3) covariates** (when appropriate) |
| 1a) Determine whether the observed genotype counts are in Hardy-Weinberg equilibrium (HWE) | Chi-Squared test | 1. Reason for test: To compare observed genotype counts with the values expected under Hardy-Weinberg<br>2. Outcome of statistical test: To test whether a population is in HWE at a locus [408, 553] |
| **Objective 2: To produce descriptive statistics for all the sample members who completed an assessment on demographics, fasting biochemical and anthropometric measurements.** | | |
| 2a) To determine the descriptive statistics of the sample members who completed an assessment on demographics, fasting biochemical and anthropometric measurements. | Descriptive statistics: Descriptives for continuous variables<br><br>Or<br><br>Descriptive statistics: Frequencies for | 1. Reason for statistical test used: To determine baseline measures of the outcomes of interest (Will be discussed in detail in the point below) in all participants of the study<br>2. Outcome of statistical test used: To determine the mean and standard deviation of the following demographic, anthropometric and biochemical variables:<br>• Age (yrs) |

| | | |
|---|---|---|
| | categorical variables | • Height (cm)<br>• Weight (kg)<br>• BMI (Kg/m$^2$)<br>• WC (cm)<br>• Hip (cm)<br>• WHR<br>• Fat (%)<br>• Fasting plasma glucose (mg/dl)<br>• Fasting serum insulin (μIU/ml)<br>• Glycated Haemoglobin (HbA1c)<br>• Glucose (mmol/l)<br>• Insulin (mg/dl)<br>• Vitamin B12 levels (pmol/L)<br>• Physical Activity Levels [Sedentary (%); Moderate (%); Vigorous (%)]<br>• Total energy (kcal/d)<br>• Protein (g)<br>• Fat (g)<br>• Carbohydrate (g)<br>• Dietary fibre (g)<br>• Polyunsaturated Fatty acid (PUFA) (g)<br>• |
| 2b) To stratify the descriptive statistics table into men and women. | Students t test | 1. Outcome of statistical test used: To identify if there are any statistically significant differences in the demographic, anthropometric and biochemical variables between the tertiles of vitamin B12 concentration between men and women in each tertile [408]. |
| **Objective 3: To test the association between the B12-GRS and metabolic-GRS on fasting biochemical/anthropometric measurements (Vitamin B12, glucose, Insulin, Hbac1, vitamin B12, BMI, Fat % and WHR).** | | |
| 3a) Testing the association between the GRSs with fasting biochemical/anthropometric measurements, whilst adjusting for covariates. | Univariate linear regression | 1) Reason for test: exposure variable (*GRS*) is a categorical variable and the outcome variable (fasting biochemical trait/anthropometric trait) is a continuous variable.<br>2) Outcome of test: To identify the impact of the genetic variants on the |

| | | levels of the fasting metabolic trait/ anthropometric trait.<br>3) Covariates to be adjusted: Age, gender, BMI (BMI was not adjusted, when BMI was the continuous outcome) [341]. |
|---|---|---|
| **Objective 4: To test the interaction between the two GRSs (B12-GRS and metabolic-GRS) and dietary factors on fasting biochemical/anthropometric measurements (Vitamin B12, glucose, Insulin, Hbac1, vitamin B12, BMI, Fat % and WHR).** | | |
| 4a) Testing the interaction between macronutrients and SNPs on fasting biochemical and anthropometric measurements. | Univariate linear regression | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and the outcome variable (fasting biochemical measurement/ anthropometric measurement) is a continuous variable.<br>2) Outcome of test: identifying the impact of the gene variants and the macronutrients: carbohydrate, protein, fat on fasting biochemical measurement/anthropometric measurement<br>3) Covariates to be adjusted: Age, gender, BMI [341] |
| 4b) Testing to find out if high, low or medium consumption of these macronutrients are causing the interaction | Univariate linear regression | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and outcome variable (fasting biochemical measurement/anthropometric measurement) is a continuous variable.<br>2) Outcome of test: identifying the impact of gene variants and the consumption of different quantities of the macronutrient on fasting biochemical measurement/anthropometric measurement<br>3) Covariates to be adjusted: Age, gender, BMI [341].<br><br>(Data split based on: Tertiles of carbohydrate, protein and fat). |
| **SPECIAL NOTES:** | | |
| When looking at carbohydrates, Proteins and Fat in grams, you will need to adjust for Kcal. If you are using the percentage energy intake of the | Compute variables | • For Carbohydrate interactions:<br>1g of Carbohydrates = 4kcal<br>• For Fat interactions:<br>1g=9 kcal<br>• For Protein interactions: |

| | | |
|---|---|---|
| macronutrients, you do not need to adjust for Kcal, as it has already been adjusted for. | | 1g= 4kcal [526] |
| When significant interactions with macronutrients are detected, further investigation will take place to underline the specific type of macronutrient responsible for the interaction. | Univariate linear regression | if interactions with fat intake are significant, then<br>• Test for interaction between SNPs and saturated fatty acid intake, SNPs and monounsaturated fatty acid intake and SNPs and polyunsaturated fatty acid intake. Tertiles will be made for each of these dietary sub-groups. |
| After carrying out the interaction analysis using Univariate linear regression and obtaining P values, make sure adjustments are made to P values when several dependent or independent statistical tests are being performed simultaneously on a single data set. | Bonferroni Correction | 2) This method adjusts for multiple comparison and reduces experiment-wise error rate in genetic association studies [527] |

## 9.3 Research analysis plan: Evidence for the association between *FTO* gene variants and vitamin B12 concentrations in an Asian Indian population

**Main study objective:**

To investigate the association of two previously studied *FTO* polymorphisms (rs8050136 and rs2388405) with vitamin B12 concentrations and metabolic disease-related outcomes and examined whether these associations were modified by dietary factors and physical activity.

**Abbreviations:**

- BMI: Body Mass Index
- WHR: Waist to Hip Ratio
- WC: Waist Circumference
- SNP: Single Nucleotide Polymorphism
- TG: Triglyceride
- HDL: High Density Lipoprotein
- LDL: Low Density Lipoprotein
- HbAC1: Glycated Haemoglobin

**Specific aims of study:**

1) Firstly, we assessed the relationship between genetic variants thought to have a role in metabolic disease related traits with biochemical and metabolic traits (Homocysteine, folic acid, obesity, BMI, waist circumference).

2) Secondly, we assessed the effect of genetic variants thought to have a role in metabolic disease-related traits on serum vitamin B12.

3) Finally, we tested the interaction between the metabolic disease-related SNPs and dietary factors on fasting vitamin B12 levels and (Homocysteine, folic acid, obesity, BMI, waist circumference).

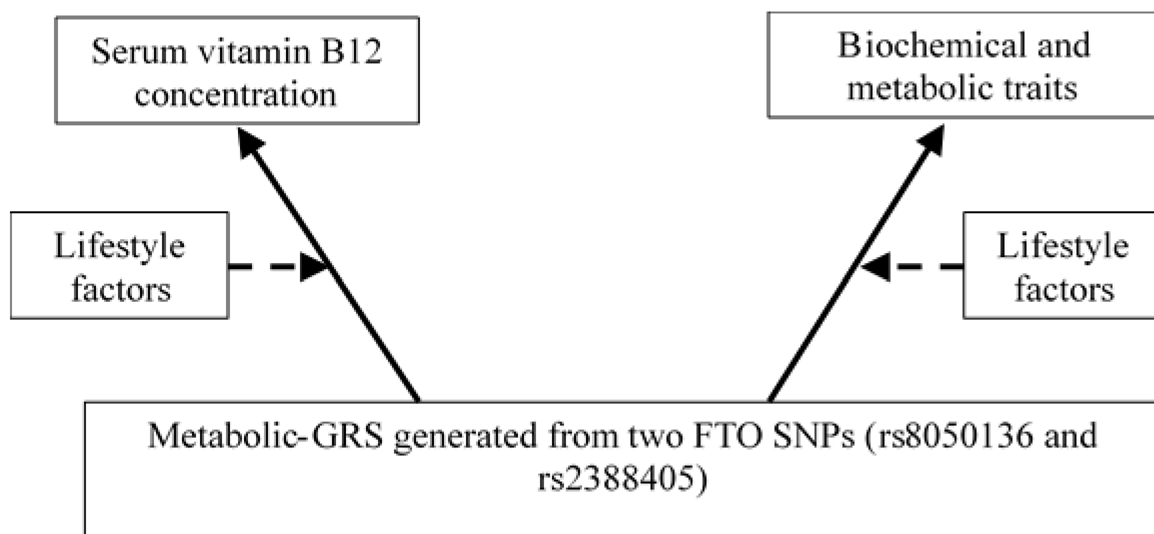


**Diagram representing the study design**

**Previous Studies looking at the association of the two SNPs with obesity related traits:**

| **Gene** | **rs number** | **Studies which have previously investigated the association of this SNP with obesity traits.** |
|---|---|---|
| *Fat mass and obesity associated (FTO)* | rs2388405 | [418] [419] [420]. |
| *Fat mass and obesity associated (FTO)* | rs8050136 | [371, 542, 544, 546] |

**Disease cut off-values:**

1. In The CURES 2003 study, diabetes was diagnosed 'based on the past medical history, drug treatment for diabetes, and/or using the ADA fasting criteria' [410].
2. Generalized obesity was defined according to the World Health Organization Asia Pacific Guidelines for Asians as non-obese (BMI < 25 kg/m$^2$ ) and obese (BMI ≥ 25 kg/m$^2$ ) [422].
3. Vitamin B12 status is defined as:

<148 pmol/L (%) – deficient individuals
148-221 pmol/L- suboptimal individuals
>221 pmol/L – normal individuals [2]

| **Objective 1: To work out whether each SNP was in Hardy-Weinberg equilibrium** | | |
|---|---|---|
| **Aims:** | **Statistical test used:** | **1) Reason for statistical test used**<br>**2) Outcome of statistical test used**<br>**3) covariates** (when appropriate) |
| 1a) Determine whether the observed genotype counts are in Hardy-Weinberg equilibrium (HWE) | Chi-Squared test | 3. Reason for test: To compare observed genotype counts with the values expected under Hardy-Weinberg<br>4. Outcome of statistical test: To test whether a population is in HWE at a locus [408, 553] |
| **Objective 2: To produce descriptive statistics for the sample members who completed an assessment on demographics, fasting biochemical and anthropometric measurements.** | | |
| 2a) To determine the descriptive statistics of sample members who completed an assessment on demographics and anthropometric measures of the outcomes of interest. | Descriptive statistics: Descriptives for continuous variables<br><br>Or<br><br>Descriptive statistics: Frequencies for | 1. Reason for statistical test used: To determine demographic and anthropometric measures of the outcomes of interest (Will be discussed in detail in the point below) in all participants of the study<br>2. Outcome of statistical test used: To determine the mean and standard deviation of the following demographic, anthropometric and biochemical variables:<br>• Age (yrs)<br>• BMI (Kg/m$^2$)<br>• WC (cm) |

| | | |
|---|---|---|
| | categorical variables | • WHR<br>• Obese cases (%)<br>• Fasting plasma glucose (mg/dl)<br>• Fasting serum insulin (µIU/ml)<br>• Glycated Haemoglobin (HbA1c)<br>• Glucose (mmol/l)<br>• Insulin (mg/dl)<br>• Vitamin B12 levels (pmol/L)<br>• PAL [Sedentary (%); Moderate (%); Vigorous (%)]<br>• Total Carbohydrate energy %<br>• Fat energy %<br>• Protein energy %<br>• Dietary fibre (g) |
| 2b) To stratify the descriptive statistics table into individuals who are diabetics, pre-diabetics and Normal Glucose tolerance individuals (NGT). | Students t test | 1. Reason for statistical test used: To compare the mean and standard deviations of demographic, anthropometric and biochemical variables between each group<br>2. Outcome of statistical test used: To identify if there are any statistically significant differences in the demographic, anthropometric and biochemical variables between each group (diabetics, pre-diabetics, NGT and combined individuals). |
| **Objective 3: To test the relationship between genetic variants thought to have a role in metabolic disease with biochemical and metabolic traits (Homocysteine, folic acid, obesity, BMI, waist circumference).** | | |
| 3a) To test for the association between the GRS and biochemical/anthropometric trait | Univariate linear regression | 1) Reason for test: The exposure variable (*GRS*) is a categorical variable and the outcome variable (biochemical trait/anthropometric trait) is a continuous variable.<br>2) Outcome of test: identifying the impact of the variants on the levels of the metabolic trait/anthropometric trait<br>3) Covariates to be adjusted: Age, gender, BMI (BMI was not adjusted, when BMI was the continuous outcome).<br>-When using the combined group (Diabetics, pre-diabetics and NGT), diabetes was adjusted as a confounder [341]. |

| | | |
|---|---|---|
| | | Special notes: For the BMI SNPs, the effect allele was the allele which raises BMI. This allele was compared with BMI raising alleles from Speliotes et al. [414, 554] |
| **Objective 4: To test the effect of genetic variants thought to have a role in metabolic disease-related traits on serum vitamin B12** | | |
| 4a) Testing the association between the GRS with fasting vitamin B12 concentrations | Univariate linear regression | 1) Reason for test: exposure variable (*GRS*) is a categorical variable and the outcome variable (vitamin B12 concentrations) is a continuous variable.<br>2) Outcome of test: identifying the impact of the variants on the levels of vitamin B12<br>3) Covariates to be adjusted: Age, gender, BMI (BMI was not adjusted, when BMI was the continuous outcome).<br>-When using the combined group (Diabetics, pre-diabetics and NGT), diabetes was adjusted as a confounder [341].<br><br>Special notes: For the BMI SNPs, the effect allele was the allele which raises BMI. This allele was compared with BMI raising alleles from Speliotes et al. [414, 554] |
| **Objective 6: Testing the interaction between the metabolic disease-related SNPs and dietary factors on vitamin B12 levels** | | |
| 6a) Testing the interaction between macronutrients and SNPs on vitamin B12 levels in each group | Univariate linear regression | 1) Reason for test: The exposure variable (*GRS*) is a categorical variable and the outcome variable (vitamin B12 levels) is a continuous variable.<br>2) Outcome of test: identifying the impact of the gene variants and the macronutrients: carbohydrate, protein, fat on vitamin B12 levels<br>3) Covariates to be adjusted: Age, gender, BMI [341]<br><br>-When using the combined group (Diabetics, pre-diabetics and NGT), diabetes was adjusted as a confounder. |
| 6b) Testing to find out if high, low or medium consumption of these macronutrients are causing the interaction | Univariate linear regression | 1) Reason for test: The exposure variable (*GRS*) is a categorical variable and the outcome variable (vitamin B12 levels) is a continuous variable.<br>2) Outcome of test: identifying the impact of gene variants and the consumption of different quantities of the macronutrient on vitamin B12 levels. |

| | | |
|---|---|---|
| | | 3) Covariates to be adjusted: Age, gender, BMI [341].<br>-When using the combined group (Diabetics, pre-diabetics and NGT), diabetes was adjusted as a confounder.<br>(Data split based on: Tertiles of carbohydrate, protein and fat) [253]. |
| **SPECIAL NOTES:** | | |
| When looking at carbohydrates, proteins and fat in grams, you will need to adjust for Kcal. If you are using the percentage energy intake of the macronutrients, you do not need to adjust for Kcal, as it has already been adjusted for. | Compute variables | • For carbohydrate interactions:<br>1g of carbohydrates = 4kcal<br>• For fat interactions:<br>1g=9 kcal<br>• For protein interactions: 1g= 4kcal [526] |
| When significant interactions with macronutrients are detected, further investigation will take place to underline the specific type of macronutrient responsible for the interaction. | Univariate linear regression | For fat interactions:<br>• To Test for the interaction between SNPs with saturated fatty acid intake, SNPs with monounsaturated fatty acid intake and SNPs with polyunsaturated fatty acid intake. Tertiles will be made for each of these sub-groups. |
| After carrying out the interaction analysis using Univariate linear regression and obtaining P values, make sure adjustments are made to P values when several dependent or independent statistical tests are being performed simultaneously on a single data set. | Bonferroni Correction | 3) This method adjusts for multiple comparison and reduces experiment-wise error rate in genetic association studies [527] |

## 9.4 Research plan – A nutrigenetic approach for investigating the relationship between vitamin B12 status and metabolic traits in Indonesian women (Replication of the Sri Lankan GOOD study)

**Main objectives:**

The aim of the present study was to explore the relationships between metabolic traits and vitamin B12 status in a cohort of healthy Indonesian women and to investigate whether these relationships were modified by dietary intake using a genetic approach.

**Hypothesis:**

I will test the hypotheses that low plasma vitamin B12 concentrations caused by genetic variants are associated with an increased risk of obesity and type 2 diabetes, and that the effect of genetic variants associated with high BMI on obesity traits is partly mediated through reduction of serum vitamin B12 concentration. The latter hypothesis requires that genetically increased obesity traits are associated with low vitamin B12 concentration and with increased risk of diabetes/obesity traits.

**Specific aims:**

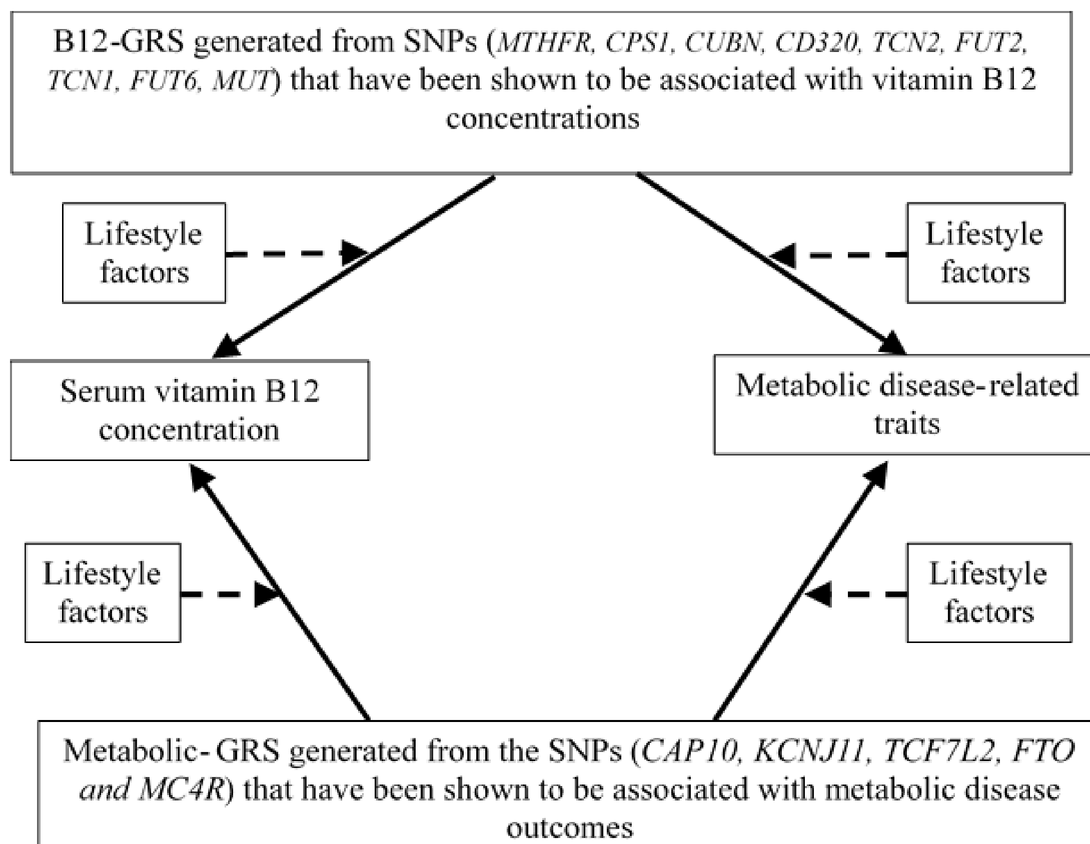


**Diagram representing the study design:** The diagram shows four possible associations, and four possible interactions. One-sided arrows with unbroken lines represent genetic associations and one-sided arrows with broken lines represent interactions between a lifestyle factor and GRS on serum vitamin B12/ metabolic traits.

1. I will test the association between the metabolic-GRS and vitamin B12 concentrations and metabolic disease-related traits (glucose, insulin, HbA1c, BMI, WC, body fat percentage)
2. I will then test the associations between the B12 –GRS and vitamin B12 status and metabolic disease related traits.
3. Lastly, I will test whether these genetic associations are modified by lifestyle factors (macronutrient intake and physical activity levels).

**Previous Studies looking at the association of the 10 B12-related SNPs with vitamin B12 concentrations:**

| **Gene** | **SNP rs number** | **Studies which show an association with vitamin B12** |
|---|---|---|
| *Methylenetetrahydrofolate reductase (MTHFR)* | rs1801133 | [288] |
| *Carbamoyl-phosphate synthase 1 (CPS1)* | rs1047891 | [205] |
| *Cubulin (CUBN)* | rs1801222 | [205, 234, 253] |
| *CD320 molecule (CD320)* | rs2336573 | [205, 237, 253] |
| *Transcobalamin 2 (TCN2)* | rs1131603 | [205, 233] |
| *Fucosyltransferase 2 (FUT2)* | rs602662 | [205, 233, 234, 253, 254, 258, 260] |
| *Transcobalamin 1 (TCN1)* | rs34324219 | [205, 233] |
| *Fucosyltransferase 6 (FUT6)* | rs778805 | [205] |
| *Methylmalonyl-CoA mutase (MUT)* | rs1141321 | [205, 206, 234] |

**Previous Studies looking at the association of the 10 metabolic disease-related SNPs with diabetes and obesity traits:**

| **Gene** | **SNP rs number** | **Studies which show its association with Type 2 diabetes in South Asian populations** | **Studies which show its association with obesity in South Asian / South East Asian populations** |
|---|---|---|---|
| *Calpain 10 (CAPN10)* | rs3792267 | [374, 528] | |
| *Calpain 10 (CAPN10)* | rs5030952 | [528] | |
| *Potassium voltage-gated channel subfamily J member 11 (KCNJ11)* | rs5219 | [529] | |
| *Transcription factor 7-like 2 (TCF7L2)* | rs12255372 | [372, 373, 530-532] | |
| *Transcription factor 7-like 2 (TCF7L2)* | rs7903146 | [372, 373, 530, 532-536] | [479, 537] |

| | | | |
|---|---|---|---|
| *Fat mass and obesity associated (FTO)* | rs9939609 | [538, 539] | [370, 479, 539-546] |
| *Melanocortin 4 Receptor (MC4R)* | rs17782313 | | [370, 545, 547-550] |
| *Fat mass and obesity associated (FTO)* | rs8050136 | | [371, 542, 544, 546] |
| *MC4R* | rs2229616 | | [551] |

**Disease cut off-values:**

4) Only normal glucose tolerant (NGT) individuals were included in this study. According to the World Health Organization (WHO) NGT individuals are those with 2-h plasma glucose value < 7.8 mmol/l (140 mg/dl) [552]

5) Generalized obesity was defined according to the World Health Organization Asia Pacific Guidelines for Asians as non-obese (BMI < 25 kg/m2 ) and obese (BMI ≥ 25 kg/m2 )[422].

Vitamin B12 status is defined as [2]:
<148 pmol/L (%) – deficient individuals
148-221 pmol/L- suboptimal individuals
>221 pmol/L – normal individuals

**Plan of action:**

| **Objective 1: To work out whether the SNPs are in Hardy Weinberg** | | |
|---|---|---|
| **Aims:** | **Statistical test used:** | **1) Reason for statistical test used**<br>**2) Outcome of statistical test used**<br>**3) covariates** (when appropriate) |
| 1a) Determine whether the observed genotype counts are in Hardy-Weinberg equilibrium (HWE) | Chi-Squared test | 1.Reason for test: To compare observed genotype counts with the values expected under Hardy-Weinberg<br>2. Outcome of statistical test: To test whether a population is in HWE at a locus [408, 553] |
| **Objective 2: To produce descriptive statistics for all the sample members who completed an assessment on demographics, fasting biochemical and anthropometric measurements.** | | |
| 2a) To determine the descriptive statistics of the sample members who completed an assessment on demographics, fasting biochemical and anthropometric measurements. | Descriptive statistics: Descriptives for continuous variables<br><br>Or<br><br>Descriptive statistics: Frequencies for categorical variables | 1. Reason for statistical test used: To determine baseline measures of the outcomes of interest (Will be discussed in detail in the point below) in all participants of the study<br>2. Outcome of statistical test used: To determine the mean and standard deviation of the following demographic, anthropometric and biochemical variables:<br>• Age (yrs)<br>• Height (cm)<br>• Weight (kg) |

| | | |
|---|---|---|
| | | • BMI (Kg/m$^2$)<br>• WC (cm)<br>• Hip (cm)<br>• WHR<br>• Fat (%)<br>• Fasting plasma glucose (mg/dl)<br>• Fasting serum insulin (μIU/ml)<br>• Glycated Haemoglobin (HbA1c)<br>• Glucose (mmol/l)<br>• Insulin (mg/dl)<br>• Vitamin B12 levels (pmol/L)<br>• Physical Activity Levels [Sedentary (%); Moderate (%); Vigorous (%)]<br>• Total energy (kcal/d)<br>• Protein (g)<br>• Fat (g)<br>• Carbohydrate (g)<br>• Dietary fibre (g)<br>• Polyunsaturated Fatty acid (PUFA) (g)<br>• |
| 2b) To stratify the descriptive statistics table into men and women. | Students t test | 1. Outcome of statistical test used: To identify if there are any statistically significant differences in the demographic, anthropometric and biochemical variables between the tertiles of vitamin B12 concentration between men and women in each tertile [408]. |
| **Objective 3: To test the association between the B12-GRS and metabolic-GRS on fasting biochemical/anthropometric measurements (Vitamin B12, glucose, insulin, HbA1c, BMI, WC, body fat percentage)** | | |
| 3a) Testing the association between the GRSs with fasting biochemical/anthropometric measurements, whilst adjusting for covariates. | Univariate linear regression | 1) Reason for test: exposure variable (*GRS*) is a categorical variable and the outcome variable (fasting biochemical trait/anthropometric trait) is a continuous variable.<br>2) Outcome of test: To identify the impact of the genetic variants on the levels of the fasting metabolic trait/ anthropometric trait. |

| | | 3) Covariates to be adjusted: Age, gender, BMI (BMI was not adjusted, when BMI was the continuous outcome) [341]. |
|---|---|---|
| **Objective 4: To test the interaction between the two GRSs (B12-GRS and metabolic-GRS) and dietary factors on fasting biochemical/anthropometric measurements (Vitamin B12, glucose, insulin, HbA1c, BMI, WC, body fat percentage)** | | |
| 4a) Testing the interaction between macronutrients and SNPs on fasting biochemical and anthropometric measurements. | Univariate linear regression | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and the outcome variable (fasting biochemical measurement/ anthropometric measurement) is a continuous variable.<br>2) Outcome of test: identifying the impact of the gene variants and the macronutrients: carbohydrate, protein, fat on fasting biochemical measurement/anthropometric measurement<br>3) Covariates to be adjusted: Age, gender, BMI [341] |
| 4b) Testing to find out if high, low or medium consumption of these macronutrients are causing the interaction | Univariate linear regression | 1) Reason for test: exposure variable (*SNP*) is a categorical variable and outcome variable (fasting biochemical measurement/anthropometric measurement) is a continuous variable.<br>2) Outcome of test: identifying the impact of gene variants and the consumption of different quantities of the macronutrient on fasting biochemical measurement/anthropometric measurement<br>3) Covariates to be adjusted: Age, gender, BMI [341].<br><br>(Data split based on: Tertiles of carbohydrate, protein and fat). |
| **SPECIAL NOTES:** | | |
| When looking at carbohydrates, Proteins and Fat in grams, you will need to adjust for Kcal. If you are using the percentage energy intake of the macronutrients, you do not | Compute variables | • For Carbohydrate interactions:<br>1g of Carbohydrates = 4kcal<br>• For Fat interactions:<br>1g=9 kcal<br>• For Protein interactions:<br>1g= 4kcal [526] |

| | | |
|---|---|---|
| need to adjust for Kcal, as it has already been adjusted for. | | |
| When significant interactions with macronutrients are detected, further investigation will take place to underline the specific type of macronutrient responsible for the interaction. | Univariate linear regression | if interactions with fat intake are significant, then<br>• Test for interaction between SNPs and saturated fatty acid intake, SNPs and monounsaturated fatty acid intake and SNPs and polyunsaturated fatty acid intake. Tertiles will be made for each of these dietary sub-groups. |
| After carrying out the interaction analysis using Univariate linear regression and obtaining P values, make sure adjustments are made to P values when several dependent or independent statistical tests are being performed simultaneously on a single data set. | Bonferroni Correction | 3. This method adjusts for multiple comparison and reduces experiment-wise error rate in genetic association studies [527] |

## 9.5 Analysis plan: A genetic approach to investigate the relationship between vitamin B12 status and cardiometabolic traits in response to changes in dietary fat composition in adults with moderate cardiovascular disease risk

**Hypothesis:**

The modification of dietary fat intake has been shown to affect vitamin B12 status in rats [472], this post-hoc analysis will present additional outcome measures from the DIVAS study exploring the effect of dietary fat composition on vitamin B12 status in individuals with moderate CVD risk. The main of this study is to investigate whether genetic polymorphisms contribute to any observable changes in vitamin B12 status and cardiometabolic disease risk markers, a retrospective post hoc analysis of the DIVAS study will be conducted. Thus, in the current study I will aim to investigate whether nine SNPs (6 metabolic SNPs and 3 vitamin B12 SNPs) modified the response of vitamin B12 concentrations and cardiometabolic traits, after substitution of SFA with MUFA or polyunsaturated fatty acids (n-6 PUFA) in 119 participants at moderate CVD risk.

**Objectives:**

To prove this hypothesis, we will

- Examine association between (6 metabolic SNPs and 3 vitamin B12 SNPs) and changes 13 phenotypic outcomes (vitamin B12, 24 h ambulatory systolic blood pressure, 24 h ambulatory diastolic blood pressure, total cholesterol, HDL cholesterol, LDL cholesterol, TAG, glucose, insulin, HOMA-IR, BMI, WC and WHR) after a 16-weeks dietary fat intervention.
- Investigate interaction between 6 metabolic SNPs and 3 vitamin B12 SNPs and the three intervention diets on changes in 13 phenotypic outcomes after 16-weeks intervention.

**Plan of action:**

| Objectives | Statistical test | Fixed factor | Dependent factor | Covariates |
|---|---|---|---|---|
| **Association** | General Linear regression | The metabolic-GRS was generated from the SNPs in the genes *FTO* (rs8050136 and rs9939609), *TCF7L2* (rs7903146 and rs12255372) and *MC4R* (rs17782313 and rs2229616).<br><br>The B12-GRS was generated from the SNPs in the gene *FUT2* (rs602662, rs492602 and rs16982241). | Changes in vitamin B12 concentrations and cardiometabolic traits (24 h ambulatory systolic blood pressure, 24 h ambulatory diastolic blood pressure, total cholesterol, HDL cholesterol, LDL cholesterol, TAG, glucose, insulin, HOMA-IR, BMI, WC and WHR) at baseline (visit 1) and after 16 weeks (visit 2) in | Age, gender, BMI, and ethnicity [13] |
| **Interaction** | General Linear regression | The metabolic-GRS was generated from the SNPs in the genes *FTO* (rs8050136 and rs9939609), *TCF7L2* (rs7903146 and rs12255372) and *MC4R* (rs17782313 and rs2229616).<br><br>The B12-GRS was generated from the SNPs in the gene *FUT2* (rs602662, rs492602 and rs16982241)<br><br>The three-intervention diet (group 1 high SFA diet, group 2 high MUFA diet, and group 3 High PUFA diet) | Changes in vitamin B12 concentrations and cardiometabolic traits at baseline (visit 1) and after 16 weeks (visit 2). | Age, gender, BMI, and ethnicity [13] |