Vitamin B12 and Folate Status during Pregnancy among Saudi Population

By

Nahla Mohammed Bawazeer

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Clinical Sciences Research Lab
Division of Metabolic & Vascular Health
Warwick Medical School
University of Warwick
England, UK

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List of Abbreviations and Acronyms

ADA	American Diabetes Association
BMI	Body Mass Index
BMR	Basal Metabolic Rate
BW	Birth Weight
Cbl	Cobalamin
Cm	Centimetre
CPT-1	Carnitine Palmitoyltranferase
CI	Confidence Interval
CV	Coefficient Variation
CVD	Cardiovascular Disease
DHF	Dihyrofolate
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
dTMP	Deoxythymidine Monophosphate
dUMP	Deoxyuridine Monophosphate
EI	Energy Intake
EI/BMR	Energy Intake/Basal Metabolic Rate Ratio
EIA	Enzyme Immunoassay
Fat %	Total Body Fat Content
FFQ	Food Frequency Questionnaire
g	Gram
GA	Gestational Age
GDM	Gestational Diabetes Mellitus
holoTC	Holotranscobalamin
HOMA	Homeostatic Model Assessment
Hcy	Homocysteine
IDF	International Diabetes Federation
IF	Intrinsic Factor
IGT	Impaired Glucose Tolerance
IUGR	Intrauterine Growth Retardation
Kcal	Kilo-calorie

Kg	Kilogram
Kg/m^2	Kilogram Per Meter Square
KSA	Kingdom of Saudi Arabia
LOA	Limit of Agreement
MBA	Microbiological Assay
MCM	Methyl Malonyl-Coa Mutase
Mg	Milligram
Mid-arm	Mid Arm Circumferences
MM-CoA	Methylmalonic-Coa
MS	Methionine Synthase
MTHF	Methyl Tetrahydrofolate
MTHFR	Methylene Tertrahydrofolate Reductase
N/A	Not Available
N/R	Not Relevant
N/R	Not Reported
NHANES	National Health and Nutrition Examination Survey
nmol/l	Nanomoles Per Litre
NTDs	Neural Tube Defects
PAL	Physical Activity level
PMNS	Pune Maternal Nutrition Study
pmol/l	Picomoles Per Liter
PP	Primipara
R	Methyl Acceptor
r	Correlation Coefficient
RA	Radioassay
R-CH3	Methylate Compound
RDA	Recommended Dietary Allowance
RDI	Recommended Dietary Intake
RIA	Radioimmunoassay
SAH	S-Adenosylhomocysteine
SALSA	Sacramento Area Latino Study on Aging
SAMe	S-Adenosylmethionine
SD	Standard Deviation

SFFQ	Semi-Food Frequency Questionnaire
SS/TR	Subscapular-Triceps Skinfold Thickness Ratio
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TCI	Transcobalamin I
TCII	Transcobalamin II
TCIII	Transcobalamin III
THF	Tetrahydrofolate
TS	Thymidylate Synthase
UK	United Kingdom
US	United State
UDC	University Diabetes Center
USA	Unites State of America
VC	Validity coefficient
WHR	Waist-Hip Ratio
wks.	Weeks
µg	Microgram
µM	Micromole
µmol/l	Micromoles Per Litre
1-C	One Carbon Unit
24 h FR	24 Hours Food Recall
95% CI	95% Confident Interval

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Declaration

I declare that this thesis is presented in accordance with the regulations for the degree of Doctor of Philosophy by the High Degree Committee at the University of Warwick. The thesis has been composed and written by myself based on my own work. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given. This thesis has not been submitted in any previous application for a higher degree.

Nahla Mohammed Bawazeer

Clinical Sciences Research Lab
Division of Metabolic & Vascular Health
Warwick Medical School
University of Warwick
England, UK

Summary

T2DM is a growing health problem worldwide. It is now increasingly being diagnosed earlier in life. The factors involved in such an epidemic are complex. The intrauterine environment has long been known as an important contributor to many diseases including metabolic disorders such as T2DM. Recently, there is emerging evidence for maternal micronutrients affecting vital developmental processes *in utero* which can adversely "programme" the offspring to develop metabolic disorders in later life. Thus, "gene-diet" interaction during foetal development is likely to be a significant contributor to the epidemic of T2DM. In particular, the intrauterine imbalance between the two related vitamins, vitamin B12 and folate, affect DNA methylation and in turn programme the foetus for the whole life. Evidence from mandatory folic acid fortification studies suggests that in the presence of adequate folate, neural tube defects due to vitamin B12 insufficiency have tripled. In India, children born to mothers with "high folate and low vitamin B12" had higher adiposity and insulin resistance. Therefore, micronutrient status during pregnancy is likely to have a significant impact on the metabolic risk of the offspring. This thesis examines whether vitamin B12 insufficiency is prevalent in pregnancy, especially in a non-vegetarian population across the world as well as the Saudi pregnant population. As estimated intake is an accepted measure for micronutrient levels, we also examined the relationship between estimated vitamin B12 and folate intake with actual levels in the blood. We have found that vitamin B12 insufficiency was not uncommon during pregnancy across the world even in the non-vegetarian population and is also common in the Saudi population. Surprisingly, vitamin B12 insufficiency was observed in 50% of the tested population even in the presence of adequate vitamin B12 intake. In addition, we have also shown for the first time in the Saudi population that maternal BMI is inversely related to

vitamin B12 levels, particularly in pregnancy. Even though we have shown a similar (or worse) picture in mothers with gestational diabetes, this study needs to be replicated, as our numbers are too small. Prospective studies linking the role of vitamin B12 insufficiency especially in the presence of high folate on birth outcomes in the Saudi population as well as intervention studies investigating the role of vitamin B12 supplementation in women of childbearing age and in pregnancy are urgently needed.

Chapter 1

Introduction

1.1 Diabetes Mellitus (DM): General background

DM is considered to be a genetically and clinically heterogeneous group of metabolic disorders that share glucose intolerance in common (Patel 2003). It is characterised by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both (ADA 2010; WHO Consultation 2011). Because of its chronic nature, DM is becoming one of the main threats to human health now-a-days. It increases the risk of coronary heart disease and stroke, and it is a leading cause of blindness, renal failure and limb amputations (Patel 2003; IDF 2011).

DM occurs in different types; the two major classes are type 1 diabetes (T1DM) and type 2 diabetes (T2DM). T1DM, a most common chronic disease of children, is an autoimmune disease caused by the destruction of pancreatic β-cell islets, leading to absolute insulin deficiency. T2DM may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance (ADA 2011). People with T1DM are treated with insulin for survival to prevent the development of ketoacidosis, while people with T2DM may require insulin only if the blood glucose level is not achieved through diet alone or with oral hypoglycaemic agents (Zimmet et al. 2001). T2DM is more prevalent and accounts for approximately 90 – 95% of all cases of DM (ADA 2011). T2DM is a condition which has a linear progression and may pre-exist for many years at least 4 - 7 years before clinical diagnosis (Harris et al. 1992). Gestational diabetes mellitus (GDM) is defined "as carbohydrate intolerance of varying severity with onset or first recognition during pregnancy" (Coustan 2000). It is the most frequent metabolic disorder occurring during pregnancy (Jimenez-Moleon et al. 2000) and is a risk factor for affected mothers to develop T2DM in the future (Dabelea et al. 2005). Indeed, a mother with a history of

GDM has at least a seven-fold increased risk of developing T2DM later in her life (Bellamy et al. 2009). Moreover, the risk of developing GDM is about 2, 4 and 8 times higher among overweight, obese and severely obese women, respectively, compared with normal-weight pregnant women (Chu et al. 2007). GDM has many deleterious effects in terms of maternal and foetal outcomes (Xiong et al. 2001). It has been found that exposure to maternal hyperglycaemia during pregnancy is associated with birth defects (Kjos 1999), effects on childhood growth and glucose regulation (Dabelea 2007). Among the Pima Indians, most of the increased prevalence of childhood T2DM over the past 30 years was attributable to increased exposure to maternal diabetes during pregnancy (Dabelea et al. 1998).

1.1.1 Epidemiology of DM

The prevalence of DM is increasing dramatically worldwide. It is expected to rise from 6.4%, affecting 285 million adults, in 2010, to 7.7%, and 439 million adults by 2030. Between 2010 and 2030, there will be a 69% increase in the numbers of adults with DM in developing countries and a 20% increase in developed countries (Shaw et al. 2010). According to the IDF, "The overall global prevalence of DM is about 4.6%." (Gadsby 2002). The ADA reported that DM is affecting 15.7 million people in the US, comprising almost 6% of the population (Nedra et al. 2000). While in the UK, it is suggested that the prevalence is increased from 3.5 to 4.7% (Gadsby 2002) and this figure could increase up to 10% over the age of 75 (Mackinnon 2002). The DM epidemic relates particularly to T2DM, and is taking place both in developed and developing nations (Zimmet 1999). It is a growing problem in the Middle East, more specifically in Saudi Arabia. Epidemiological studies in Saudi Arabia have shown that in 1982, the prevalence was 2.5%, and it increased dramatically to 13% in 1996. The

latest epidemiological survey conducted in Saudi Arabia showed that the prevalence has increased to 23.7% (Al-Nozha et al. 2004; Elhadd et al. 2007). Also, there is a parallel increase in GDM along with T2DM and its prevalence may range from 1 to 16% of all pregnancies, depending on the type of population and the diagnostic criteria used (Jimenez-Moleon et al. 2000). In Saudi Arabia, the overall prevalence is about 12% with a significant morbidity and mortality (Ardawi et al. 2000; Elhadd et al. 2007). This is significantly higher compared to US where about 135,000 cases of GDM (3 - 8 % of all pregnancies) are diagnosed annually (Dabelea et al. 2005).

1.1.2 T2DM in children

T2DM was considered to be a disease for adults. However, it is now being diagnosed earlier in life and the incidence of T2DM in children is also increasing at an alarming rate (Rocchini 2002). This may partly be due to a higher incidence of childhood obesity owing to changes in lifestyle, diet, and reduction in physical activity (Miller et al. 2004). It is becoming the major form of DM in young people in several non-white populations, such as Native Americans, Asian and Pacific Islanders, and African-Americans, with the highest rates observed among the adolescent (The Writing Group for the Search for Diabetes in Youth Study Group 2007). An approximate fourfold rise in the incidence of T2DM in 6 to 15 year olds has been shown in Japan, and between 8 and 45% of newly presenting children and adolescents in the US have T2DM (Alberti et al. 2004). This is not only a serious public health problem for health care professionals, it may burden the future generation with significant morbidity and mortality at the peak of their working lives and productivity. This is likely to affect the workforce of countries across the world. This in turn will add to the economic burden

for both developed and low and middle income countries across the globe. (Alberti et al. 2004).

1.1.3 Factors involved in T2DM epidemics

The factors involved in the T2DM epidemic are complex. Genetic susceptibility of certain ethnic groups, environmental and behavioural factors such as a sedentary lifestyle, nutrition and obesity are clearly important risk factors of the prevalence of DM (Patel 2003). Furthermore, population growth, ageing, urbanization and other factors also contribute to the DM epidemic (Wild et al. 2004). However, the intrauterine environment, due to maternal malnutrition (thrifty phenotype hypothesis) (Hales and Barker 1992), and genetic controls in the foetus of glucose sensing, insulin secretion and insulin resistance (foetal insulin hypothesis) (Hattersley and Tooke 1999) must also contribute to the increasing prevalence of childhood obesity as well as T2DM and cardiovascular disease (CVD) in adult life (Saravanan and Yajnik 2010). Indeed, there is emerging evidence that a cluster of metabolic risk factors are present at birth in the offspring born to mothers with a higher metabolic risk (Catalano et al. 2009; Saravanan and Yajnik 2010).

1.1.3.1 Thrifty phenotype hypothesis

The concept of this hypothesis was put forward about 20 years ago after reviewing the aetiology of T2DM (Hales and Barker 1992). The thrifty phenotype hypothesis proposes that poor foetal nutrition *in utero* changes glucose-insulin metabolism leading to the development of T2DM in adult life. These changes include a reduced capacity for insulin secretion and insulin resistance, due to the poor development of pancreatic β-cell mass and function, which combined with the effects of

obesity, ageing and physical inactivity, are the most important factors in determining T2DM (**Figure 1.1**) (Hales and Barker 2001).

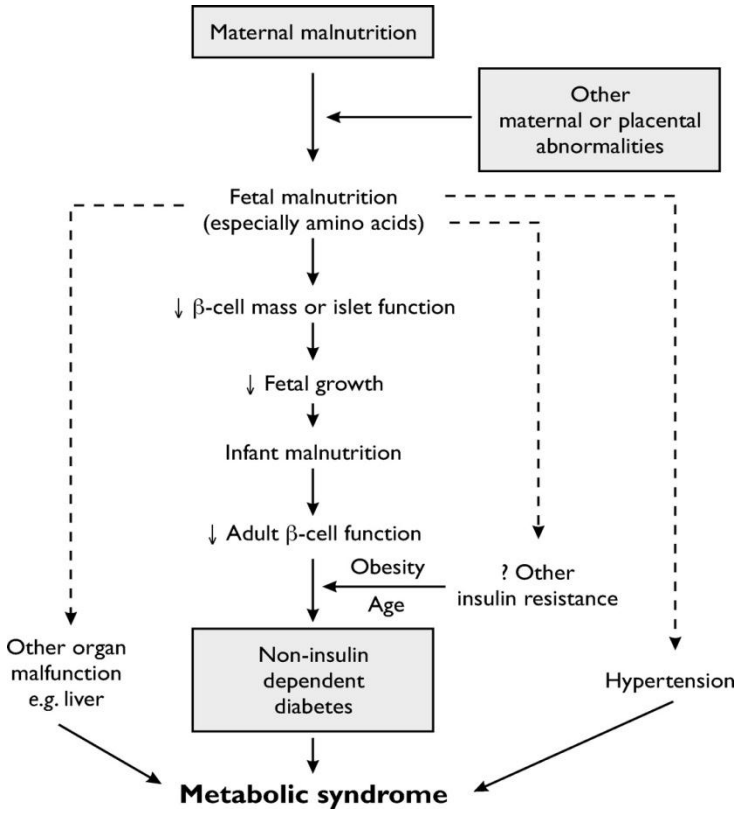
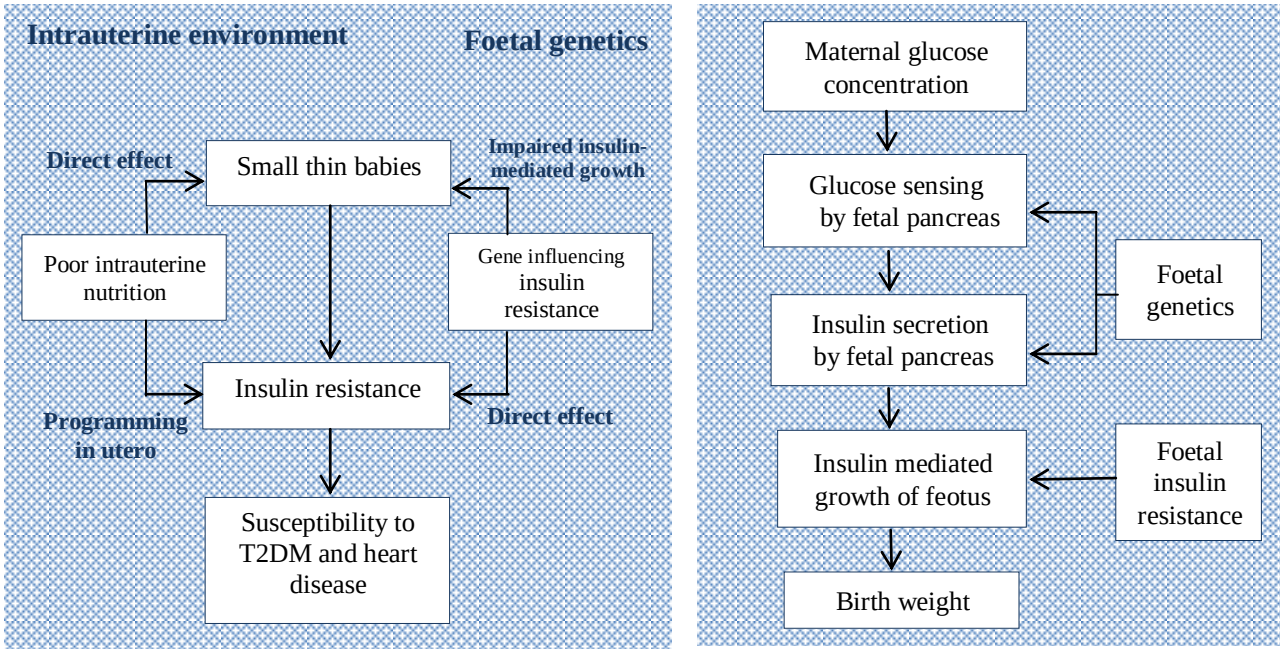


Figure 1.1: Diagram represent the key features of the "thrifty phenotype" hypothesis of the aetiology of T2DM (non-insulin-dependent diabetes). It proposes that poor foetal nutrition affects the growth of different organs and thus produces permanent changes in the structure and function of the body. One of the major long-term consequences of inadequate early nutrition is impaired development of the endocrine pancreas. Also outlined is the suggestion that the features of Metabolic syndrome may have closely related origins in failures of early growth and development. Not shown for the sake of simplicity and clarity are the additional possibilities that (i) an early reduction of insulin production could have secondary consequences for the growth and development of other organs involved in Metabolic syndrome; (ii) infant malnutrition may be involved in processes contributing to components of Metabolic syndrome (Hales and Barker 1992).

1.1.3.2 Foetal insulin hypothesis

The foetal insulin hypothesis offers an alternative explanation for the consistent association between impaired foetal growth and insulin resistance during life and the link with hypertension and vascular disease (Hattersley and Tooke 1999). It proposes that the association between low birth weight and adult insulin resistance is principally genetically mediated (**Figure 1.2A**). The concept is that insulin-mediated foetal growth will be affected by foetal genetic factors that regulate either foetal insulin secretion by the foetal pancreas or the sensitivity of foetal tissues to the effects of insulin (Hattersley and Tooke 1999) (**Figure 1.2B**).



(A) **(B)**

Figure 1.2: Foetal insulin hypothesis. **(A)** shows two alternative explanations for the association of small, thin babies with insulin resistance, T2DM, and ischaemic heart disease - intrauterine environment and foetal genetics. **(B)** shows a simplified representation of the hypothesis: foetal genetics could lead to defects in glucose sensing, insulin secretion, or the response to insulin of insulin-sensitive tissues (insulin resistance) thus impaired foetal growth (Hattersley and Tooke 1999).

Though, evidence from monogenic disorders supports the foetal insulin hypothesis, this affects a relatively small number of people and is therefore an unlikely contributor to the epidemic of T2DM and CVD (Saravanan and Yajnik 2010). On the contrary, several studies across different populations confirmed that the future metabolic risk (T2DM and CVD) is high in people born with low birth weight, especially if they become overweight adults (Barker 2004), suggesting this association is much more common and therefore unlikely to be due to genetic factors alone (Saravanan and Yajnik 2010). 'Gene-diet' interaction during foetal development is more likely to contribute to the epidemic of T2DM. Several micronutrients and vitamin are involved in the metabolic pathways that regulate DNA synthesis and/or repair and the expression of genes (Fenech and Ferguson 2001). The deficiency of such nutrients may result in impaired enzyme activity, disruption of genomic integrity and alteration of DNA methylation. Therefore, gene-diet interactions seem to explain the different response to environmental/diet exposure at the molecular level (Friso and Choi 2002). Such interaction is likely to 'programme the foetus' for the rest of its life. An example on such interaction on foetal programming is given in (**Figure 1.3**). However, the factors and the mechanism by which such programming results in low birth weight is not clear.

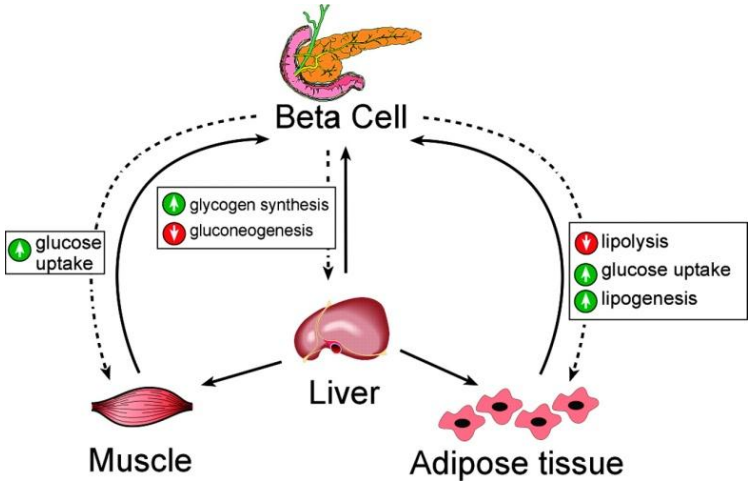
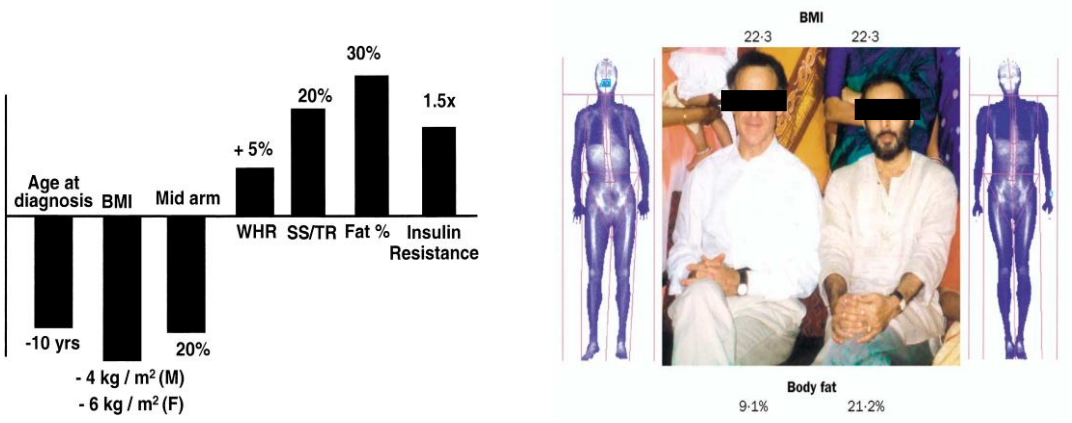


Figure 1.3: Demonstrates the feedback loop of the insulin involvement in a number of different tissues. During foetal development, these tissues learn to communicate with each other. The signalling from the beta cells of the pancreas is via insulin (dotted lines). This acts on the liver, muscle and adipose tissue to change levels of glucose in the circulation (solid lines). The development of liver, muscle and adipose tissue is regulated by gene-nutrient interactions. If the maternal diet is nutritionally imbalanced, the development of these tissues will change, thus altering the characteristics of this feedback loop. The communication between these organs is complex where any changes in just one component can influence the behaviour of the whole system. For example, maternal malnutrition may suppress beta cell development resulting in fewer foetal beta cells, resulting in reduced insulin release, requiring a corresponding increase in insulin sensitivity of the foetal liver, muscle or adipose tissue to maintain glucose homeostasis (Maloney and Rees 2005).

Understanding ethnic variations could provide us with some explanation to the susceptibility to T2DM and CVD. The South Asian population (Indian) is a good example for this, where they are known to be at higher risk of these conditions. They have a distinctive adverse anthropometric (**Figure 1.4**) and biochemical profile (Yajnik 2001; Yajnik and Yudkin 2004) compared to the UK counterparts, which are reflected in different patterns of DM. Indian diabetes patients are diagnosed a decade earlier and are considerably thinner than their UK counterparts (BMI 23.9 and 28.5 kg/m2, respectively). Despite their thinness, they are centrally obese, with a higher waist-hip ratio and a higher subscapular-triceps skinfold ratio than their UK counterparts (Yajnik

2001). They also develop T2DM and CVD at an earlier age (Yajnik 2001) and have worse adverse outcomes (Forouhi et al. 2006; Gunarathne et al. 2008).



(A) **(B)**

Figure 1.4: (A) The characteristics of adverse anthropometry of South Asians with newly diagnosed T2DM. Bars above the line indicate higher levels. Insulin resistance was calculated from the homeostatic model assessment (HOMA) model. BMI = body mass index, Fat % = total body fat content, mid-arm = mid arm circumference, SS/TR = subscapular-triceps skinfold thickness ratio, WHR = waist-hip ratio. Retreived from (Yajnik 2001) **(B)** Y-Y paradox: demonstrates the central adiposity and higher body fat content of a South Asian compared with a European with the same BMI. (Yajnik and Yudkin 2004; Saravanan and Yajnik 2010).

Similar adverse profiles seem to be present at birth (**Figure 1.5**). Indian babies were small, had small abdominal viscera and low muscle mass, but preserved body fat (Yajnik et al. 2002; Yajnik et al. 2003). This body composition may persist postnatally and predispose the person to an insulin-resistant state suggesting that genetic factors that contribute to the higher metabolic risk of South Asians undoubtedly influence body size and composition of a developing foetus and may well be due to adverse prenatal or intrauterine environment.

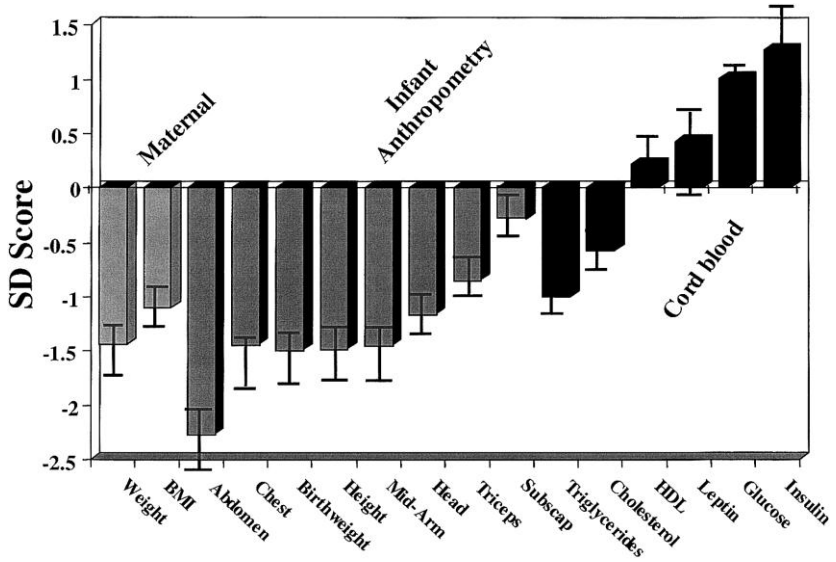


Figure 1.5: Demonstrates the adverse anthropometry and cardiovascular risk factors of South Asian children compared with white Caucasian children. Mean and 95% CI for maternal pre-pregnancy weight and height and for birth measurements and cord blood concentrations are shown. The white Caucasian group mean is represented by 0 and the mean difference for each measurement is shown. All SD scores are negative, suggesting that the Indian babies are smaller than the white Caucasian babies (Yajnik et al. 2002).

Recent evidence from a well-designed longitudinal observational study suggests that micronutrients, especially folic acid and vitamin B12 may play a crucial role and could contribute to the epidemic of adiposity and T2DM in India (Yajnik et al. 2008). The possible mechanism of this contribution will be discussed later in the chapter.

1.2 Folate

Folate is used as the generic descriptor for all derivatives of pteroic acid that demonstrate vitamin activity in humans. It refers to both the endogenous form of the vitamin which can occur naturally in food and the synthetic form - folic acid - found in supplements and fortified food. Folic acid differs chemically from folate in that there is only one glutamic acid (monoglutamate) attached to the pteridine ring unit and it is the

most oxidized and stable form of the vitamin (**Figure 1.6**). While naturally accruing folate contains one to six glutamate molecules (polyglutamates) joined in peptide linkage (Bender 2003; Bailey 2004), in most natural foods, the pteridine ring is reduced to give either the 7,8-dihydrofolate (DHF) or 5,6,7,8-tetrahydrofolate (THF). In the cells, the reduced form of folate conjugates to the polyglutamate chain. Polyglutamates have a single type of action in that they can accept so called one-carbon (1-C) units from various donors and pass them in various biosynthetic reactions. Thus, in the cells folate will be a mixture of tetrahydrofolate (e.g. 10-formyl-, 5,10-methylene- and 5-methyltetrahydrofolate) depending on which C group attached to them (Scott 1999).

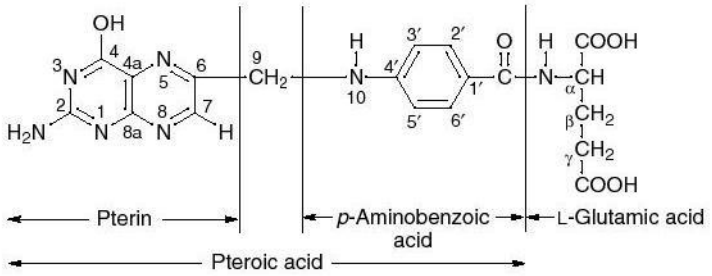


Figure 1.6: Structure of a folic acid compound**.** It comprises a bicyclic Pterin ring joined by a methylene bridge to *p*-Aminobenzoic acid, which in turn attached via α-peptide to a single molecule of L-Glutamic acid. Figure obtained from (Ball 2004a).

1.2.1 Metabolism of folate

1.2.1.1 Digestion, absorption and transport of folate

Folate cannot be synthesized in the body, thus it must be obtained from exogenous sources. There are two sources of folate in the intestine: dietary folate and folate synthesized by bacteria in the large intestine. The latter source is directly absorbed in the colon (Ball 2004a). Before the absorption of dietary folate, the polyglutamate chain must be broken down in the brush border of the mucosal cells by

the enzyme conjugase (pteroylpolyglutamate hydrolase) and form the monoglutamate, which is taken up by a specific carrier in the cell membrane of the small intestine. Most of the dietary folate undergoes reduction and methylation within the intestinal mucosa, thus the only form entering the human circulation from the intestinal cells is 5-methyl-tetrahydrofolate monoglutamate (Scott 1999). Approximately two-third of folate in the plasma are protein bound. About 10 - 20% of methyl monoglutamate is taken up by the liver during the first pass, and the remainder is rapidly cleared by peripheral tissues. The majority of 5-methyltetrahydrofolate arriving at the liver from the intestine and taken up is unchanged (not demethylated). It enters the enterohepatic cycle where it metabolized to polyglutamate derivatives and retained or secreted in the bile to be reabsorbed in the small intestine together with food folate before re-entering the circulation. The kidneys play a role in conserving body folate by actively reabsorbing folate from the glomerular filtrate (Steinberg 1984).

1.2.1.2 Metabolism and excretion

Before being stored in tissue or used as a coenzyme, folate monoglutamate is converted to the polyglutamate form by the enzyme polyglutamate synthetase. When released back to the circulation, reconversion to the monoglutamate form is required. Folate must be reduced enzymatically and resynthesized to the polyglutamate form to function in a single-carbon atom (1-C) transfer reaction (Institute of Medicine 1998a). Both folate and vitamin B12 are metabolically interrelated, which may explain why a single deficiency of either vitamin leads to the same haematological changes (Institute of Medicine 1998a). The mechanism of action will be discussed later in the chapter.

Except in cases of malabsorption, the body conserves dietary folate efficiently. Folate secreted into the bile is reabsorbed in the small intestine; much of the circulating

folate binds to plasma proteins and so cannot be filtered in the kidneys; and the free circulating folate that is filtered is captured by the folate receptors in the renal tubules and reabsorbed into the bloodstream. The result is very little folate loss by faecal or urinary excretion (Bender 2003).

1.2.2 Dietary sources and bioavailability

Dietary folate is mainly found in dark green leafy vegetables and fresh fruits. It is also present in liver products. Folic acid is fortified in wheat flour in many countries (Center for Disease Control and Prevention 2008) mandatorily, while in many other countries voluntary fortification is in practice, in a variety of foods products such as breakfast cereals and bread.

The bioavailability of folate depends on whether the folate is in the polyglutamate form (food folate) or in the monoglutamate form (synthetic folic acid). All naturally-occurring folates are chemically unstable, which leads to significant loss of activities during harvesting, storage, processing, and preparation. About half or perhaps three-quarters of initial folate activity may be lost during these processes. In contrast, folic acid contains pteridine in unreduced form, so they are very resistant to chemical oxidation (Scott 1999). Food folates, unlike folic acid, must undergo enzymatic deconjugation in the small intestine before they can be absorbed. Thus, folic acid is significantly more bioavailable than food folate (Bailey 2004). Various factors may affect the folate bioavailability. For example, organic acids present in many foods, such as orange juice and tomato, can inhibit conjugase activity (Wei and Gregory 1998). This may partly explain why the polyglutamyl folate present in natural food sources is less bioavailable than the folic acid present in fortified foods and supplements (Cuskelly et al. 1996). When folic acid is consumed as a supplement without food, it is

100% bioavailable compared to 85% when it is consumed in food as in fortified products (Pfeiffer et al. 1997). Food folate is not more than 50% bioavailable (Bailey 2004).

1.2.3 Dietary intake of folate

1.2.3.1 Dietary requirement

Based on the controlled metabolic study data, the recommended dietary allowance (RDA) of folate for an adult aged 19 years or more is 400 μg per day (Institute of Medicine 1998a). For pregnant women, approximately 600 μg per day folate is adequate to maintain normal folate status, based on erythrocyte (RBC) and serum folate concentrations during pregnancy (Bailey 2000). For lactating women, the RDA is 500 μg per day based on estimates of the folate intake needed to replace the folate secreted in human milk (Bailey 2000).

1.2.3.2 Effects of high intake

It was believed that there were no adverse effects associated with the consumption of excess folate from food (Butterworth and Tamura 1989). However, there are several reports suggesting excess folate may cause harm especially certain types of cancer (Ulrich 2008). In addition data from the USA, a decade after the mandatory folic acid fortification was introduced in 1997, shows that high folate levels particularly in the presence of vitamin B12 insufficiency is associated with higher homocysteine and methylmalonic acid (MMA) levels (Selhub et al. 2007) and a higher incidence of anaemia and cognitive dysfunction in the elderly (Morris et al. 2007).

1.3 Vitamin B12

Vitamin B12 is the generic descriptor for all cobalamin. It is involved in the cell metabolisms particularly in the DNA synthesis and regulation and fatty acid synthesis and energy production. Vitamin B12 can be converted to either two cobalamin coenzymes that are active in human metabolism, namely methylcobalamin and 5-deoxyadenosylcobalamin.

Vitamin B12 molecules refer to a group of cobalt-containing compounds (corrinoids), that have a lower axial ligand that contains the cobalt-coordinated nucleotide (5, 6-dimethylbenzimidazole as a base; **Figure 1.7**) (Bender 2003). The common synthetic form of vitamin B12 is cyanocobalamin. It is the most stable form of the vitamin, and is commonly used in pharmaceutical preparation and food supplementation (Ball 1998; Scott 1999).

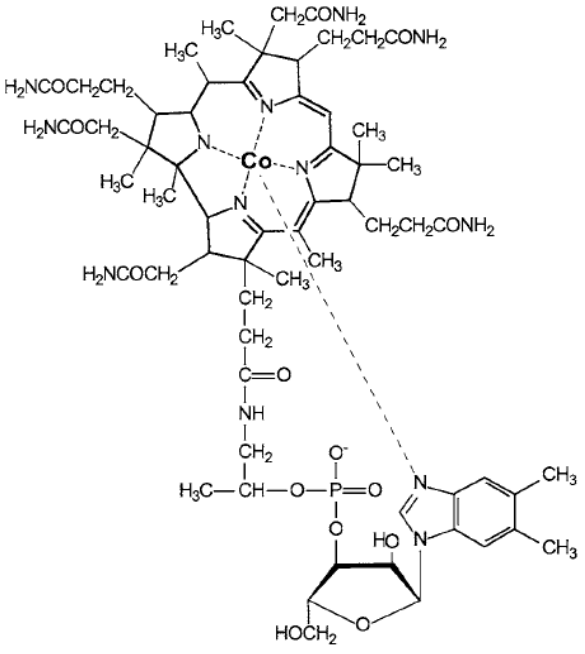


Figure 1.7: Structure of vitamin B12 compounds. Four coordination sites on the central cobalt atom are occupied by the nitrogen atoms of the corrin ring, and one by the nitrogen of the dimethylbenzimidazole nucleotide. The six coordination sites may be occupied by CN- cyanocobalamin; OH- hydroxocobalamin; H2O- aquocobalamin; CH3- methylcobalamin; and 5′deoxyadenosylcobalamin. Figure obtained from (Bender 2003).

In plasma, methylcobalamin accounts approximately 60 – 80% of plasma vitamin B12, adenosylcobalamin accounts up to 20% and the reminder mainly hydroxycobalamin. The major vitamin found in tissue is adenosylcobalamin, which accounts about 70% in the liver, then hydroxycobalamin accounts about 25% and less than 5% as methylcobalamin (Bender 2003).

1.3.1 Metabolism of vitamin B12

1.3.1.1 Digestion and absorption of vitamin B12

Vitamin B12 digestion and absorption from food in human subjects are complex as shown in (**Figure 1.8**). It requires many processes, and any interruption of one or any combinations of these processes could place individuals at risk of developing deficiency (Oh and Brown 2003). Very small amounts of free vitamin B12 (between 1 – 5%) can be absorbed by passive diffusion across the intestinal mucosa (Andres et al. 2004). This explains the mechanism of treating deficiencies associated with pernicious anaemia and food-cobalamin malabsorption (Kuzminski et al. 1998; Carmel 2000).

Once vitamin B12 is absorbed, it binds to transport proteins known as transcobalamin I, II, or III (TCI, TCII, or TCIII) and then transported throughout the body (Andres et al. 2004). TCI circulates an estimated 80% of vitamin B12. Apparently, it does not seem to be involved in cellular uptake of the vitamin. However, it prevents circulating vitamin B12 from being filtered by the kidneys (Ball 2004b). TCII is the form that delivers vitamin B12 to the tissues through specific receptors. The liver takes up to approximately 50% of vitamin B12 and the remaining amount is transported to other tissues (Institute of Medicine 1998b).

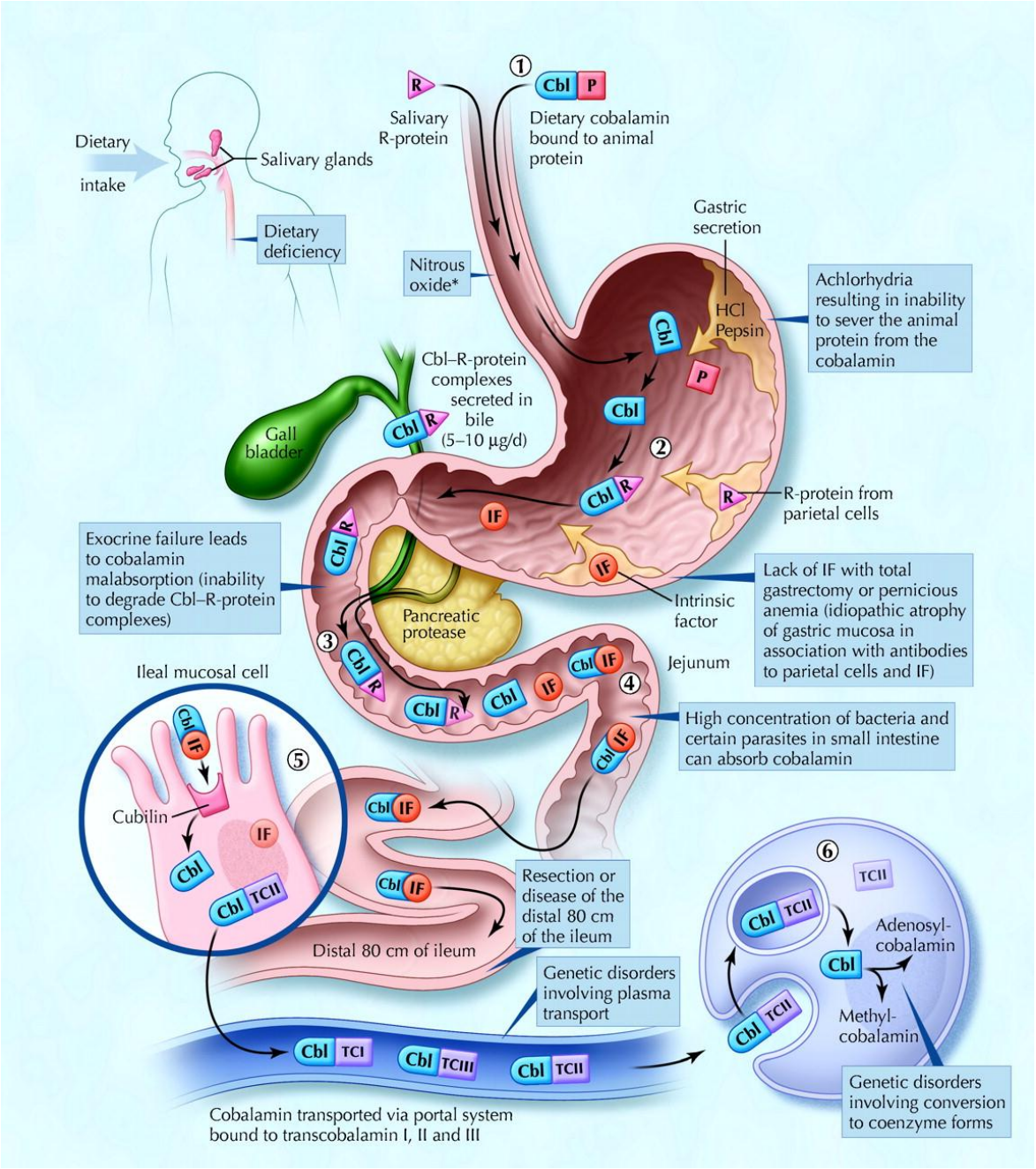


Figure 1.8: The metabolic pathway of vitamin B12 (cobalamin or Cbl) and corresponding causes of deficiency. (1) Dietary intake of vitamin B12 enters the stomach bound to food protein (P). (2) In the stomach, the acidic environment (pepsin and hydrochloric acid (HCL)) facilitates the cleavage of vitamin B12 that is bound to P, giving the free form of the vitamin. The released form of vitamin B12 is immediately passed to a mixture of glycoprotein such as R-protein (R), which is primarily produced by the parietal and salivary cells, to protect vitamin B12 from chemical denaturation in the stomach. Intrinsic factor (IF) is also secreted by parietal cells in the stomach, but its binding to vitamin B12 is weak in the presence of gastric and salivary R-protein. (3) Then, vitamin B12 leaves the stomach and enters the duodenum bound to R-protein and accompanied by IF and by vitamin B12-R-protein complex that have been secreted from the bile. (4) In the mildly alkaline environment of the jejunum, pancreatic enzymes degrade both biliary and dietary vitamin B12-R-protien complexes, releasing free vitamin B12. IF is resistant to proteolysis and binds to the released vitamin B12 to facilitate its active absorption in the ileum. (5) The IF-B12 complex is carried down

until the distal 80 cm of the ileum where it binds to mucosal cell receptors (cubilin), then vitamin B12 is bound to transport proteins known as transcobalamin I, II and III (TCI, TCII and TCIII). Vitamin B12 is subsequently transported systemically via the portal system. (6) Within each cell, the TCII–vitamin B12 complex is taken up by means of endocytosis and vitamin B12 is liberated and then converted enzymatically into its two coenzyme forms, methylcobalamin and adenosylcobalamin.*Nitrous oxide, a general anaesthetic, causes multiple defects in vitamin B12 use, most of which are intracellular and clinically relevant only in people who have low or borderline-low serum B12 level. Figure obtained from (Andres et al. 2004).

At the cellular level, lysosomal degradation of TCII occurs, resulting in released vitamin B12 into cytoplasm in the form of hydroxocobalamin. Hydroxocobalamin is either converted to methylcobalamin in the cytoplasm or to adenosylcobalamin in the mitochondria (Andres et al. 2004). TCIII is rapidly cleared by the liver. It seems to provide a mechanism for returning vitamin B12 from peripheral tissues to the liver, as well as for clearance of other corrinoids. These corrinoids are then secreted into the bile, in the form of Cbl-R protein complexes (**Figure 1.9**) (Bender 2003).

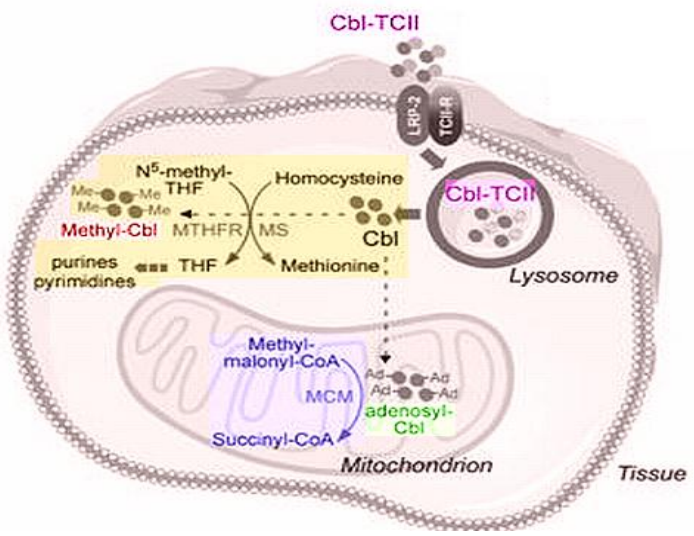


Figure 1.9: Diagram highlighting the cellular uptake and processing of vitamin B12 (Cbl). Vitamin B12 (Cbl) is bound to transcobalmin II (TCII). Cbl-TCII enters cells by means of TCII receptor-mediated endocytosis. Lysosomal enzymes degrade the TCII, resulting in free Cbl. In the mitochondria, Cbl is converted to adenosylcobalamin, a coenzyme involved in the conversion of methylmalonyl-CoA to succinyl-CoA. In the cytoplasm, Cbl is converted to methylcobalamin, a coenzyme involved in the conversion of homocysteine to methionine. MS: methionine synthase, THF: tetrahydrofolate, MTHFR: methyltetrahydrofolate, MCM: methylmalonyl CoA mutase. Figure obtained from (Dali-Youcef and Andrès 2009).

1.3.1.2 Post-absorptive metabolism, storage and excretion

Unlike the other water-soluble vitamins, the body can efficiently conserve vitamin B12. In adults, it stores approximately 2000 - 5000 µg, of which about 80% of vitamin B12 is stored in the liver in the form of adenosylcobalamin. The amount stored in the liver increases with age. The remainder is located in muscle, skin, and blood plasma. About 2 - 5 µg of vitamin B12 are lost daily through metabolic turnover, regardless of the amount stored in the body (Ball 2004b). Only after an injection of vitamin B12 and when the circulating vitamin B12 exceeds the protein-binding capacity will the excess be excreted in the urine. Thus, the binding with plasma proteins, negligible urinary loss and an efficient enterohepatic circulation, together with the slow rate of turnover, all explains why strict vegetarians, with normal absorptive capacity, take 20 years or more to develop signs of deficiency. People with absorptive malfunction develop deficiency signs within 2 - 3 years (Ball 2004b).

1.3.2 Dietary sources and bioavailability

1.3.2.1 Dietary sources

Vitamin B12 naturally originates from synthesis by bacteria and other microorganisms growing in soil or water, in sewage, and in the rumen and intestinal tract of animals. It cannot be made by plants. If there is a small amount detected in plants this may be due to microbial contamination from the soil or manure, or due to bacterial synthesis in the root modules of certain type of legumes (Ball 2004b). Thus, vitamin B12 is provided from any foods of animal origin including fish, meat, poultry, eggs, and milk products (Watanabe 2007), or from microbiologically contaminated plants. An outstanding dietary source of vitamin B12 is liver, followed by kidney and

heart (Ball 2004b). Fortified foods such as breakfast cereals contain crystalline vitamin B12 (cyanocobalamin) (Bailey 2004).

1.3.2.2 Bioavailability

Individuals depend entirely on the dietary intake of vitamin B12. Though vitamin B12 can be synthesized in the human colon, it is apparently not absorbed. Thus, strict vegetarians may have vitamin B12 insufficiency owing limited amounts ingested from the vitamin (Ball 2004b).

The bioavailability of dietary vitamin B12 is estimated to be about 50% in healthy adults with normal gastric function. If a person consumes a large portion of food rich in vitamin B12 in a meal, less absorption take place (Institute of Medicine 1998b). For example, in an intake of 0.5 µg or less, about 70% of available vitamin B12 is absorbed. At 5.0 µg intake of the vitamin, a mean of 28% is absorbed (range 2 - 50%), while an intake of 10 µg has a mean of 16% absorption (0 - 34%) of vitamin B12. These limits were derived from single oral doses of radioactive vitamin B12 (Herbert 1987). When large doses of crystalline vitamin B12 (100 µg) are taken, the absorption drops to approximately 1% of the dose, and the excess is secreted in the urine (Ball 2004b). Different levels of absorption assumed under various conditions are presented in **Table 1.1**.

Table 1.1: Assumed vitamin B12 absorption under different conditions. Obtained from (Institute of Medicine 1998b)

Form of vitamin B12	Natural Gastric Function (%)	Pernicious Anaemia (%) [a]
Naturally occurring	50	0
Crystalline, low dose (< 5 µg)	60	0
Crystalline, high dose (≥ 500 µg) with water	1	1
Crystalline, high dose with food	0.5	≤ 0.5

[a] A disorder in which lack of intrinsic factor severely limits the absorption of vitamin B12

The approximate percentage absorption of vitamin B12 from a few foods is shown in **Table 1.2**. In normal healthy adults, vitamin B12 in lean mutton (Heyssel et al. 1966), chicken meat (Doscherholmen et al. 1978), milk (Russell et al. 2001) and fish is efficiently absorbed as a comparable amount of crystalline vitamin B12 (Institute of Medicine 1998b). In contrast, vitamin B12 in eggs is poorly absorbed due to the presence of a different vitamin B12 binding proteins in egg white and egg yolk (Levine and Doscherholmen 1983). The absorption of vitamin B12 from liver is low (Heyssel et al. 1966) because of its high content of vitamin B12 (Scott 1997).

One of the reasons that could explain why a high intake of vitamin B12 does not necessarily result in higher absorption is the refractory nature of vitamin B12 absorption or on other word the limited capacity of the gastric IF-mediated absorption process (Carmel 2008). The absorption of vitamin B12 enters a refractory phase of about 3 h after ingestion. Thus, the specific IF-mediated absorption is saturated at certain level. An additional dose of vitamin B12 is absorbed normally when given 4 – 6 h after the

initial intake (Bor et al. 2004). Approximately only 1 - 1.5 µg per meal can be absorbed at any one time of a single dose of vitamin B12 (Herbert 1987).

Table 1.2: Percentage absorption of vitamin B12 from foods by healthy adults. Obtained from (Institute of Medicine 1998b)

Reference	Food	Absorption (%)
Heyssel et al., 1966	Mutton	65
Doscherholmen et al., 1978	Chicken	60
Russell et al., 2001	Milk	55 - 65
Doscherholmen et al., 1981	Trout	25 - 47
Doscherholmen et al., 1975	Eggs	24 - 36
Heyssel et al., 1966	Liver	11

Another important factor that could affect the vitamin B12 bioavailability is changing in the type of intestinal flora and/or it's activity. For example, overgrowth of intestinal bacteria, as a result of decrease gastric acidity, leads to a competition for uptake of vitamin B12 and thus interferes with vitamin B12 availability (Baik and Russell 1999, Wolters et al. 2004). Other factors that stimulate or inhibit growth of different types of intestinal flora include poor dietary intake, stress and use of medication. Furthermore, alteration in intestinal flora (quantitatively and qualitatively) could contribute to a wide range of diseases, including obesity, DM and metabolic syndrome (Diamant et al. 2011). Interest has recently been directed towards establishing a potential association between intestinal flora and T2DM. T2DM was associated with compositional changes in the intestinal microbiota (Larsen et al. 2010).

1.3.3 Dietary intake of vitamin B12

1.3.3.1 Dietary requirement

The human need for vitamin B12 is extremely small. Based on the haematological evidence and serum vitamin B12 values and to ensure normal serum concentrations and adequate stores, the RDA for vitamin B12 is calculated to be 2.4 μg per day for adults 19 years or over. Because of the estimated decrease of vitamin B12 bioavailability in people older than 50 years old, it is advisable to have most of the recommended vitamin B12 intake from fortified foods with vitamin B12 or vitamin B12 supplement (Institute of Medicine 1998b). For pregnant women, the RDA is increased to 2.6 μg per day to allow for foetal deposition throughout pregnancy and evidence that maternal absorption of the vitamin becomes more efficient during pregnancy. During lactating, the RDA is further increased to 2.8 μg per day, based on non-lactating women's requirements and the amount of vitamin B12 secreted in the milk (Bailey 2004).

1.3.3.2 Effects of high intake

Ingested amounts that exceed the limited binding capacity in plasma and tissue are excreted unchanged in the urine and faeces (Ball 2004b). Thus, it is believed that there are no adverse effects associated with excess vitamin B12 from food or from supplements in healthy individuals (Institute of Medicine 1998b; Bailey 2004). However, the studies in which excess intakes were reported were not designed to assess the adverse effects of the vitamin (Institute of Medicine 1998b; Carmel 2008).

1.4 Mechanism of actions of folic acid and vitamin B12

Folic acid and vitamin B12 are essential nutrients for humans as they are necessary for new cell formation and maintenance by participating in several vital reactions (Kamen 1997). Folic acid is involved in nucleic acid synthesis, methionine regeneration, and in the activation, oxidation and reduction of one-carbon units, referred to as 1-C metabolism, required for normal metabolism and regulation (Bailey and Gregory 1999). Vitamin B12 is an important co-factor involved in only two enzymatic reactions in the cell metabolism; methionine synthase (MS) and methylmalonyl-CoA mutase (MCM).

MS converts homocysteine to methionine and then to S-adenosylmethionine (SAMe) in the presence of vitamin B12 (in the form of methylcobalamin) and folic acid (in the form of tertrahydrofolate). Lack of MS from vitamin B12 insufficiency will lead to decreased synthesis of methionine and 5-tetrahydrofolate (5THF), as well as the accumulation of homocysteine and 5-methyl-THF (5MTHF). Less methionine synthesis will lead to less SAMe production, which is a common methyl donor required for the maintenance of methylation patterns in DNA that determine gene expression (Fenech 2001). SAMe normally suppresses methylene-THF reductase (MTHFR), thus an impaired production of SAMe will reduce this suppression and result in the irreversible conversion of 5,10-methylene-THF to 5-methyl-THF. The 5-methyl-THF then becomes metabolically trapped owing vitamin B12 insufficiency. Furthermore, the reduction in 5THF leads to reduced availability of 5,10-methylene-THF, which is needed to convert deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) in the presence of thymiylate synthase. Under conditions of folic acid insufficiency, dUMP accumulates and as a result uracil is incorporated into DNA instead of thymine (Blount et al. 1997).

MCM is required for degradation of odd-chain fatty acids and branched-chain amino acids, in particular conversion of methylmalonic-CoA (MM-CoA) in the presence of vitamin B12 (in the form of adenosylcobalamin) to succinyl-CoA, which is an important substrate in the Krebs cycle. MM-CoA is derived from propionyl-CoA, which enter the pathway via degradation of several amino acids including methionine and beta oxidation of odd-chain fatty acids. While the actions of MS (which happen inside the cell cytosol) are dependent on folic acid in addition to vitamin B12, the actions of MCM (which occur inside the mitochondria) are dependent only on vitamin B12 (**Figure 1.10**) (Rosenberg 2008).

SAM-e is the common methyl donor required for methylation reaction including methylation of DNA. In addition, the folic acid derivative, 5,10 methylene tetrahydrofolate is involved in DNA synthesis and repair (**Figure 1.10**). Thus, both vitamin B12 and folic acid play crucial roles in the genomic stability of human cells by preventing chromosomal breakage and hypomethylation of DNA (Fenech 2001) as well as being involved in several other critical pathways during development and adult life (Saravanan and Yajnik 2010).

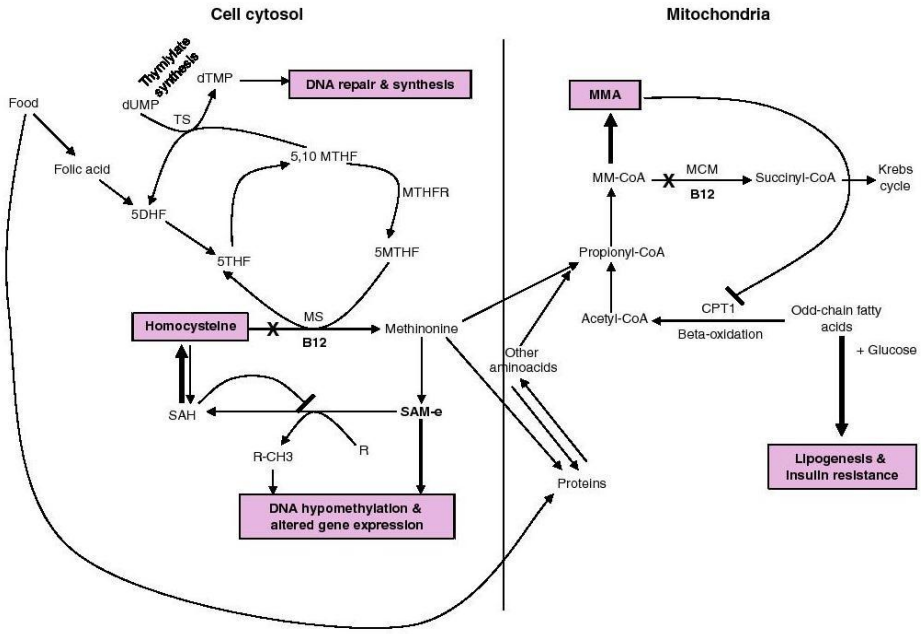


Figure 1.10: Pathways that involve vitamin B12 and the suggested mechanism of increased adiposity and insulin resistance. Vitamin B12 insufficiency traps folic acid as 5MTHF. This results in reduced synthesis of methionine and SAMe inside the cell cytosol. Inside the mitochondria, MM-CoA is converted into MMA, which is a potent inhibitor of beta-oxidation (Yajnik et al. 2008; Saravanan and Yajnik 2010). Key: dTmP = deoxythymidine monophosphate; dUmP = deoxyuridine monophosphate; TS = thymidylate synthase; MTHF = methyl tetrahydrofolate; MTHFR = methylene tetrahydrofolate reductase; DHF = dihydrofolate; THF = tetrahydrofolate; MS = methionine synthase; SAH = S-adenosylhomocysteine; SAM-e = S-adenosyl methionine; R = methyl acceptor; R-CH3 = Methylate compound; MM-CoA = methylmalonyl-CoA; MCM = methylmalonyl-CoA mutase; CPT1 = carnitine palmitoyltransferase-1.

1.5 Clinical manifestations of folic acid and vitamin B12 insufficiency

1-C metabolism can be impaired in the absence of folate insufficiency. The availability of several nutrients affects the efficiency of folate-mediated 1-C metabolism, resulting in the accumulation of various substrates and metabolic intermediates which may have negative consequences (Bailey and Gregory 1999). As mentioned previously, the enzyme MS catalyzes the vitamin B12–dependent conversion of homocysteine and 5-methyl THF to methionine and 5THF (**Figure 1.10**). Because the MTHFR enzyme catalyzed conversion of 5,10-methyl THF to 5-methyl THF is essentially irreversible, loss of MS activity due to vitamin B12 insufficiency or

genetic mutation results in an accumulation of cellular folate as 5-methyl THF, referred to as a folate "methyl trap". The accumulation of 5-methyl THF depletes the concentration of other forms of folate and thereby inhibits nucleo-protein synthesis (Herbig et al. 2002), resulting in vitamin B12-responsive megaloblastic anemia (Carmel and Karnaze 1985). Thus, overt deficiency of either vitamin B12 and folate results in megaloblastic anaemia as rapid cell division in the bone marrow is impaired, especially erythropoiesis, and thus release immature erythrocyte into the circulation (Bender 2003).

In addition, methylation is a very important process in protecting nerves myelin. One important methylation is that of myelin basic protein. It makes up one-third of myelin, the insulation cover on nerves. In the presence of vitamin B12 insufficiency, the methylation process is interrupted which leads to demyelination of the nerves, resulting in a neuropathy which leads to ataxia, paralysis and if untreated, ultimately death (Scott 1999). Neuropathy generally begins in the peripheral nerves and progresses to the spinal cord and brain (Ball 2004b). Severe vitamin B12 insufficiency results in cognitive impairment especially in elderly and rarely subacute combined degeneration of the spinal cord (Wilson and Langman 1966; Saravanan and Yajnik 2010). However, overt deficiencies of these vitamins are rare and are diagnosed early (Saravanan and Yajnik 2010).

Vitamin B12 insufficiency is not rare in populations, especially among vegans because the predominant dietary sources of vitamin B12 are meat, poultry, seafood and dairy products (Watanabe 2007). Malabsorption is a leading cause of vitamin B12 insufficiency (Fernández-Bañares et al. 2009), which is common in the elderly people (Matthews 1995). About 60 – 70% of elderly people suffer from malabsorption

(Fernández-Bañares et al. 2009). If untreated, vitamin B12 insufficiency results in an irreversible decline in cognitive function and memory (Malouf et al. 2003).

Folic acid insufficiency in mothers is one of the major risk factors for NTDs (Lumley et al. 2002). It has been assumed that folate-responsive NTDs result from impaired maternal and/or foetal folate metabolism, with the affected pathways being either dTMP or methionine (SAM) syntheses, and that these metabolic disruptions can be resolved by increasing maternal folate status. The suggested mechanisms include accumulation of homocysteine, decreased rates of DNA synthesis due to impaired dTMP synthesis, and elevations in the SAH/SAM ratio (Stover 2004). Introducing periconceptional supplementation with folate and/or multivitamins reduces the NTDs risk by 50% (Lumley et al. 2002). This has resulted in mandatory folic acid fortification of wheat flour in many countries such as the US. The list of countries that have a mandatory folic acid food fortification programme are shown in a figure in **Appendix I**. In the UK, although it is not mandatory, most breakfast cereals have fortification of folic acid (Saravanan and Yajnik 2010). However, foetal NTDs continue to affect about 6 to 12 in every 10,000 pregnancies (Ray et al. 2007). A systematic review of previously published data suggests that maternal vitamin B12 insufficiency is an independent risk factor for NTDs (Ray and Blom 2003). In addition, it has been observed that the risk of NTDs was higher in the presence of low maternal vitamin B12 in folic acid sufficient individuals (Ray et al. 2007). As of the fortification of various foods, folic acid insufficiency is increasingly rare and vitamin B12 insufficiency has become the major modifiable risk factor for NTDs (Ray et al. 2007). Several recent studies report that vitamin B12 insufficiency is common in infants too (Schulpis et al. 2004; Jones et al. 2007; Hay et al. 2010; Saravanan and Yajnik 2010). Low maternal vitamin B12 levels can result in low levels in the infants with resultant poor brain

development (Molloy et al. 2008) and cognitive impairment in children (Bhate et al. 2008). Furthermore, a combination of both low folate and vitamin B12 is associated with abnormal behaviour and development (Black 2008). Another aspect of the interrelationship between folate and vitamin B12 is the increased plasma homocysteine concentrations as a result of folate and/or vitamin B12 insufficiencies. The possible mechanism includes lack of re-methylation of homocysteine to methionine. Though, mutations in the enzymes and/or nutritional deficiencies in B-vitamins required for homocysteine metabolism can induce hyperhomocysteinemia (Wald et al. 2002).

1.6 Homocysteine, vitamin B12 and metabolic risks in offspring

Despite folate status being known as major determinant of homocysteine concentration in non-folic acid supplemented populations, subgroups within such populations may exhibit dependence of homocysteine on vitamin B12 status rather than folate (Strain et al. 2004). In a study comparing different habitual diets in free-living Australians it was found that meat-eaters have lower homocysteine and higher serum vitamin B12 concentrations compared to ovo-lacto vegetarians and vegans. Interestingly, ovo-lacto vegetarians have much lower methionine intakes than the habitual meat-eaters (Mann et al. 1999). In addition, it has been reported that there are higher homocysteine and serum folate levels in Taiwanese vegetarians compared to meat-eaters (Hung et al. 2002). Furthermore, in countries implementing folic acid fortification of flour, vitamin B12 insufficiency has emerged as the most common modifiable risk factor for hyperhomocysteinemia (Green 2009). Homocysteine is shown to be an independent risk factor for CVD, including ischemic heart disease, stroke, and peripheral vascular disease (Wald et al. 2002). It is believed that there is a causal relationship between hyperhomocysteinemia and atherosclerosis (Zhou and Austin

2009). A recent systematic review did not support the effect of vitamin B12 insufficiency on increasing the risk of CVD in adults (Rafnsson et al. 2010). In addition, B-vitamins supplementation in a high risk population did not reduce CVD, despite significant homocysteine lowering (Bazzano et al. 2006; Albert et al. 2008). However, these results do not rule out the causal link between the B-vitamins and CVD (Saravanan and Yajnik 2010). Saravanan and Yajnik listed several factors to support this link: "(a) these studies were performed in non-deficient populations; (b) these vitamins, especially vitamin B12, may have other effects independent of homocysteine and (c) they may play a more crucial role during development rather than during adulthood" (Saravanan and Yajnik 2010). Thus, the effect of vitamin B12 insufficiency, if any, on the risk of chronic disease such as CVD and DM may occur earlier in life (Yajnik et al. 2008; Rafnsson et al. 2010). In fact, homocysteine correlates strongly with low birth weight while folic acid has the opposite effect (Yajnik et al. 2008). The folic acid (independent effects of vitamin B12), in insufficient states may also cause increased MMA resulting in increased lipogenesis due to the inhibition of beta-oxidation by inhibiting CPT-1, an enzyme that helps in the change of fat to energy (**Figure 1.10**) (Saravanan and Yajnik 2010). Also in the presence of vitamin B12 insufficiency, methyl folate trap will adversely affect methionine production from homocysteine, thus leading to less amino acid production, and therefore potentially reducing protein synthesis and lean tissue deposition (Yajnik et al. 2008). Such interactions in the intrauterine environment could result in 'thin-fat' babies with higher body fat and lower muscle mass (**Figure 1.11**). These babies will in turn have higher insulin resistance and therefore be at high risk of CVD in later life (Yajnik et al. 2003; Bhargava et al. 2004). Thus, lowering the homocysteine by using vitamin B12 and folic

acid during pregnancy is likely to have a significant impact on the metabolic risk of the offspring (Saravanan and Yajnik 2010).

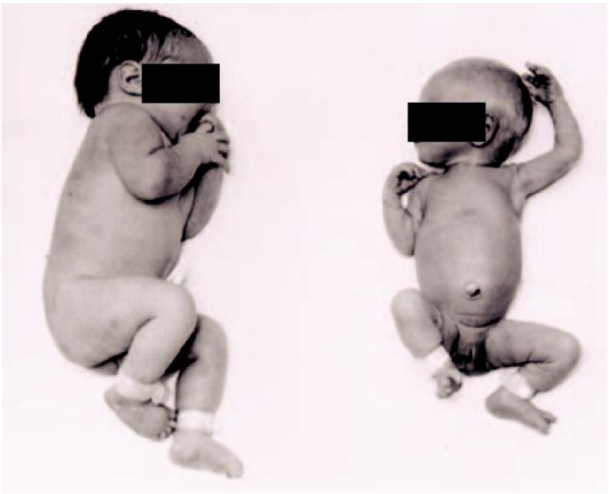


Figure 1.11: The characteristics phenotype of a South Asian compared to Caucasian baby. Compared with UK white babies, Indian babies are small. There is a substantial deficit in non-fat soft tissues, while subcutaneous fat was preserved (Yajnik et al. 2003).

1.7 Attentions from folic acid fortification studies

Since January of 1998, the US Food and Drug Administration has required the folic acid fortification of all enriched cereal grain products. The main motivation behind fortification is the prevention of the NTDs' birth. In this regard, the folic acid fortification programme has been effective in terms of reducing the incidence of NTDs by 20 – 50 % (Honein et al. 2001; De Wals et al. 2007). Parallel to the NTDs' birth reduction, additional benefits have been observed. The prevalence of both low folate and high homocysteine has been reduced in the general population (Jacques et al. 1999); in addition the epidemiological evidence showed a decline in the rate of stroke mortality in the US and Canada which may be related to the folic acid fortification (Yang et al. 2006). However, some concern has been raised. Geometric mean folate concentration has increased from ~ 12 to ~ 30 nmol/l (Ganji and Kafai 2006) and some of the serum folate was detectable as unmetabolized folic acid (Troen et al. 2006). More important,

those who were exposed to folic acid fortification and used vitamin supplements were consuming an amount exceeding the folate upper tolerable limit of 1 mg/d (Choumenkovitch et al. 2002). Folic acid is a synthetic form and not found generally in food; a high intake of this unnatural substance could lead to unintended consequences affecting at least a part of the population (Miller et al. 2009).

Before the folic acid fortification programme started, the possibility of an increased folic acid intake that might mask haematological signs related to vitamin B12 insufficiency or even aggravate its neurologic and neuropsychiatric effects was the main concern (Morris et al. 2007). However, the adverse effects of excess folic acid intake especially in the presence of vitamin B12 insufficiency have only recently been studied. After folic acid fortification, data from the National Health and Nutrition Examination Survey (NHANES III) reported both a 'good' and a 'not-so-good' side of folate (Morris et al. 2007; Smith 2007). The 'good news' is that, in subjects with a normal vitamin B12 status, high serum folate was associated with protection against cognitive impairment of the elderly. A similar result was reported for Latinos living in California, where higher red blood cell folate concentrations after folic acid fortification were associated with protection from cognitive impairment and dementia (Ramos et al. 2005). The 'not-so-good' news is that the relation between high serum folate and cognitive impairment was reversed in subjects who had a low vitamin B12 status (Morris et al. 2007; Smith 2007). Thus, simply we can say that folic acid supplementation is not good for everyone.

In support of the periconceptional supplementation with folic acid, population-wide fortification of wheat flour achieved similar reductions in NTDs (Lumley et al. 2002; Saravanan and Yajnik 2010). The prevalence of vitamin B12 insufficiency has increased in Canada, especially in the era after folic acid flour fortification. In addition,

NTDs attributable to vitamin B12 insufficiency have tripled in the same period (Ray and Blom 2003; Ray et al. 2007). The current data suggest that 1 in 20 women in Ontario may be vitamin B12 insufficient in the critical period of the embryonic neural tube closure. Thus, determining the prevalence of vitamin B12 insufficiency in this crucial period may inform future programme designed to lower the risk of NTD (Ray et al. 2008).

In the Pune Maternal Nutrition Study (PMNS), the participants were known to be vegetarian and using folic acid as a standard care (500 µg /day) during pregnancy. Thus, the majority of pregnant women (> 60%) had low vitamin B12 and only one woman had a low folate level. After a follow up, it has been shown that the children born to mothers with the combination of 'high folic acid and low vitamin B12' had higher adiposity and insulin resistance (**Figure 1.12**) (Yajnik et al. 2008). Thus, an intrauterine imbalance between the two related vitamins (vitamin B12 and folate) may increase NTDs, increase the risk of disease such DM and CVD in later life, as well as increasing the risk of cognitive impairment in the elderly population (Saravanan and Yajnik 2010).

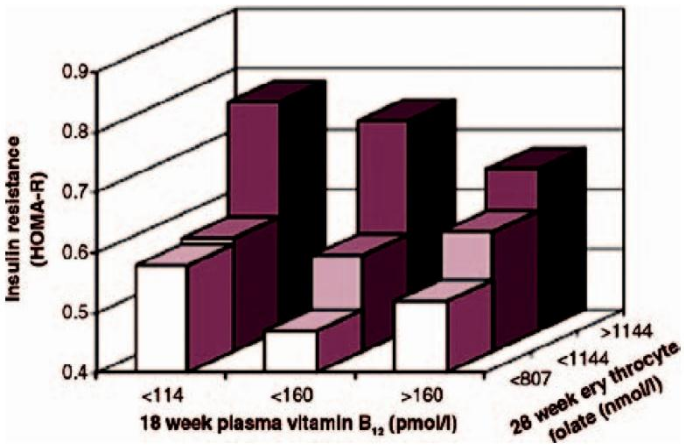


Figure 1.12: The impact of maternal "high folate and low vitamin B12" in the offspring. Insulin resistance in children at 6 years of age in relation to maternal vitamin B12 (18 weeks) and erythrocyte folate (28 weeks). Children born to mothers who had the lowest vitamin B12 and highest folic acid levels during pregnancy were the most

insulin resistant (Yajnik et al. 2008). Insulin resistance was calculated using the fasting insulin and glucose concentration (homeostatic model assessment of insulin resistance [HOMA-R]).

1.8 Possible mechanisms of "imbalance of vitamin B12/folate" on the metabolic risk of offspring

It is well known that influences linked to early growth and development have an important effect on the risk of T2DM and CVD. The intrauterine environment, particularly maternal nutrition, can alter the vital development process *in utero* and during infancy and continue if exposed to an adverse environment in childhood and adult life (Godfrey and Barker 2000; Barker 2004; Mcmillen and Robinson 2005). However, until recently the mechanism of specific nutrients on 'nutrient programming' was not known (Sinclair and Singh 2007; Saravanan and Yajnik 2010). The DNA methylation that occurs initially at gametes phase and then at pre-implantation embryos highlight the possibility that periconceptional availability of methyl groups might influence such programming which leads to several alterations at adult status (Santos and Dean 2004). Furthermore, early nutrition can influence the DNA methylation because the mammalian 1-C metabolism, which provides the methyl groups for all biological methylation reactions, is highly dependent on dietary methyl donors (methionine) and cofactors (vitamin B12 and folate) (Waterland and Jirtle 2004). The genome of the pre-implantation mammalian embryo undergoes extensive demethylation. The patterns of DNA methylation must then be maintained over many rounds of rapid cellular proliferation during foetal and early postnatal development. Thus, the availability of dietary methyl donors and cofactors during this critical period might influence cell differentiation, development and function, then enhance the susceptibility to chronic disease in later life (Waterland and Jirtle 2004). The effects of

unbalanced maternal nutrition on the methylation status were studied in animals by using diets providing methyl groups which contain both vitamin B12 and folic acid. Studies in rodents showed that such diets during pregnancy altered the DNA methylation of candidate genes (agouti, peroxisomal proliferator-activated receptor (PPAR-α) and glucocorticoid receptor) which in turn affected the regulatory mechanisms of these genes throughout the life of these animals (Waterland and Jirtle 2003; Lillycrop et al. 2005). More recently, the effect of a diet restricting the supply of vitamin B12, folate and methionine were investigated in mature female sheep. Interestingly, the results showed that lambs born to sheep fed a 'methyl-deficient' diet had higher adiposity, higher insulin resistance and higher blood pressure (Sinclair et al. 2007). Thus, the results from animal studies can provide clear evidence for epigenetic alterations of DNA methylation by these nutrients during the periconceptional period. Furthermore, evidence from humans in regard to this issue is very limited and only comes from the PMNS study . However, the causality from this study cannot be proven (this would need intervention studies) and need to be urgently replicated in other populations, given the potential huge impact of such intervention (Yajnik et al. 2008; Saravanan and Yajnik 2010). As subclinical vitamin B12 insufficiency is not rare (Allen 2009), many research questions have been raised which need to be considered as to whether or not to introduce mandatory vitamin B12 fortification in the population (Green 2009). However, given the strong influence on the DNA methylation with potential lifelong epigenetic programming, such measures should only be taken after careful evaluation of such an intervention (Saravanan and Yajnik 2010).

1.9 Thesis aims

It is well known that T2DM is a growing health problem worldwide. It is now being diagnosed earlier in life. Thus, preventing the epidemic of such a disease is a huge public health challenge that needs careful long term planning and implementation. As discussed previously, 'gene-diet' interaction during foetal development is likely to contribute to the epidemic of T2DM and CVD. More important, the intrauterine imbalance between the two related vitamins, vitamin B12 and folate, altered DNA methylation and could programme the foetus for the whole life. Thus, micronutrient status during pregnancy is likely to have a significant impact on the metabolic risk of the offspring. This thesis will predominately focus on vitamin B12 status during pregnancy among Saudi population. This will be shown, firstly, by understanding the worldwide prevalence of vitamin B12 insufficiency, particularly in the non-vegetarian population. Secondly, by investigating the possible role of maternal vitamin B12 in blood, as an independent risk factor, in predicting low birth weight in the offspring. Thirdly, by investigating the correlation between dietary intake of vitamin B12 and folate and their nutritional biomarker. Finally, by providing prevalence of vitamin B12 insufficiency among Saudi pregnant women. This will help us to identify the importance of maternal vitamin B12 status during the periconceptional period.

Chapter 2

Worldwide prevalence of vitamin B12 insufficiency during pregnancy and it's relation to birth weight

Systematic review (A)

"Worldwide prevalence of vitamin B12 insufficiency during Pregnancy"

Systematic review (B)

"Does maternal vitamin B12 level independently predict low birth weight?"

2.1. Introduction

Pregnant women in developing countries are vulnerable to nutritional deficiencies. This is due to the increased nutritional and physiological demands imposed by pregnancy involving a growing placenta, foetus and maternal tissue, which could be associated with maternal and foetal morbidity (Baker et al. 1975). An average of 20 - 30% of pregnant women suffer from a vitamin deficiency. Without supplementation, about 75% would show a deficiency of at least one vitamin (Baker et al. 2002). The importance of the different micronutrients deficiency may vary depending on regional and cultural differences (Bondevik et al. 2001). Thus, there is widespread recognition of the importance of adequate maternal nutrition during pregnancy in developing countries where profound malnutrition is uncommon (Mathews et al. 1999). The frequencies of low birth weight infants, still births, and babies born with congenital disorders are high among malnourished mothers (Leader et al. 1981; Pitkin 1981). As nutritional requirements are high, the effects of malnutrition can be severe and long-lasting in pregnant mothers (Ackurt et al. 1995). Nutritional status during pregnancy is thus implicated as a significant contributory factor in maternal and infant morbidity and mortality (Kafatos et al. 1989).

The associations between low birth weight and the risk of later chronic disease such as CVD and DM has been strongly addressed by Barker and his colleagues, where poor maternal nutrition has been implicated as one of the key "adverse environmental influences *in utero*," which could lead to compromised foetal growth and adverse long term consequences (Barker 2004). Moreover, low birth weight is associated with insulin resistance and related condition, including T2DM, but the underlying mechanisms are not clear (Levy Marchal and Jaquet 2004). The thrifty phenotype hypothesis proposes that foetal malnutrition *in utero* results in reduced foetal growth and programming of

the foetus to be insulin resistant postnatally (Barker 1995). In addition, "Foetal programming" is likely to be mediated through DNA methylation which involves B-vitamins such as folic acid and vitamin B12 in such reactions. This is called epigenetic regulation and may provide some clues to the epidemic of T2DM and CVD (Saravanan and Yajnik 2010).

In the presence of folic acid fortification of various foods and the recommendation for preconception folic acid supplementation, folate insufficiency has become rare in the general population as well as in pregnant women. Indeed, vitamin B12 insufficiency has become a potential major modifiable risk factor for metabolic diseases as well as neural tube defects (NTDs) (Morris et al. 2007). In fact, 10 years after mandatory folic acid fortification in Canada, NTDs due to vitamin B12 insufficiency have tripled (Ray et al. 2007). In addition, vitamin B12 insufficiency has been reported to be common in pregnancy. Approximately 20% of women show a physiologic drop in serum vitamin B12 level during pregnancy (Pardo et al. 2000). In India, where vitamin B12 insufficiency is common, authors of PMNS provided a strong stimulus towards further understanding of the possibly fundamental relationship between vitamin B12 insufficiency in mothers and metabolic outcomes in offspring, which seriously overlap with the growing global concern about obesity and adiposity (Rosenberg 2008). The PMNS study has children with a low mean birth weight; children are short and thin but relatively adipose compared to white children, and the results showed that children born to mothers with low vitamin B12 but high folate concentrations were the most insulin resistant (Yajnik et al. 2008). Thus, vitamin B12 insufficiency during the periconceptional period, especially in the presence of high folic acid levels, may increase NTDs as well as the offspring's future risk of T2DM and CVD (Saravanan and Yajnik 2010).

It is perceived that vitamin B12 insufficiency will be rare in populations with a high intake of animal protein such as Saudi Arabia. However, the results from the National Diet and Nutrition Survey (NDNS) data of the UK adult population showed the presence of vitamin B12 insufficiency in 10.5% compared to 2.1% folate insufficiency, with an even higher prevalence of 11.8% in women of childbearing age (Saravanan et al. 2010a). We therefore aimed to conduct a systematic review trying to answer the following questions: (A) What is the worldwide prevalence of vitamin B12 insufficiency during pregnancy, particularly in the non-vegetarian population? and (B) Does the vitamin B12 level during pregnancy predict low birth weight in the offspring? Thus, we identify all relevant observational studies investigating the worldwide prevalence of vitamin B12 insufficiency and the possible role of maternal vitamin B12, as an independent risk factor, in predicting low birth weight in the offspring.

2.2 Methods

2.2.1 Study search

We conducted a comprehensive literature search for publications on vitamin B12 during pregnancy in MEDLINE/PUBMED (National Library of Medicine and National Institute of Health), and EMBASE (The Excerpta Medica database), Global health, CAB (Commonwealth Agricultural Bureau database) and CINAHL (The Nursing & Allied Health database). All databases were searched from inception. We applied a search strategy based on a combination of keywords and medical subject headings (MeSH) (e.g. cobalamin, vitamin B12, vitamin B12 insufficiency, methylmalonic acid, holotranscobalamin, homocysteine, pregnancy or pregnant women and birth weight). The study search for the two systematic reviews was conducted separately by different reviewers and at different times, using slightly different

eligibility criteria and search keywords relevant to the aim of the reviews. All search results were limited to studies conducted on humans, and published in the English language. We also examined the reference lists of key publications for other potential citations.

2.2.2 Study eligibility

Study eligibility criteria included publications of observational studies (longitudinal or cross-sectional studies) on normal adult pregnancy (18 years old or more). Studies that were designed specifically to look for NTDs' birth, or case-control studies on low birth weight, studies on adolescent pregnancy, anaemic pregnancy, studies on vitamin B12 and early pregnancy loss, or studies designed to look for the effect of smoking in vitamin B12 level were excluded. The eligibility criteria for each systematic review A and B were specified in (**Figure 2.1** and **Figure 2.3)** respectively. Results for some subjects were reported in more than one paper. In these cases, we included the papers with the most complete information relevant to the aim of the reviews.

2.2.3. Study selection and data extraction

All initial data searches and screening of titles and abstracts of identified studies were independently done for eligibility according to the pre-specified inclusion and exclusion criteria by two reviewers. Potentially relevant articles were retrieved and full-texts were evaluated against the same criteria. Each accepted study was extracted by a single reviewer, and a second reviewer independently verified the extracted data. Data extraction issues were resolved by consensus. When disagreements could not be resolved, a third reviewer was consulted for a final judgement. Data was subsequently

extracted onto pre-designed forms by the two reviewers. For each study, we extracted information on the study site and population, maternal age, gestational age at blood collection, vitamin B12 measurement method, use of supplement prior to blood sampling, and results. When the study design was not specified in the article, it was assigned according to the type of study design and method used in subject collection and agreed by the two reviewers. In addition, Vitamin B12 and folate levels are presented in the International System of Units (SI) (pmol/l and nmol/l) respectively. When levels in an article were presented in conventional units (e.g. pg/ml for vitamin B12 and ng/ml for folate), calculations were made to convert them in to SI units using the conversion table for chemical compounds (conventional unit => SI unit: multiply by conversion factor; 0.738 and 2.266 for vitamin B12 and folate respectively).

2.2.4. Study quality assessment

Studies included in the reviews were assessed independently by the two reviewers for methodological quality using assessment checklist criteria according to the type of study design (cross-sectional or longitudinal observational study design). Differences in methodological quality across studies can indicate that the results of some studies are more likely to be affected by bias than others. Therefore, the quality of a study should be taken into account when the potential causal distribution of risk factors is being evaluated. The results were compared and disagreements between the reviewers on individual items were resolved by discussion. When disagreements could not be resolved, a third party was consulted for a final judgment.

2.2.4.1. Longitudinal studies

An assessment checklist of criteria provided in the NICE guidance for public health (National Institute for Health and Clinical Excellence 2009), which was adapted from the Tooth et al. checklist criteria of reporting of observational longitudinal studies (Tooth et al. 2005) were used. The quality assessment focused on appraising the internal (study design and quality) and external (how well it can be applied to the population in question) validity of the studies. The criteria reflect design and interpretation aspects covering the study rationale and population, recruitment, measurements and biases, data analysis, and generalizability of the results. The overall assessment for each article in the review was concluded with an evidence statement, as adapted from the Scottish Intercollegiate Guidelines Network (SIGN) (www.sign.ac.uk), which reflects the strength of the study. The results of the quality assessment were used for descriptive purposes to provide an overall evaluation of the studies included in the review. More specifically, the overall assessment per paper is based on the number of relevant items addressed in each paper. The ranking system used for the overall assessment was 1st tertile (< 33% "yes") = few criteria met, 2nd tertile (33% - 66% "yes") = some criteria met, and 3rd tertile (> 66% "yes") = most criteria met. However, when comparing different papers (e.g. high quality versus low quality), we looked within the overall assessment and focus on which specific items (e.g. focusing on the key 10 relevant items of internal validity; which are questions 1, 4, 7, 9, 17, 18, 27, 28, 29, and 30 in the checklist) were addressed or not since it is unlikely that they are all equal.

2.2.4.2. Cross-sectional studies

An assessment checklist of criteria for cross-sectional studies provided in (Ariens et al. 2000; van der Windt et al. 2000) was used with a slight modification (in

some items' statement) to suit the aim of the present review. The list consisted of different items in 5 categories on information, validity and precision (study objective, study population, exposure and outcome assessment, and analysis/data presentation). Each item was scored as "1" when a study positively provided the information on an item, "0" when a study did not provide that information, or "don't know" (unclear) if the paper provided insufficient information on a specific item. Subsequently, total quality scores were calculated by counting the number of positively scored items over the total number of applicable items. The maximum score given was 8. The studies were categorized as either high or low in methodological quality according to their total score computed. A high quality study was defined as a study that scored positively on at least 75% (≥ 6) of the total methodological quality assessment score.

2.2.5. Study heterogeneity

Substantial study heterogeneity was observed because of differences in sample characteristics, study design (including: gestational age and use of vitamin supplement at blood collection) and data analyses performed (including: measurement and adjustment for confounding factors). In addition, study heterogeneity arose from differences between studies in biological sample characteristics: plasma vs. serum and fasting vs. non-fasting, and vitamin B12 assay measurement methods (microbial assay, immunoassay, radioimmunoassay, enzyme assay, etc.).

2.3 Systematic review (A)

"Worldwide Prevalence of Vitamin B12 Insufficiency during Pregnancy"

2.3.1. Results

2.3.1.1. Study characteristics

A total of 4187 relavent articles were identified in bibographic databases, from inception to August 2011, for title and abstract screening. According to the pre-specified inclusion and exclusion criteria, only 251 articles were selected and initially retrieved for further review and inspection. Detailed evaluation of the full text was reviewed for 122 articles. Sixty five articles were excluded because of duplicate studies or same study population (n = 12), no prevalence of vitamin B12 insufficiency or clear vitamin B12 cut-off level was reported (n = 28), or has anaemic subjects (n = 3), studies on lactating mother (n = 1), non-pregnant sample (n = 2), reviews (n = 5), small sample size (n = 11) or conference papers (n = 3) (**Figure 2.1**). A total of 57 articles were included in the review. The studies have been categorized according to the geographic distribution into different continents. In addition to the usual list of continents, 2 additional categories have been added (Middle Eastern and South Asian countries) as previously used (Stabler and Allen 2004), as we believe that countries in these areas could share similar dietary habits and food consumption patterns that may affect their total nutrients intake and actual levels in the blood. The list and number of studies included in each category are as follows: American continent (North American; n = 7 and Central and South American; n = 6), Australian continent (n = 2), European continent (n = 11), African continent (n = 7), Asian continent (n = 7), Middle Eastern countries (n = 6), and South Asian countries (n = 11). The methodological overview of the studies included in this review are shown depending on each continent from (**Table**

2.1 to **Table 2.7)**. A brief description on the studies included in each continent are given as follows.

2.3.1.1.1. American continent

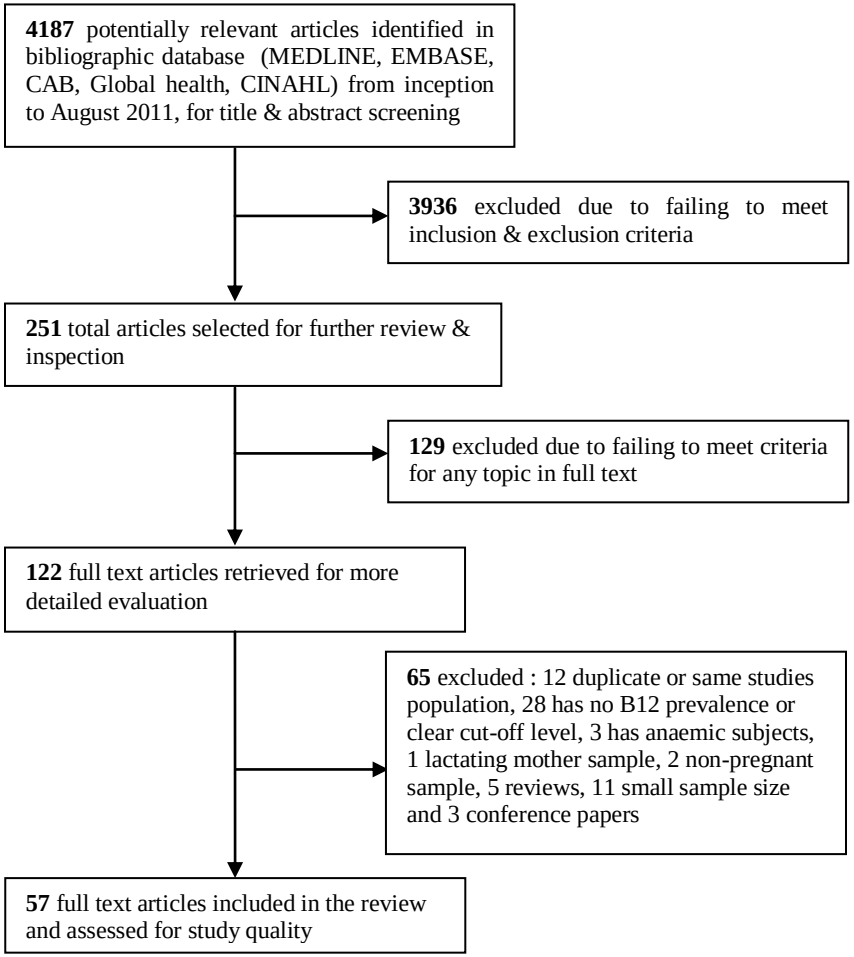
Thirteen studies, 7 from North American: Canada (Lowenstein et al. 1960; House et al. 2000; Ray et al. 2008), USA (Baker et al. 1975; Jacob et al. 1976; Knight et al. 1991; Pagán et al. 2002) and 6 from Central and South American: Mexico (Black et al. 1994), Latin America (Cook et al. 1971), Venezuela (Garcia-Casal et al. 2005) and Brazil (Giugliani et al. 1984; Guerra-Shinohara et al. 2004; Barbosa et al. 2008), were included in the review; 3 longitudinal and 10 cross-sectional studies. The number of participants ranged from 51 to 10622 pregnant and the mean maternal age ranged from 25 to 30.5. Only 4 studies reported the use of multivitamins at blood collection (**Table 2.1**).

2.3.1.1.2. Australian continent

Only 2 studies were included in this continent (Whiteside et al. 1968; Cole et al. 1974). One is a longitudinal study followed the participants at the 3 trimesters and the other one is cross-sectional study screened the participants at the third trimester. The number of participants ranged from 60 to 147 pregnant (**Table 2.2**).

2.3.1.1.3. European continent

Eleven studies from Denmark (Zachau-Christiansen et al. 1962; Milman et al. 2006), UK (Ball and Giles 1964; Roberts et al. 1973), Scotland (Shields et al. 2011), France (Frery et al. 1992), Norway (Bjorke et al. 2001), Netherland (Bruinse and Van den Berg 1995), Amesterdam (Goedhart et al. 2011) , Spain (Murphy et al. 2007) and



Inclusion Criteria:

- Studies on normal adult pregnancy.
- Studies identify number or % of low vitamin B12 level.

Exclusion Criteria

- No cut-off level identify for low vitamin B12 level.
- Case-control studies that were designed to look for LBW.
- Adolescents' pregnancy
- Pregnancy and NTDs or IUGR infants
- Vitamin B12 and early pregnancy loss.
- Studies designed to look for the effect of smoking on vitamin B12
- Anaemia.

Figure 2.1 Flow diagram of studies included in the systematic review (A). All initial data searches and screening of titles and abstracts of identified studies were independently done for eligibility according to the pre-specified inclusion and exclusion criteria by 2 reviewers – agreement was (97%).

Table 2.1: Shows the methodological overview of studies included in the review: American continent.

References	Country or city/ time period	Study Design	No. of subjects	Period of blood collection	Maternal age (years)	Vitamin B12 measurement method/ blood sample	B12 supplement prior to blood sample	Q/A score L	Q/A score CS
Lowenstein et al., 1960	Canada	L	345	2nd trimester 3rdtrimester	N/R	MBA/ Serum	N/R	(-)	
Cook et al., 1971	Latin America 1968-1970	CS	899	35 (32-40) ‡	(15-40)	N/R	N/R		6
Baker et al., 1975	New Jersey	CS	174	Delivery Cord	(20-37)	MBA/Serum	Yes (76.4% used multivitamin)		7
Jacob et al., 1976	Los Angeles 1972-1974	CS	301 Mexican	2nd trimester (n= 257)	25 ± 6 (15-42)	MBA/Serum	Yes (20% used multivitamin)		5
Giugliani et al., 1984	Brazil	CS	51	Delivery (38-42)	N/R	RIA/Serum	Yes (51%)		5
Knight et al., 1991	Washington DC 1985-1990	L	600	14 - 27wks (n=109) > 27wks (n=117) Delivery (n=101) Cord (n=91)	(16-35)	RIA/Fasting serum	Yes (90%used multivitamin)	(+)	
Black et al. ,1994	Mexico 1985-1987	CS	85	5-8 months of pregnancy	30.5 ± 7	RA/Fasting serum	N/R		5
House et al., 2000	Newfoundland 1996-1997	CS	1424	~16 wks.	28 (15-47)‡	EIA (IMx B12 system)/Serum	N/R		5
Pagan et al., 2002	USA 1986-1988	L	285	18 wks. 30 wks.	25.5 (18-38)	RBA/Non-fasting serum	N/R	(+)	
Guerra-Shinohara et al., 2004	Brazil	CS	119	Delivery (37-42wks.) Newborn	25.5 ± 6.7 (15-44)	IMx system & Immulite kits/ Serum	N/R		6
Garcia-Casal et al., 2005	Venezuela 2001- 2002	CS	1283	1st trimester 2nd trimester 3rd trimester	19-25 (n=571) 26^{+} (n=449)	RIA/Fasting serum	N/R		5
Ray et al., 2008	Canada Jan - July 2007	CS	10622	1st trimester	29.9 ± 7.1 (15-46)	N/R	N/R		5
Barbosa et al., 2008	Brazil Apr- May 2001	CS	275	Delivery (39 ± 1.2)	25.2 ± 6.5	Immulite Kit/Serum	No		6

Values represent are mean ± SD (range), ‡ median (range), MBA = microbiological assay, RIA = radioimmunoassay, RA = radioassay, EIA = enzyme immunoassay, RBA = Radiobinding assay, CS = cross-sectional study, L= longitudinal study, Q/A = quality assessment, quality score for L are: (-) = few criteria fulfilled, (+) some criteria fulfilled and (++) most criteria fulfilled, quality score for CS are ≥ 6 = high quality and < 6 = low quality, N/R = not reported.

Table 2.2: Shows the methodological overview of studies included in the review : Australian continent

References	Country or city/ time period	Study Design	Subjects	Period of blood collection	Maternal age (years)	Vitamin B12 measurement method/ blood sample	B12 supplement prior to blood sample	Q/A score	
								L	CS
Whiteside et al., 1968	Australia	L	60 pregnant	12 wks. 26 wks. 38 wks.	N/R	MBA/Serum	N/R	(-)	
Cole et al., 1974	South Australia	CS	147 pregnant	3rd trimester	23 (15 - 28)	MBA/Serum	N/R		3

Values represent are mean (range), MBA = microbiological assay, CS = cross-sectional study, L = longitudinal study, Q/A = quality assessment, quality score for L are: (-) = few criteria fulfilled, (+) some criteria fulfilled and (++) most criteria fulfilled, quality score for CS are ≥ 6 = high quality and < 6 = low quality, N/R = not reported.

Table 2.3: Shows the methodological overview of studies included in the review : European continent

References	Country or city/ time period	Study Design	Subjects	Period of blood collection	Maternal age (years)	Vitamin B12 measurement method/ blood sample	B12 supplement prior to blood sample	Q/A score L	Q/A score CS
Zachau-Christiansen et al., 1962	Denmark	CS	367 pregnant	Delivery Cord	N/R	MBA/Serum	N/R		6
Ball et al., 1964*	Stoke-on-Trent, UK. 1957- 1964	CS	320 pregnant	9 wks. to full term	N/R	MBA/Serum	N/R		3
Robert et al., 1973	England Feb - July 1971	L	322 immigrant pregnant	2nd trimester 3rd trimester	N/R	MBA/Serum	N/R	(+)	
Frey et al., 1992	France	CS	188 pregnant 154 newborn	Delivery (39.5 ± 1.3) Cord	29.2 ± 5 (19 - 43)	RA/ Plasma	N/R		7
Bruinse et al., 1995*	Netherland	L	116 pregnant	16 wks., 28 wks. 34 wks., delivery	N/R	CPB-assay with intrinsic factor /Fasting serum	No	(+)	
Bjorke-Monsen et al., 2001	Norway 1996 – 1997	CS	169 mothers 173 newborn	Delivery [39 - 41] ‡ Cord	29 [26-34] ‡	MBA/Serum	36% used multivitamin		7
Schulpis et al., 2004	Greek 1999 – 2002	CS	1933 pregnant; 1025 Greeks, 908 Albanian	Delivery Cord	Greeks 27.5± 3.4 Albanian 26.8 ± 2.5	CIA/ Serum	No		5
Milman et al., 2006	Denmark 1995 – 1997	L	406 pregnant	18 wks. 32 wks. 39 wks.	29.6 ± 4	EIA/Non-fasting plasma	34% used multivitamin prior to inclusion	(+)	
Murphy et al., 2007*	Spain	L	92 pregnant	8 wks., 20 wks. 32 wks., Delivery cord	18 – 35	MBA /Serum	27% used multivitamin	(+)	
Goedhart et al., 2011	Amsterdam 2003 – 2004	CS	4389 pregnant	2nd trimester (median 13 wks.)	N/R	CIA/Serum	N/R		6
Shield et al., 2011	Scotland 2008 – 2009	CS	190 pregnant	1st trimester (n = 113) 3rd trimester (n = 77)	29 (17 - 42) 29 (18 - 40)	N/R/Serum	N/R		5

Values represent are mean ± SD (range), ‡ median [25th – 75th], MBA = microbiological assay, RA = radioassay, EIA = enzyme immunoassay, CIA = Chemiluminescence immuneassay, CS = cross-sectional study, L= longitudinal study, Q/A = quality assessment, quality score for L are: (-) = few criteria fulfilled, (+) some criteria fulfilled and (++) most criteria fulfilled, quality score for CS are ≥ 6 = high quality and < 6 = low quality, N/R = not reported, *provided vitamin B12 insufficiency (%) in total during pregnancy.

Greek (Schulpis et al. 2004) were included in the review; 4 longitudinal and 7 cross-sectional studies (**Table 2.3**).The number of participants ranged from 92 to 4389 pregnant and the mean maternal age ranged from 26.8 to 29.6 years old. Only 3 studies reported the use of multivitamins at the time of blood collection (**Table 2.3**).

2.3.1.1.4. African continent

Seven studies from Nigeria (Osifo and Onifade 1976; Vanderjagt et al. 2007; Vanderjagt et al. 2009), Kenya (Brabin et al. 1986), South Africa (Colman et al. 1975), Tanzania (Hinderaker et al. 2002) and Ethiopia (Gibson et al. 2008) were included in the review; 1 longitudinal and 6 cross-sectional studies. The number of participants ranged from 50 to 2547 pregnant and the mean maternal age ranged from 17.7 to 27.8 years old. Only 1 study reported the use of multivitamins at the time of blood collection (**Table 2.4**).

2.3.1.1.5. Asian continent

Seven studies from Taiwan (Ho et al. 1987), South Korea (Park et al. 2004), China (Ma et al. 2004), Japan (Takimoto et al. 2007), Philippine (Marzan et al. 1971) and Thailand (Areekul et al. 1976; Srisupandit et al. 1983) were included in the review; 1 longitudinal and 6 cross-sectional studies. The number of participants ranged from 92 to 1163 pregnant and the mean maternal age ranged from 25 to 30.5 years old. Only 1 study reported the use of multivitamins at the time of blood collection (**Table 2.5**).

Table 2.4: Shows the methodological overview of studies included in the review : African continent

References	Country or city/ time period	Study Design	Subjects	Period of blood collection	Maternal age (years)	Vitamin B12 measurement method/ blood sample	B12 supplement prior to blood sample	Q/A score	
								L	CS
Colman et al., 1975	South Africa	CS	144 pregnant	3rd trimester	23.5 (16 - 45)	RID/Serum	N/R		5
Osifo et al., 1976	Nigeria	CS	50 mother-infant pairs	Delivery Cord	N/R	MBA/ Non-fastingserum	N/R		4
Barbin et al., 1986	Kenya Jan - Apr. 1981	L	291 pregnant; 119 primigravida 172 multigravida	1st trimester 2nd trimester 3rd trimester	17.7 ± 2.0 24 ± 5	RA/Serum	N/R	(+)	
Hinderaker et al., 2002	Tanzania 1995 - 1996	CS	2547 pregnant; 155 non-anemic	Median 5 months	Median 25	Magnetic separation assay/ Serum	N/R		6
Vanderjagat et al., 2007*	Nigeria Jun - Aug. 2003	CS	146 pregnant	2nd trimester (n = 59) 3rd trimester (n = 79)	26.5 (15 - 45)	CIA/ Non-fasting serum	Yes (33% used multivitamin)		6
Gibson et al., 2008	Ethiopia	CS	99 pregnant	3rd trimester	27.8 ± 4.6	RA/Non-fasting serum	N/R		6
Vanderjagat et al., 2009	Nigeria	CS	98 pregnant	33.7 ± 3.8 wks.	27.4 ± 5.4	CIA/ Non-fasting serum	No		5

Values represent are mean ± SD (range), MBA = microbiological assay, RA = radioassay, RID = Radioisotope dilution, CIA = Chemiluminescence immuneassay, CS = cross-sectional study, L= longitudinal study, Q/A = quality assessment, quality score for L are: (-) = few criteria fulfilled, (+) some criteria fulfilled and (++) most criteria fulfilled, quality score for CS are ≥ 6 = high quality and < 6 = low quality, N/R = not reported, *provided vitamin B12 insufficiency (%) in total during pregnancy.

Table 2.5: Shows the methodological overview of studies included in the review : Asian continent

References	Country/ time period	Study Design	Subjects	Period of blood collection	Maternal age (years)	Vitamin B12 measurement method/ blood sample	B12 supplement prior to blood sample	Q/A score	
								L	CS
Marzan et al., 1971	Philippine	CS	250 pregnant	2nd trimester 3rd trimester	25 26	RID/Serum	No		5
Areekul et al. ,1976*	Thailand	CS	216 pregnant; 169 non-anemic	1st trimester 2nd trimester 3rd trimester	16 - 42	RAD/ Non-fasting serum	N/R		6
Srisupandit et al., 1983*	Thailand	CS	568 pregnant	2nd trimester 3rd trimester	16 - 30	RID / Serum	N/R		6
Ho et al ., 1987	Taiwan	CS	221 Chinese pregnant; 198 non-anemic	Delivery 39.5 (32 - 43)	27.6 (18 - 42)	RA/Serum	No		6
Park et al., 2004	South Korea	CS	92 pregnant	24-28 wks.	30.5 ± 3.7 (18 - 39)	RIA/Fasting serum	Yes (39% used multivitamin)		5
Ma et al., 2004	China 1999 - 2000	CS	1163 pregnant; 512 non-anemic	3rd trimester	(20 - 35)	RIA/Serum	No		6
Takimoto et al., 2007	Japan 2001 - 2003	L	94 pregnant; only 39 pregnant provide blood sample at all 3 trimesters	7-14 wks. (n = 51) 26-29 wks. (n = 77) 34-36 wks. (n = 82)	29 ± 4.7	CIA/ Non-fasting serum	N/R	(++)	

Values represent are mean ± SD (range), RIA = radioimmunoassay, RA = radioassay, RID = Radioisotope dilution, RAD = Radioactive dilution, CIA = Chemiluminescence immunoassay, CS = cross-sectional study, L= longitudinal study, Q/A = quality assessment, quality score for L are: (-) = few criteria fulfilled, (+) some criteria fulfilled and (++) most criteria fulfilled, quality score for CS are ≥ 6 = high quality and < 6 = low quality, N/R = not reported, *provided vitamin B12 insufficiency (%) in total during pregnancy.

2.3.1.1.6. Middle Eastern countries

Six studies from Turkey (Ackurt et al. 1995; Koc et al. 2006; Ipciglu et al. 2007; Haliloglu et al. 2010), Sudan (Abdelrahim et al. 2009) and Egypt (Hussein et al. 2009) were included in the review; 2 longitudinal and 4 cross-sectional studies. The number of participants ranged from 68 to 279 pregnant and the mean maternal age ranged from 23.7 to 27 years old. Only 1 study reported the use of multivitamins at the time of blood collection (**Table 2.6**).

2.3.1.1.7. South Asian countries

Eleven studies from India (Yusufji et al. 1973; Pathak et al. 2007; Yajnik et al. 2008; Krishnaveni et al. 2009; Katre et al. 2010; Veena et al. 2010), Nepal (Bondevik et al. 2001; Jiang et al. 2005) and Bangladesh (Hall et al. 2007; Li et al. 2008; Lindstrom et al. 2011) were included in the review; 3 longitudinal and 8 cross-sectional studies. The number of participants ranged from 101 to 1165 pregnant and the mean maternal age ranged from 22.6 to 27 years old. Only 2 studies reported the use of multivitamins at the time of blood collection. (**Table 2.7**).

Table 2.6: Shows the methodological overview of studies included in the review : Middle Eastern countries

References	Country/ time period	Study Design	Subjects	Period of blood collection	Maternal age (years)	Vitamin B12 measurement method/ blood sample	B12 supplement prior to blood sample	Q/A score L	Q/A score CS
Ackurt et al., 1995	Turkey Feb - Dec 1991	L	130 pregnant	13-17 wks. (n = 130) 28-32wks. (n = 88)	23.7 ± 4 (16 - 35)	RA/ Non-fasting plasma	N/R	(+)	
Koc et al., 2006	Turkey Before 2004	CS	180 parturient	Delivery (38 - 42 wks.) Cord	27 ± 5.8	electrochemiluminiscence method/ Non-fasting serum	19% used multivitamins		6
Ipciglu et al., 2007*	Turkey	CS	186 pregnant	2nd trimester (n = 58) 3rd trimester (n = 98)	19 - 35	Microparticles enzyme immunoassay/ Fasting serum	N/R		5
Abdelrahim et al., 2009	Sudan 2007-2008	CS	279 pregnant; 55 non-anemic	Delivery	25.9 ± 6.4	Immunofluorescent assay/ Serum	N/R		6
Hussein et al., 2009	Egypt	CS	84 mother-newborn pairs	Delivery Cord	26 ± 0.5 (16 - 42)	Automated Chemiluminescent/ Serum	N/R		6
Haliloglu et al., 2010*	Turkey	L	68 pregnant	1st trimester (11 wks.) 2nd trimester (25 wks.) 3rd trimester (32 wks.)	23 - 31	Iluminescence method/ Fasting serum	No	(+)	

Values represent are mean ± SD (range), RA = radioassay, CS = cross-sectional study, L= longitudinal study, Q/A = quality assessment, quality score for L are: (-) = few criteria fulfilled, (+) some criteria fulfilled and (++) most criteria fulfilled, quality score for CS are ≥ 6 = high quality and < 6 = low quality, N/R = not reported, *provided vitamin B12 insufficiency (%) in total during pregnancy.

Table 2.7: Shows the methodological overview of studies included in the review : South Asian countries

References	Country/ time period	Study Design	Subjects	Period of blood collection	Maternal age (years)	Vitamin B12 measurement method/ blood sample	B12 supplement prior to blood sample	Q/A score	
								L	CS
Yusufji et al., 1973	India 1966-1968	CS	1000 pregnant	3rd trimester	25.3 (15 - 35)	MBA/ Serum	N/R		6
Bondevik et al., 2001	Nepal 1994-1995	CS	328 pregnant	18.5 wks.	22.6 (15 - 36)	MSA/ Non-fasting serum	No		7
Jiang et al., 2005	Nepal 1998-2001	CS	1165 pregnant	10.9 ± 4.6	23.6 ± 6.0 (15 - 45)	MBA/Serum	No		7
Pathak et al., 2007	India	CS	283 pregnant	≥ 28 wks	22.9 ± 3.3	MBA/Serum	N/R		6
Hall et al., 2007	Bangladesh 2004-2005	CS	101 pregnant	Delivery Cord	26 ± 5.3 (16.7 - 39)	RIA/Plasma	N/R		5
Yajnik et al., 2008	Indian 1994-1996	L	797 pregnant	18 ± 2 wks. 28 ± 2 wks.	21 [19, 23]‡	MBA/ Fasting plasma	N/R	(++)	
Li et al., 2008	Bangladesh Jan. - Dec. 2002	L	753 pregnant	8 wks. † 14 wks.	27 (14 - 44)	RA/Plasma	N/R	(+)	
Krishnaveni et al., 2009	India 1997-1998	CS	774 pregnant; 35 GDM	30 ± 2 wks.	N/R	MBA/Fasting serum	Yes (30%)		7
Veena et al., 2010	India 1997-1998	CS	830 pregnant	30 ± 2 wks.	23.9 ± 4.2	MBA/Fasting plasma	N/R		7
Katre et al., 2010	India 2004-2006	L	184 pregnant; 94 rural, 90 urban	Mean 17 (12 - 22) Mean 28 (24 - 30) † Mean 34 (32 - 36) †	22.8 ± 3.7	MBA/Fasting plasma	Yes (45%)	(++)	
Lindstrom et al., 2011	Bangladesh Jan. - Dec. 2002	CS	738 pregnant; 523 non-anemic	14.5 ± 1.8	26.6 ± 5.9	RIA/Plasma	N/R		7

Values represent are mean ± SD (range), ‡ median [25^{th} – 75^{th}], MBA = microbiological assay, RIA = radioimmunoassay, RA = radioassay, MSA = Magnatic separation assay, CS = cross-sectional study, L= longitudinal study, Q/A = quality assessment, quality score for L are: (-) = few criteria fulfilled, (+) some criteria fulfilled and (++) most criteria fulfilled, quality score for CS are ≥ 6 = high quality and < 6 = low quality, N/R = not reported, † B12 insufficiency (%) was not presented at this period.

2.3.1.2. Study quality assessment

The present review included 15 longitudinal and 42 cross-sectional studies. According to the type of study design, we used a suitable quality assessment checklist, as previously discussed in the Method, to identify the level of methodological quality. The last column of the methodological overview tables (**Tables 2.1** to **2.7**) present the total quality score for studies included in the review.

From the total longitudinal studies included in the review, 3 studies (Takimoto et al. 2007; Yajnik et al. 2008; Katre et al. 2010) met most of the quality assessment criteria, 10 studies met some and 2 studies (Lowenstein et al. 1960; Whiteside et al. 1968) met few of the assessment criteria. Details of the quality assessment of these studies are presented in **Tables 1** and **2** in **Appendix III**. Most of the studies reported a clear research question, defined target and study population, location, stated eligibility criteria, number of participants at the beginning of the study, issues of selection in to the study methods of data collection, type of analyses conducted, reported the absolute effect size and related their results back to the target population. While issues related to consenting (number of consenters and reasons for non-consenting, comparisons of consenters with non-consenters in terms of baseline demographic or clinical features) and analysis (loss of follow up, missing data and confounders accounted into the analysis) were poorly reported. Other items such as reliability and validity of measurement method were both reported only in (Lowenstein et al. 1960; Brabin et al. 1986; Murphy et al. 2007), while reliability alone was reported in (Knight et al. 1991; Katre et al. 2010) and validity was reported only in (Pagán et al. 2002; Takimoto et al. 2007). Furthermore, relative effect size was only reported in (Pagán et al. 2002; Takimoto et al. 2007; Katre et al. 2010). In terms of assessing the bias, no study reported the impact of biases

quantitatively; however, only 4 studies reported it qualitatively (Takimoto et al. 2007; Li et al. 2008; Yajnik et al. 2008; Katre et al. 2010). Finally, issues on the generalizability of the results was only discussed in (Ackurt et al. 1995; Milman et al. 2006; Yajnik et al. 2008).

From the total cross-sectional studies included in the review, 60% (n = 25) of the included studies scored positively on more than 75% on the quality items in the assessment checklist and were rated as a high quality studies. Fifteen studies scored between 44% and < 75% and only 2 studies scored 43%, though both were rated as low quality studies. Details of the quality assessment of these studies are presented in **Tables 1** to **7** in **Appendix IV**. Most of the studies clearly defined their objectives, issues related to the study population and outcome measures were also well identified, while adjusting the results for possible confounders in the analysis were varied and assessed in 14 studies only. Six studies (Ball and Giles 1964; House et al. 2000; Schulpis et al. 2004; Garcia-Casal et al. 2005; Hall et al. 2007; Ray et al. 2008) did not assess the outcome measures (serum vitamin B12 and folate) from new participants. In addition, the exposure assessment item was not applicable for all the included studies according to the aim of the present review.

2.3.1.3. Prevalence of vitamin B12 and folate insufficiency during pregnancy

Studies in the review used different cut-off values to identify vitamin B12 and folate insufficiency during pregnancy. About 50% of them used a cut-off value of < 148 or < 150 pmol/l for vitamin B12 insufficiency, while the cut-off value used for folate varied between the studies. Furthermore, gestational age at blood collection varied from follow up at each trimesters to delivery time. Thus, the prevalence of vitamin B12 and folate insufficiency in this review are presented

separately for each trimester and delivery. In addition, the studies included in each trimester were ordered into continent, then the studies included in each continent were ascendingly ordered according to the cut-off level used. The data in this review was presented only when the sample size per trimester was ≥ 50. The results from each trimester, delivery vs. cord and during pregnancy were given as follows.

2.3.1.3.1. Vitamin B12 and folate status at first trimester

About 7 studies reported the level of vitamin B12 and folate at the first trimester. The overall prevalence of vitamin B12 insufficiency ranged from 0 (Brabin et al. 1986) to 44.2% (Garcia-Casal et al. 2005). Africa, Australia, Europe, and South Asia showed a prevalence of 0, 5, 16 and 28.3% respectively. In addition, America showed a prevalence range between 5.2 to 44.2%. Furthermore, the overall prevalence of folate insufficiency ranged from about 11 (Jiang et al. 2005) to 39.5% (Garcia-Casal et al. 2005). South Asia, Australia and America showed a prevalence of 11, 26 and 39.5% respectively, while it was ranged between 25 to 28.6 in Africa (**Table 2.8**).

2.3.1.3.2. Vitamin B12 and folate status at second trimester

About 21 studies reported the level of vitamin B12 and folate at the second trimester. The overall prevalence of vitamin B12 insufficiency was ranged from 0.35% (Pagán et al. 2002) to 73% (Katre et al. 2010). Australia, and the Middle East showed a prevalence of 25 and about 49% respectively. In addition, America, Europe, Africa, Asia and South Asia showed a prevalence range between 0.35 to 58.8%, 15 to 35%, 6.1 to 14.4%, 1.5 to 46.1% and 42 to 73% respectively. The overall prevalence of folate insufficiency ranged from 0.2% (Yajnik et al. 2008) to

69% (Jacob et al. 1976). Note that Yajnik et al. used RBC folate to assess the folate level. The Middle East and Australia showed a prevalence of about 59.7 and 68% respectively, while it was ranged between 3.3 and 69% in America, 19.7 and 34% in Europe, 13.7 and 31.2% in Africa, 8 and 31% in Asia and 0.2 and 40% in South Asia. At the second trimester, the South Asian countries share a similar pattern of high serum vitamin B12 and low folate levels compared to other countries. In contrast, a study from Newfoundland and Kenya showed the opposite, low vitamin B12 insufficiency but high folate levels. Furthermore, Turkey showed high vitamin B12 and folate insufficiency (**Table 2.9**).

2.3.1.3.3. Vitamin B12 and folate status at third trimester

About 24 studies reported the level of vitamin B12 and folate at the third trimester. The overall prevalence of vitamin B12 insufficiency ranged from 0% (Brabin et al. 1986) to about 81% (Ackurt et al. 1995). The Middle East showed a prevalence of 80.9%, while Asia, Africa, Australia, America, Europe and South Asia showed a prevalence range between 0 to 16%, 0 to 23%, 12.3 to 20%, 2.1 to 69.2%, 42.6 to 60% and 42.5 to 74.1% respectively. In addition, the overall prevalence of folate insufficiency was ranged from 0.2% (Yajnik et al. 2008) to 73% (Yusufji et al. 1973). The Middle East showed a prevalence of 76.4%. In addition, the folate insufficiency was ranged between 2 to 43.8% in Africa, 10.1 to 35.5% in America, 16.9 to 38% in Asia and 0.2 to 73% in South Asia. Similar to the second trimester, the South Asian countries had high vitamin B12 insufficiency and low folate levels compared to other countries, except in Yusufji et al. study (Yusufji et al. 1973) where both vitamin B12 and folate insufficiency were high (**Table 2.10**).

Table 2.8: Shows the prevalence of vitamin B12 and folate insufficiency of the studies included in the review : at 1st trimester

Continent	Reference	Year	Country	No. of subject	Serum B12 cut-off level (pmol/l)	% of B12 insufficiency	Serum folate cut-off level (nmol/l)	% of folate insufficiency
Australia	Whiteside et al.	1968	Australia	60	< 74	5	< 11.3	26
America	Ray et al.	2008	Canada					
			≤ 28 days gest.	1244	< 125	5.2	N/R	N/R
			> 28 days gest.	2490		10.1		
	Garcia-Casal et al.	2005	Venezuela					
			19-25yrs	86	< 148	44.2	< 6.8	39.5
Europe	Shield et al.	2011	Scotland	113	< 156	16	N/R	N/R
Africa	Barbin et al.	1986	Kenya					
			Primipara	119	125 – 161	0	< 4.5	28.6
			Multipara	172		0		25.0
Asia	Takimoto et al.	2007	Japan	51	< 148	N/R	< 12	N/R
South Asia	Jiang et al.	2005	Nepal	B12 data (1158) Folate data (1164)	< 150	28.3	< 6.7	11.1

N/R = not reported, N/M = not measured in the study, gest. = gestation

Table 2.9: Shows the prevalence of vitamin B12 and folate insufficiency of the studies included in the review : at 2nd trimester

Continent	References	Year	Country	No. of subjects	Serum B12 cut-off level (pmol/l)	% of B12 insufficiency	Serum folate cut-off level (nmol/l)	% of folate insufficiency
Australia	Whiteside et al.	1968	Australia	60	< 74	25	< 11.3	68
America	Jacob et al.	1976	Los Angeles	B12 data (182)	< 74	0.5	< 6.8	18
				Folate data (300)	74 – 110	4	6.8 - 13.4	51
	House et al.	2000	Newfoundland	1424	< 130	25.3	< 6	1.9
					130- 160	18.3	6 - 7	1.4
	Knight et al.	1991	Washington DC	108	< 148	7	< 13.6	26
	Lowenstein et al.	1960	Canada	59	< 148	15.2	N/M	N/M
	Pagan et al.	2002	USA	285	< 148	0.35	< 11	5
	Garcia-Casal et al.	2005	Venezuela					
			19-25yrs.	243	<148	58.8	< 6.8	41.1
			26^{+}yrs.	187		58.3		29.4
Europe	Robert et al.	1973	England	320	< 118	35	< 13.6	34
	Milman et al.	2006	Denmark	406	< 150	15	N/R	N/R
	Goedhart et al.	2011	Amsterdam	B12 data (2921)	< 185	20.1	< 19.2	19.7
				Folate data (2622)				
Africa	Barbin et al.	1986	Kenya					
			Primipara	119	125 – 161	6.1	< 4.5	30
			Multipara	172		6.1		31.2
	Hinderaker et al.	2002	Tanzania	153	< 150	14.4	< 4.5	13.7
Middle East	Ackurt et al.	1995	Turkey	130	< 110.7	48.8	≤ 13.5	59.7
Asia	Marzan et al.	1971	Philippine	100	< 59	1.5	< 6.8	21
	Takimoto et al.	2007	Japan	77	< 148	8	< 12	31
	Park et al.	2004	South Korea	89	< 258	46.1	< 6.8	8
South Asia	Yajnik et al.	2008	India	638	< 150	60	< 283†	0.2
	Katre et al.	2010	India	163	< 150	73	< 7	1
	Bondevik et al.	2001	Nepal	328	< 150	49	≤ 4.5	4.5
	Lindstrom et al.	2011	Bangladesh	523	< 150	42	< 6.8	15
	Li et al.	2008	Bangladesh	753	<185	60	< 9	40

N/R = not reported, N/M = not measured in the study, † RBC folate

Table 2.10: Shows the prevalence of vitamin B12 and folate insufficiency of the studies included in the review : at 3rd trimester

Continent	Reference	Year	Country	No. of subjects	Serum B12 cut-off level (pmol/l)	% of B12 insufficiency	Serum folate cut-off level (nmol/l)	% of folate insufficiency
Australia	Whiteside et al.	1968	Australia	60	< 74	20	< 11.3	54
	Cole et al.	1974	South Australia	B12 data (130) Folate data (136)	< 148	12.3	< 6.8	26.5
America	Cook et al.	1971	Latin America	899	< 59	15.4	< 6.8	10.1
	Knight et al.	1991	Washington DC	117	< 148	14	< 13.6	17
	Lowenstein et al.	1960	Canada	252	< 148	19	N/M	N/M
	Pagan et al.	2002	USA	285	< 148	2.1	< 11	13
	Garcia-Casal, et al.	2005	Venezuela					
			19-25yrs	234	< 148	69.2	< 6.8	35.5
			26^{+}yrs	204		68.1		30.9
Europe	Robert et al.	1973	England	119	< 118	48	< 13.6	N/R
	Milman et al.	2006	Denmark	406	< 150	42.6	N/R	N/R
	Shield et al.	2011	Scotland	77	< 156	60	N/R	N/R
Africa	Barbin et al.	1986	Kenya					
			Primipara	119	125 – 161	6.9	< 4.5	24.1
			Multipara	172		0		31.2
	Vanderjagat et al.	2009	Nigeria	98	< 148	12.2	< 7.7	3.06
	Gibson et al.	2008	Ethiopia	B12 data (83) Folate data (94)	< 150	23	< 6.8	2
	Colman et al.	1975	South Africa	B12 data (106) Folate data (144)	< 295	0.9	< 263†	43.8
Middle East	Ackurt et al.	1995	Turkey	88	< 110.7	80.9	≤ 13.5	76.4
Asia	Marzan et al.	1971	Philippine	57	< 59	0	< 6.8	37.5
	Takimoto et al.	2007	Japan	82	< 148	16	< 12	38
	Ma et al.	2004	China	B12 data (512) Folate data (573)	< 148	8.4	< 6.8	16.9
South Asia	Yusufji et al.	1973	India	492	< 103	52	< 13.6	73
	Pathak et al.	2007	India	266	< 148	74.1	< 6.8	26.3
	Yajnik et al.	2008	India	594	< 150	71	< 283†	0.2
	Veena et al.	2010	India	536	< 150	42.5	< 7	4.1
	Krishnaveni et al.	2009	India	780	< 150	43	< 7	4.0
	Christain et al.	2006	Nepal	173	< 150	69.9	< 6.8	22.5

N/R = not reported, N/M = not measured in the study, † RBC folate

2.3.1.3.4. **Vitamin B12 and folate status at delivery**

About 15 studies reported the level of vitamin B12 and folate at delivery time; 8 out of the 15 studies reported vitamin B12 insufficency in both the maternal and cord levels. The overall prevalence of maternal vitamin B12 insufficiency ranged from 1.5% (Ho et al. 1987) to 88% (Schulpis et al. 2004). Asia showed a prevalence of 1.5%, while Africa and South Asian showed a prevalence of 40% and about 59% respectively. In addition, vitamin B12 insufficiency was ranged between 1.8 and 72% in the Middle East, 15 and 88% in Europe and 23 and 75% in America. The overall prevalence of maternal folate insufficiency ranged from 0% (Giugliani et al. 1984) to 66.2% (Zachau-Christiansen et al. 1962). Asia showed a prevalence of 1.5%, while it ranged between 0 and 36% in America, 2.6 and 66.2% in Europe, and 12 and 47.2% in the Middle East. Moreover, the cord vitamin B12 and folate insufficiency were generally lower compared to the maternal level. (**Table 2.11**).

2.3.1.4. Trends of vitamin B12 and folate insufficiency throughout pregnancy

Thirteen studies reported the level of vitamin B12 and folate at 2 or 3 trimesters during pregnancy. The prevalence of vitamin B12 and folate insufficiency at the 3 trimesters were only available in 3 studies. The trend of vitamin B12 and folate insufficiency per trimester varied between different countries as shown in **Table 2.12**.

About 12 studies provided evidence of the prevalence of vitamin B12 and folate insufficiency generally during pregnancy. The prevalence of vitamin B12 insufficiency showed a higher range (0 to 61.3%) compared to folate insufficiency (0 to 36.3%) during pregnancy (**Table 2.13**).

Table 2.11: Shows the prevalence of vitamin B12 and folate insufficiency of the studies included in the review : at delivery

Continent	References	Year	Country	No. of subjects	Serum B12 cut-off level (pmol/l)	% of B12 insufficiency		Serum Folate cut-off level (nmol/l)	% of folate insufficiency	
						Mother	Cord		Mother	Cord
America	Baker et al.	1975	New Jersey	174	< 59	23	N/R	< 9	36	N/R
	Guerra-Shinohara et al.	2004	Brazil	119	Mother < 132 Placenta* < 194	52.9	49.5*	N/R	N/R	N/R
	Knight et al.	1991	Washington DC	Mother/cord B12 data (101/99) Folate data (91/84)	< 148	8	2	< 13.6	18	4
	Giugliani et al.	1984	Brazil	51	< 165	21.6	N/M	< 3	0	N/M
	Barbosa et al.	2008	Brazil	275	< 179	75	N/M	N/R	N/R	N/M
Europe	Zachau-Christiansen et al.	1962	Denmark	B12 data (365) Folate data (349)	< 147	41.4	12.8	< 6.5	66.2	N/R
	Frey et al	1992	France	188	< 148	27.6	N/R	< 5.6	N/R	N/R
	Bjorke-Monsen et al.	2001	Norway	Mother /cord (169/173)	< 150	15	8	N/R	N/R	N/R
	Schulpis et al.	2004	Athens							
			Greek	1025	< 170	21.4	7.3	< 10.2	2.6	0
			Albanian	908		88	72		2.8	1.8
Africa	Osifo et al.	1976	Nigeria	50	< 148	40	20	N/M	N/M	N/M
Middle East	Abdelrahim et al.	2009	Sudan	55	< 110.7	1.8	N/M	< 15	47.2	N/M
	Koc et al.	2006	Turkey	180	< 118	72	41	< 9.0	12	0
	Hussein et al.	2009	Egypt	84	< 150	46.4	N/R	N/M	N/M	N/M
Asia	Ho et al.	1987	Taiwan	198	< 125	1.5	N/M	< 5.5	1.5	N/M
South Asia	Hall et al.	2007	Bangladesh	B12 data (95) Folate data (99)	< 185	58.9	N/R	< 6.8 < 9	1 10.1	N/R N/R

N/R = not reported, N/M = not measured

Table 2.12: Shows the trend of vitamin B12 and folate insufficiency of the studies included in the review : throughout pregnancy

References	Year	Country	No. of subjects	Serum B12 cut-off level (pmol/l)	Prevalence of B12 insufficiency (%) per trimester			Serum folate cut-off level (nmol/l)	Prevalence of folate insufficiency (%) per trimester		
					1st	2nd	3rd		1st	2nd	3rd
Marzan et al.	1971	Philippine	250	< 60	s	1.5	0	< 6.8	s	21	37.5
Whiteside et al.	1968	Australia	60	< 74	5	25	20	< 11.3	26	68	54
Ackurt et al.	1995	Turkey	130	< 110.7	N/M	48.8	80.9	≤ 13.5	N/M	59	76.4
Robert et al.	1973	England	322	< 118	N/M	35	48	< 13.6	N/M	34	N/R
Barbin et al.	1986	Kenya									
		Primipara	119	125 - 161	0	6.1	6.9	< 4.5	0	1.5	0
		Multipara	172		0	6.1	0		0	3.7	15.6
Knight et al.	1991	Washington DC	600	< 148	s	7	14	< 13.6	s	26	17
Lowenstein et al.	1960	Canada	345	< 148	s	15.2	19	N/M	s	N/M	N/M
Pagan et al.	2002	USA	285	< 148	N/M	0.35	2.1	< 11	N/M	5	13
Garcia-Casal et al.	2005	Venezuela									
		19-25yrs.	571	< 148	44.2	58.8	69.2	< 6.8	39.5	41.1	35.5
		26+ yrs.	449		41.9	58.3	68.1		44.2	29.4	30.9
Takimoto et al.	2007	Japan	94	< 148	N/R	8	16	< 12	N/R	31	38
Milman et al.	2006	Denmark	406	< 150	N/M	15	42.6	N/R	N/M	N/R	N/R
Yajnik et al.	2008	India	797	< 150	N/M	60	71	< 283†	N/M	0.2	0.2
Shield et al.	2011	Scotland	190	< 156	28.3	N/M	60	N/R	N/R	N/M	N/R

Values present as % (n), N/R = not reported, N/M = not measured. s = small sample size, †RBC folate.

Table 2.13 shows the vitamin B12 and folate insufficiency of the studies included in the review : generally during pregnancy

Reference	Year	Country	No. of subjects	Serum B12 cut-off level (pmol/l)	% of B12 insufficiency in pregnancy	Serum folate cut-off level (nmol/l)	% of folate insufficiency in pregnancy
Marzan et al.	1971	Philippine	187	< 59	0.8	< 6.8	24.7
Black et al.	1994	Mexico	85	< 74	15	< 6.1	0
Haliloglu et al.	2010	Turkey	68	< 99	0	< 6.8	0
Srisupandit et al.	1983	Thailand	568	< 110	0	< 6.8	15.5
Areekul et al.	1976	Thailand	B12 data (150) Folate data (164)	< 110	7.3	< 6.8	25.6
Ipciglu et al.	2007	Turkey	186	< 133	53	N/R	N/R
Ball et al.	1964	UK	320	< 148	33	N/R	N/R
Garcia-Casal et al.	2005	Venezuela	1283	< 148	61.3	< 6.8	36.3
Vanderjagat et al	2007	Nigeria	146	< 148	9	< 7.7	8.9
Murphy et al.	2007	Spain	92	< 150	0	N/M	N/M
Shield et al.	2011	Scotland	190	< 156	33.7	N/R	N/R
Bruinse et al.	1995	Netherland	70	< 180	0	N/R	N/R

Values present as % (n), N/R = not reported, N/M = not measured

2.3.2. Discussion

This is the first systematic review investigating the worldwide prevalence of vitamin B12 insufficiency during pregnancy. Generally, the results supported our hypothesis that vitamin B12 insufficiency during pregnancy is not rare, even in a non-vegetarian populations. According to the assessment checklist criteria used in the present review, about half of the included studies were rated as high methodological quality studies; therefore it is unlikely that our conclusion is affected by bias from the included studies. The results of vitamin B12 insufficiency from generally known non-vegetarian population were varied: from 0% in Kenya at the first trimester (Brabin et al. 1986) to about 44% at the first trimester from Venezuela (Garcia-Casal et al. 2005), 60% at the second trimester from Bangladesh (Li et al. 2008), 81% at the third trimester from Turkey (Ackurt et al. 1995) and 88% at delivery time from the Albanian population (Schulpis et al. 2004). In addition, using the cut-off value of around < 148 pmol/l showed that the average prevalence of vitamin B12 insufficiency at the second trimester was 8%, 14%, 15%, and 28% in Asia, Africa, Europe and America respectively (with a total average worldwide prevalence of 16% at the second trimester), while at the third trimester, it was 12%, 18%, 34% and 43% in Australia and Asia, Africa, America and Europe respectively (with a total average worldwide prevalence of 24% at the third trimester). The Middle East showed a prevalence of 46% at delivery time. Generally, the prevalence of vitamin B12 insufficiency was 9% in Nigeria (Vanderjagt et al. 2007), 33% in England (Ball and Giles 1964) and Scotland (Shields et al. 2011) and 61% in Venezuela (Garcia-Casal et al. 2005) during pregnancy. Similarly, the folate insufficiency was observed among the studies included in the review, although, the cut-off value used to define the folate insufficiency was much varied compared to the vitamin B12.

Similar to an earlier report, the prevalence of vitamin B12 insufficiency (Stabler and Allen 2004) was low on the Asian continent and rated as least prevalent of vitamin B12 insufficiency, except in the Park et al. study from South Korea where it showed a high prevalence owing to the high cut-off level (< 258 pmol/l) used to define vitamin B12 insufficiency (Park et al. 2004). This could be related to the greater use of all forms of seafood and invertebrate and reptile animal foods as well as soy products, algae and fish sauces compared to other countries (Stabler and Allen 2004). Our review was comprehensive, including all the publications reporting vitamin B12 levels during pregnancy including Northern American (US, Canada) and European. As expected, the high prevalence of vitamin B12 insufficiency was observed in studies from known vegetarian populations such as India and Nepal at both the second and third trimester, mostly with high quality on our assessment criteria (see **Table 2.7**), with an average prevalence of about 60%. Furthermore, the combination of low vitamin B12 and high folate levels were also observed in these studies, except in Yusufji et al. study where it showed high a prevalence of folate insufficiency (73%) (Yusufji et al. 1973). This is could be due to the high cut-off value used to define the folate insufficiency (< 13.6 nmol/l), as well as because this study was published a long time ago before the recommendation of the preconception folic acid supplement started. Though such a combination is likely to be common mainly in a vegetarian population such as India, the prevalence of low vitamin B12 and high folate levels in this review were also observed in non-vegetarian population, particularly from American continent such as the studies from Newfoundland (43.6% vs. 3.3%) at the second trimester (House et al. 2000), from Mexico (15% vs. 0%) at about the third trimester (Black et al. 1994) and from Venezuela (61.3% vs. 36.3 %) throughout pregnancy (Garcia-Casal et al. 2005). In a subsample of the latter study from Mexico, a higher cut-off level of vitamin B12

insufficiency (< 149 pmol/l) was studied and a prevalence of 62% vs. 0% was observed (Allen et al. 1995). This could be related to the mandatory folic acid food fortification and/or the preconception folic acid supplementation in these countries. Even in countries such as the UK, where only voluntary folic acid fortification of cereals exists, the combination of low vitamin B12 and high folate levels was also observed in the general adult population 10.5% vs. 2.1% (Saravanan et al. 2010a) as well as in pregnant women 20% vs. 6% (Saravanan et al. 2010b). Therefore, the presence of maternal low vitamin B12 and adequate folate levels is becoming the main concern (Glew et al. 2006). As previously discussed, folic acid insufficiency is increasingly rare, while the prevalence of vitamin B12 insufficiency has increased especially after the introduction of mandatory folic acid fortification. In addition, the NTDs attributable to vitamin B12 insufficiency have tripled (Ray and Blom 2003; Ray et al. 2007). Furthermore, excess folic acid intake in the presence of maternal low vitamin B12 could contribute to the increased risk of chronic disease such DM and CVD in the offspring (Rosenberg 2008; Yajnik et al. 2008). The potential mechanism of the action of such imbalance was previously discussed in the Introduction (**Chapter 1**). Thus to understand the pattern of vitamin B12 status during pregnancy before and after the initiation of folic acid fortification, an average prevalence of low vitamin B12 from the included studies in the review, as well as other studies from Malaysia (0%), Venezuela (0%), Israel (22%), New Jersey (10%) and England (20%) (Diez-Ewald and Molina 1972; Ali et al. 1982; Pardo et al. 2000; Baker et al. 2002; Saravanan et al. 2010b) respectively, were plotted on a world map to demonstrate the presence of maternal vitamin B12 insufficiency, as well as to note the change in vitamin B12 level before and after the year 1997 (**Figure 2.2**). The trend in wheat flour fortification with folic acid worldwide from 2004 to 2007 was given in **Appendix II**). From the present review, vitamin B12 insufficiency before

and after the year of fortification ranged from 0 to 23% vs. 10 to 64% in America, 0 to 41.4% vs. 15 to 88% in Europe, 0.9 to 40% vs. 12.2 to 23% in Africa, 49% vs. 1.8 to 72% in the Middle East, 0 to 7% vs. 8 to 46% in Asia, and 52% vs. 49 to 60% in South Asia.

In conclusion**,** this is the first systematic review that investigates the worldwide prevalence of vitamin B12 insufficiency during pregnancy. Much heterogeneity in the study's methodology and quality was observed. Mixed results were identified owing to the different cut-off levels used to define vitamin B12 and folate insufficiency. Despite the fact that the prevalence of vitamin B12 insufficiency varied between different countries, the overall results showed that vitamin B12 insufficiency is not rare during pregnancy, particularly in non-vegetarian populations. Moreover, the trend in vitamin B12 insufficiency change before and after folic acid fortification was difficult to identify owing to the limited number of studies included. However the trend of change in America can be observed.

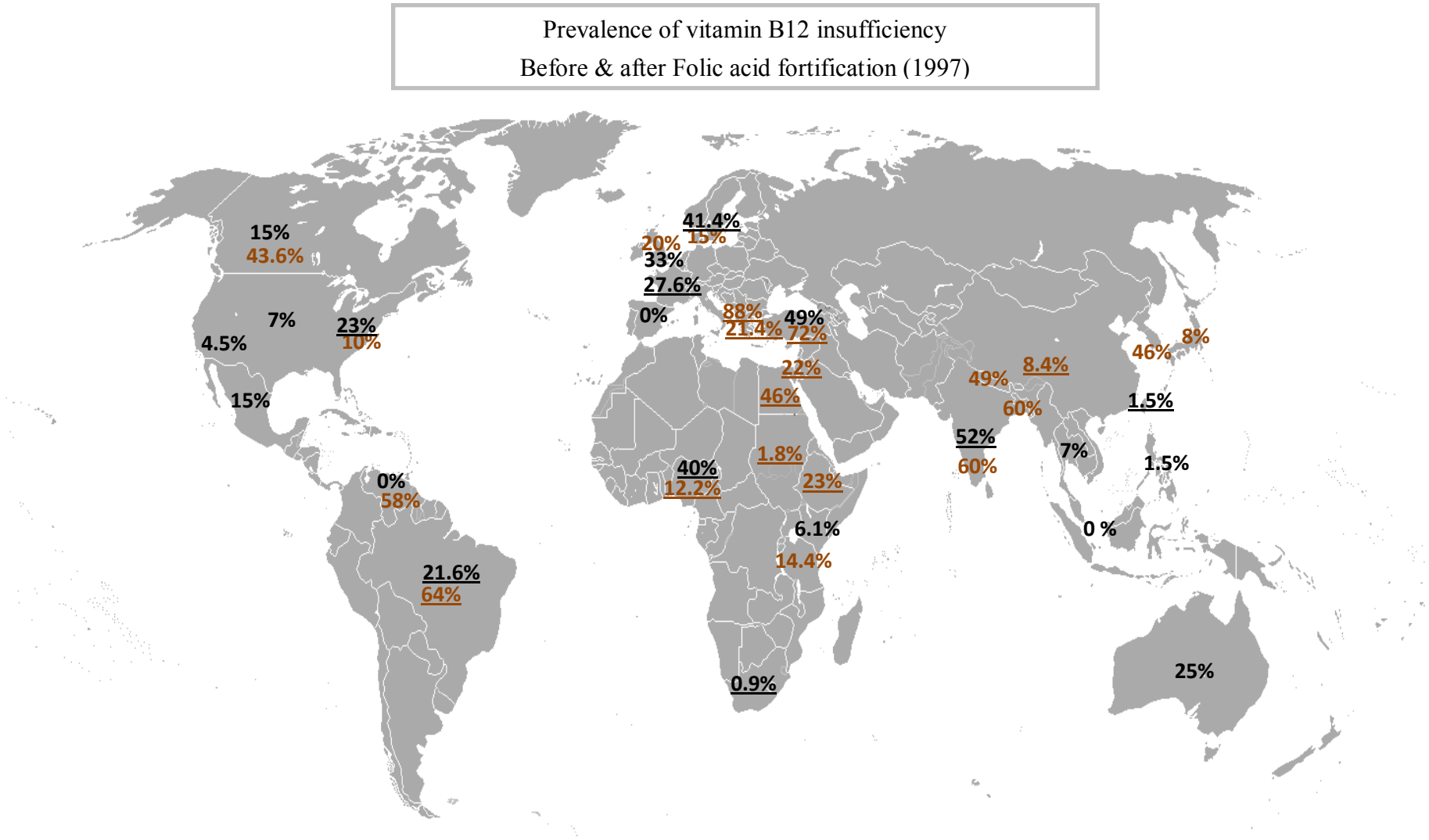


Figure 2.2: Shows the prevalence of vitamin B12 insufficiency before & after folic acid fortification in 1997. Studies before 1997 **%** in **black colour**, after 1997 **%** in **brown colour** at 1st or 2nd trimester, those with underline at third trimester or delivery

2.4 Systematic review (B)

"Does maternal vitamin B12 level independently predict low birth weight?"

2.4.1. Results

2.4.1.1. Study characteristics

A total of 189 articles were initially retrieved, along with several other cited references. Then, 19 full text articles were reviewed for more a detailed evaluation. Five articles were excluded because no mean levels of vitamin B12, folate, and BW (Bondevik et al. 2001), and gestational age (Martin et al. 1967) were reported and no statistical analysis (e.g. correlation, regression) were done between maternal vitamin B12 levels and BW (Glorimar et al. 2004; Milman et al. 2007; Wallace et al. 2008). Only 14 articles met the inclusion criteria (**Figure 2.3**). Details of the study populations and methods are presented in **Table 2.14**. The study population were from Australia (Whiteside et al. 1968), USA (Knight et al. 1991; Tamura et al. 1994), UK (Mathews et al. 2004; Relton et al. 2005), the Netherlands (Hogeveen et al. 2010), Norway (Hay et al. 2010), Turkey (Ackurt et al. 1995), South Africa (Mamabolo et al. 2006), Japan (Takimoto et al. 2007; Takimoto et al. 2011), and India (Muthayya et al. 2006b; Yajnik et al. 2008; Veena et al. 2010). The type of study design was not stated in (Whiteside et al. 1968; Tamura et al. 1994; Relton et al. 2005; Takimoto et al. 2007; Yajnik et al. 2008), so we assigned their design accordingly depending on the method used for data collection in the study. Studies (Whiteside et al. 1968; Relton et al. 2005; Takimoto et al. 2007; Yajnik et al. 2008) followed the participants at different times during pregnancy, so we assigned a longitudinal type of study design accordingly.

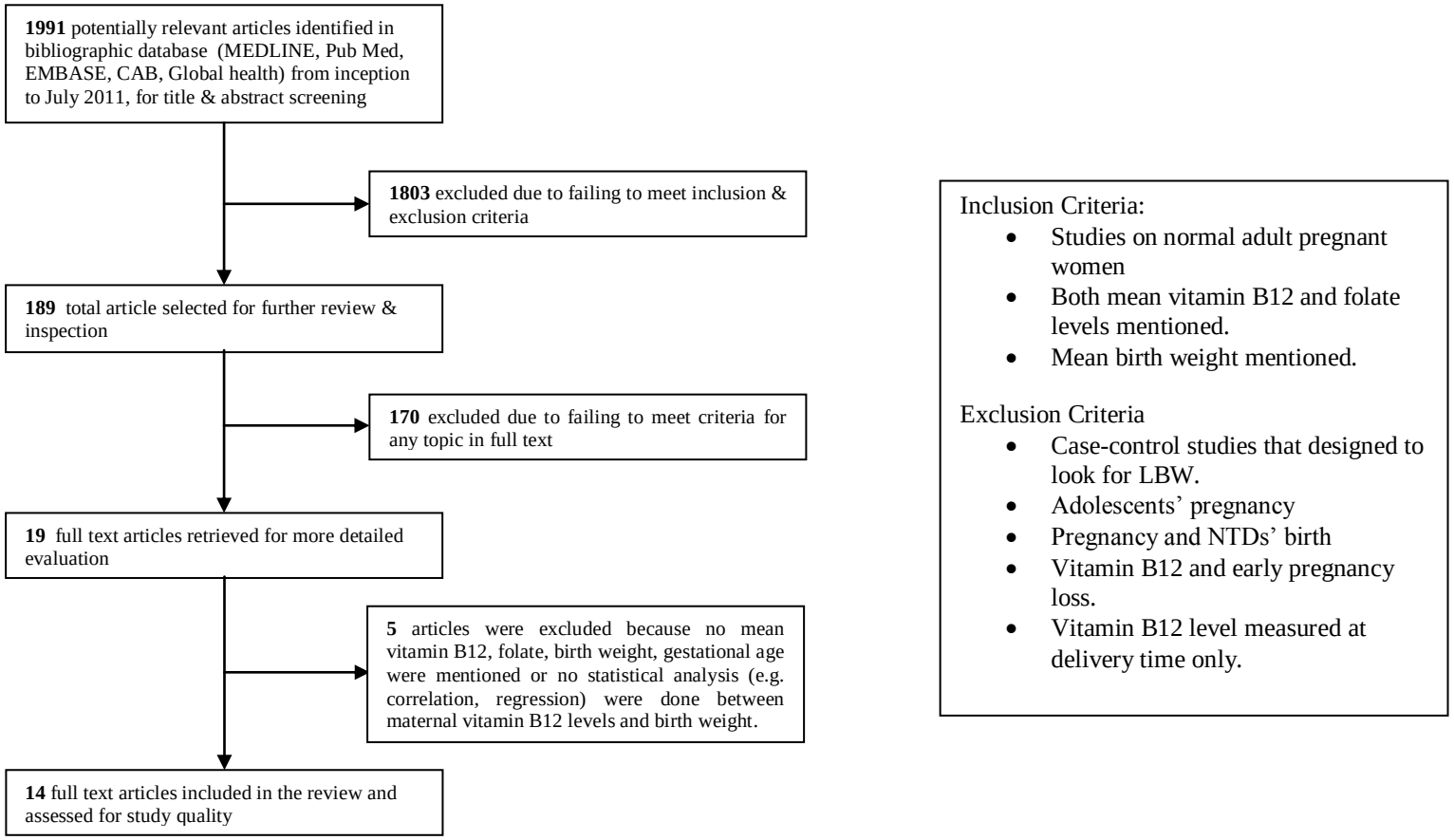


Figure 2.3: Flow diagram of studies included in the systematic review (B). All initial data searches and screening of titles and abstracts of identified studies were independently done for eligibility according to the pre-specified inclusion and exclusion criteria by 2 reviewers – agreement was (88.6%).

In contrary, in (Tamura et al. 1994) the participants were planned to be seen once during the study period, so we assigned a cross-sectional type of study accordingly. Furthermore, most investigations used a prospective observational study design (longitudinal study) where study subjects were followed at different times during pregnancy. Four studies followed the participants at three different points (first, second, and third trimesters) during pregnancy (Whiteside et al. 1968; Muthayya et al. 2006b; Takimoto et al. 2007) as well as delivery (Knight et al. 1991). Another 3 studies followed the participants at 2 different points (second and third trimesters) during pregnancy (Ackurt et al. 1995; Mathews et al. 2004; Yajnik et al. 2008). One study measured the vitamin B12 level at the first trimester (Relton et al. 2005), 2 studies at the second trimester (Tamura et al. 1994; Hay et al. 2010) and 4 studies at the third trimester (Mamabolo et al. 2006; Hogeveen et al. 2010; Veena et al. 2010; Takimoto et al. 2011). The mean maternal age at enrolment ranged from 23.4 to 34.1 years (Tamura et al. 1994; Ackurt et al. 1995) respectively. Only 2 studies used primipara subjects (100%) (Knight et al. 1991; Mathews et al. 2004). The mean gestational age at delivery ranged from 36.79 to 40.2 weeks (Knight et al. 1991; Mathews et al. 2004).

Most studies determined the vitamin B12 level from blood samples using widely available methods such as microbiological assay (Whiteside et al. 1968; Yajnik et al. 2008; Hay et al. 2010; Hogeveen et al. 2010; Veena et al. 2010) or radioassay methods (Knight et al. 1991; Tamura et al. 1994; Ackurt et al. 1995) or enzyme immunoassay (Mathews et al. 2004; Relton et al. 2005) or chemiluminescence assay (Takimoto et al. 2007; Takimoto et al. 2011). In addition, only 2 studies reported clearly about vitamin B12 supplementation prior to blood sampling (Hay et al. 2010; Hogeveen et al. 2010). Some of the studies used iron

(Whiteside et al. 1968) and folic acid (Muthayya et al. 2006b; Yajnik et al. 2008) as routine practice, while the rest had mixed reported supplementation.

2.4.1.2. Study quality assessment

The NICE checklist criteria was used as discussed in the Method in all included studies to obtain comparable levels of methodological quality. The included studies differed substantially in the methodological quality (see **Appendix V**). Six studies (Mathews et al. 2004; Muthayya et al. 2006b; Takimoto et al. 2007; Yajnik et al. 2008; Hogeveen et al. 2010; Veena et al. 2010) met most of the quality assessment criteria, another 6 studies met some (Knight et al. 1991; Ackurt et al. 1995; Relton et al. 2005; Mamabolo et al. 2006; Hay et al. 2010; Takimoto et al. 2011), while only 2 studies met few of the assessment criteria (Whiteside et al. 1968; Tamura et al. 1994). Most of the studies reported a clear research question, defined target and study population, location, stated eligibility criteria, number of participants at the beginning of the study, issues of selection in to the study methods of data collection, type of analyses conducted, reported the absolute effect size and related their results back to the target population. While issues in consenting (number of consenters and reasons for non-consenting, comparisons of consenters with non-consenters in terms of baseline demographic or clinical features), reliability and validity of measurement method and loss to follow up and missing data account in to the analysis were poorly reported. The participant was justified only in one study (Mathews et al. 2004), while the relative effect size was only reported in the (Muthayya et al. 2006b) study. In addition, issues on the generalizability of the results was only discussed in (Ackurt et al. 1995; Yajnik et al. 2008) study.

Table 2.14: Shows the methodological overview of studies included in the review

References	Country/ time period	Study design	Subjects	Age (years) Parity GA at delivery	GA at blood collection (n)	Vitamin B12 measurement method/ blood sample	Use Supplement prior to blood sample		
							B12	Folic acid	Iron & other
Whiteside et al., 1968	Australia	L	60 pregnant	N/A	12 wks. 26 wks. 38wks.	MBA / Serum	N/A	N/A	Yes (as routine practice; ferrous sulphate, 200 mg, 1-3 times per day)
Knight et al., 1991	Washington, DC, USA 1985 - 1990	L	600 African American pregnant	(16 - 35) PP 100% Group I (> 2500g) 40.25 ± 0.08, Group II (< 2500) 36.79 ± 0.45	1 - 13 wks.(22) 14 - 27 wks. (109) > 27 wks. (117) Delivery (101)	RIA / Fasting serum	N/A	N/A	90% received iron & vitamin supplement
Tamura et al., 1994	Birmingham, USA	CS	76 pregnant (60 whites, 16 black)	N/A	17.1 ± 1.3 wks. (15 - 22 wks.)	RA / Plasma	N/A	83% were taking prenatal suppl., providing 800-1000µg folic acid/day	N/A
Ackurt et al., 1995	Turkey Feb - Dec 1991	L	130 pregnant	N/A PP 48% N/A	13 - 17 wks. (130) 28 - 32 wks. (88)	RA / Non-fasting plasma	N/A	N/A	35.4% were regularly taking iron and/or multivitamins, 6.1% took suppl. on a sporadic basis
Mathews et al., 2004	Portsmouth, UK 1994 - 1996	L	798 pregnant (n = 661, 716)*	25.4 ± 4.9 PP 100% M 40.0 ± 1.4 F 40.2 ± 1.4	~ 16 wks. ~ 28 wks.**	EIA / Serum	N/A	N/A	N/A
Relton et al., 2005	Cumbria, UK 2000 - 2002	L	998 pregnant; only 315 mother-child pairs (n = 504, 683)*	27.8 ± 5.83 PP 43% N/A	11.5 ± 5.8 wks.	EIA / Non-fasting serum	N/A	N/A	N/A
Muthayya et al., 2006b	Bangalore, India 2001 - 2003	L	377 pregnant (n = 136, 139)*	24.6 ± 4.1 PP 58.9% N/A	12.9 ± 3.3 wks. 24.1 ± 2.0 wks. 33.9 ± 1.2 wks. Delivery	CIA / Fasting serum	N/A	Yes (as standard care, 500µg folic acid)	Yes (as standard care, 60 mg iron)

Data are presented as mean ± SD (range), * number of the available sample for serum vitamin B12 & folic acid data (n= B12, folic acid), ** No data available on vitamin B12 at this time, GA = gestational age, N/A= Not Available, PP = primipara, M = male, F = female, MBA = microbiological assay, RIA = radioimmunoassay, RA = Radioassay, EIA = Enzyme immunoassay, CIA = Chemiluminescence assay.

Cont. Table 2.14

References	Country/ time period	Study design	Subjects	Age (years) Parity GA at delivery	GA at blood collection (n)	Vitamin B12 measurement method/ blood sample	Use Supplement prior to blood sample		
							B12	Folic acid	Iron & other
Mamabolo et al., 2006	South Africa 1999 - 2000	L	219 pregnant 8.8% GDM (1.5% GDM, 7.3% IGT)	25.4 ± 7.0 1.53 ± 1.86 N/A	28 - 36 wks.	Immunoassay / Fasting serum	N/A	N/A	N/A
Takimoto et al., 2007	Tokyo, Japan 2001 - 2003	L	94 pregnant, only 39 pregnant provide blood sample at all 3 trimesters	29 ± 4.7 PP 77% 39.6 ± 1.0	7 - 14 wks. (51) 26 - 29 wks. (77) 34 - 36 wks. (82)	CIA / Non-fasting serum	N/A	Only 2 pregnant took folic acid at 1st trimester & 4 pregnant at 2nd trimester	N/A
Yajnik et al., 2008	Pune, Indian 1994 - 1996	L	700 pregnant 653 children (n = 638, 618)*	Median 21 [19,23] PP 31.6% N/A	18 ± 2 wks. (638) 28 ± 2 wks. (594)	MBA / Fasting plasma	N/A	Yes (as a standard care, 500µg folic acid) started from 18 wks. Gestation	Yes (as a standard care, 60 mg iron) started from 18 wks. Gestation
Hogeveen et al., 2010	Netherlands 2002 - 2004	L	366 pregnant and newborn	33.3 (30.7 - 35.5) PP 48% 39.7 (38.4 - 40.7)	30 - 34 wks.	MBA / Non-fasting plasma	No	Yes (57%)	N/A
Hay et al., 2010	Oslo, Norway Apr - Jun 1997	L	149 pregnant 361 newborn	29.9 ± 4.4 PP 49.5% 40.1 ± 1.2	17 - 19 wks.	MBA / Plasma	Yes	Yes	N/A
Veena et al., 2010	Mysore, India 1997 - 1998	L	674 pregnant 536 children	23.9 ± 4.2 PP 50.8% 39.3 ± 1.6	30 ± 2 wks.	MBA / Fasting plasma	N/A	N/A	29% women reported taking multi-vitamin at recruitment phase.
Takimoto et al., 2011	Tokyo, Japan 2007 - 2008	L	42 pregnant;14 folic acid users vs. 19 non-folic acid users	33.1 ± 5 vs. 32.7 ± 4.1 PP 87.9% N/A	3rd trimester	CIA / Non-fasting serum	N/A	Yes (14 women; 8 at 1st trimester and 9 at 2nd trimester)	N/A

Data are presented as mean ± SD or median [interquartile range], * number of the available sample for serum vitamin B12 & folic acid data (n= B12, folic acid), GA = gestational age, N/A = Not Available, PP = primipara, MBA = microbiological assay, CIA = Chemiluminescence assay, IGT= Impaired Glucose Tolerance

2.4.1.3. Association between maternal vitamin B12 and BW

The results of the association between maternal vitamin B12 and BW from the studies included in the review are presented in **Table 2.15**. Only 3 studies showed a significant correlation at the second trimester between vitamin B12 and BW. In one study (Whiteside et al. 1968), vitamin B12 showed a positive correlation with BW after computing regression equation using iron, vitamin B12 and folate as independent variables and BW as dependent variable (p-value not reported). In the other study (Muthayya et al. 2006b), a low maternal vitamin B12 concentration through pregnancy was independently associated with the increased risk of IUGR after controlling for maternal age, parity, maternal education level, and body weight in early pregnancy, and after additionally controlling for weight gain in the second trimester. The higher the association was found at second trimester ($p < 0.001$). In contrast, Hay et al. found that birth weight was negatively associated with cord vitamin B12 ($p = 0.02$). In addition, a positive association between maternal holoTC and cord B12 ($p < 0.0001$) was found. No statistical correlation were made directly between maternal vitamin B12 and BW in this study (Hay et al. 2010).

2.4.1.4. Association between maternal folate and BW

The results of the association between maternal folate and BW from the studies included in the review are presented in **Table 2.16**. Five studies showed a significant positive correlation between folate and BW; at the first trimester ($p < 0.001$) (Relton et al. 2005), at the second trimester (Whiteside et al. 1968; Knight et al. 1991; Mathews et al. 2004) (p = n/a, 0.02, and 0.045 respectively) and at the third trimester ($p < 0.05$) (Veena et al. 2010). Only 1 study found a negative association between serum folate and BW ($p < 0.01$) at the third trimester (Takimoto et al. 2011).

Table 2.15: Results of the association between vitamin B12 & BW from the studies included in the review

References	B12 levels (pmol/l) per trimester	BW (g)	Association under study and effect size (per trimester)			Variables controlled in multivariate analysis
			1st trimester	2nd trimester	3rd trimester	
Whiteside et al., 1968	1st: 217 2nd: 127 3rd: 115.1	B12 < 73.8, 3250g B12 73.8-146.9, 3460g B12 ≥ 174, 3630g	No significant association	Positive correlation (combined with iron & folate), p = n/a	No significant association	Vitamin B12, folate, and iron.
Knight et al., 1991	1st: 337.6 ± 23.9 2nd: 340 ± 14.8 3rd: 297 ± 11.4	Normal 3274.4 ± 26.3 Low 2159.9 ± 42.6	No significant association	No significant association	No significant association	Ferritin, folate, vitamin B12, and selected CBC data
Tamura et al., 1994	2nd:: 320 ± 130	3511 ± 536 (2470 - 4790)	N/R	No significant association	N/R	N/A
Ackurt et al., 1995	2nd: 140.8 ±105 3rd: 94.6 ± 107.7	3510 ± 560	N/R	p > 0.05	P > 0.05	N/A
Mathews et al., 2004	2nd: 207.4 [121.8, 379.3]¹	M 3402 ± 489 F 3285 ± 431	N/R	β = 0.024, 95% CI (- 0.22, 0.27), p = 0.85	N/A	Maternal height, smoking
Relton et al., 2005	1st: 239.1 ± 97.4	3430 ± 470	β = 0.03, 95% CI (- 0.05, 0.12), R^2 0.001 , p=0.41	N/R	N/R	N/A
Muthayya et al., 2006b	1st : 115, 156, 224 ² 2nd : 113, 149, 210 3rd : 111, 142, 181	2400 ± 340 2850 ± 450	Adjusted OR (95% CI) 5.98 (1.72, 20.74) P = 0.006 N/A	Adjusted OR (95% CI) 9.28 (2.90, 29.68) p < 0.001 N/A	Adjusted OR (95% CI) 2.81 (1.01, 7.87) p = 0.059 N/A	Maternal age quartile, education, parity, baseline (1st trimester) weight quartile N/A
Mamabolo et al., 2006	3rd: 174.8 ± 76.6	3120 ± 550	N/R	N/R	No significant Association	N/A

Values are mean or mean ± SD (range) or ¹median [5th, 95th centile] or ² medians (interquartile range) or ³median [25th , 75th centile] or ⁴geometric means (95% CI) or ⁵median, N/R = not relevant, (N/A, n/a) = not available, FA users = folic acid users, Non-users = non-folic acid users, † Whole blood folate, *RBC folate.

Cont. Table 2.15

References	B12 levels (pmol/l) per trimester	BW (g)	Association under study and effect size (per trimester)			Variables controlled in multivariate analysis
			1st trimester	2nd trimester	3rd trimester	
Takimoto et al., 2007	1st : 405 ± 146 2nd : 301 ± 96 3rd :265 ± 95	3120 ± 411	Effect size - 1.05, p = 0.08	Effect size - 5.349, p = 0.38	Effect size 0.776, p = 0.44	Maternal age, pre-pregnancy BMI and parity
Yajnik et al., 2008	2nd : 135 [103, 175]³ 3rd : 122 [94, 160]	2665 ± 358 ‡	N/R	No significant association	No significant Association	N/A
Hogeveen et al., 2010	3rd : 179 (134 - 219)	3425 (3075 - 3855)	N/R	N/R	R = - 0.06, P > 0.15	Gestational age, smoking, and infant sex.
Hay et al., 2010	2nd : 282 (269, 295)⁴	3673 ± 455	N/R	Negative association between BW and cord B12; Partial r = - 0.19, P = 0.021. Positive association between maternal holoTC and cord B12; Partial r = 0.50, P < 0.0001.	N/R	Maternal factors, newborn anthropometric data with P< 0.1 by univariate analyses and maternal plasma vitamin indexes
Veena et al., 2010	3rd :162.5 (124, 220)²	2873 ± 454	N/R	N/R	β = - 0.01, 95% CI (- 0.09, 0.06), P > 0.05	Child's sex and current age
Takimoto et al., 2011	3rd : FA users 262.6 ± 106 Non-FA users 288.8 ± 104.6	 2.894 ± 318 3.154 ± 230	N/R	N/R	No significant Association	Maternal age, height, pre-pregnancy BMI, weight gain, gestational length, and infant sex.

Values are mean or mean ± SD (range) or ¹median [5th, 95th centile] or ² medians (interquartile range) or ³median [25th , 75th centile] or ⁴geometric means (95% CI) or ⁵median, N/R = not relevant, (N/A, n/a) = not available, FA = folic acid, ‡ BW obtained from (Rao et al. 2001).

Table 2.16 Results of the association between folate & BW from the studies included in the review.

References	Folate levels (nmol/l) per trimester	BW (g)	Association under study and effect size (per trimester)			Variables controlled in multivariate analysis
			1st trimester	2nd trimester	3rd trimester	
Whiteside et al., 1968	1st: 25.1 2nd: 11.8 3rd:19.0	Folate < 11.3, 3430g Folate ≥ 11.3, 3710g	N/A	Positive correlation (combined with iron & folate) p = n/a	N/A	Vitamin B12, folate, and iron.
Knight et al., 1991	1st: 18.8 ± 2.0† 2nd: 21.7 ± 1.4† 3rd:24 ± 2.0†	Normal 3274.4 ± 26.3 Low 2159.9 ± 42.6	No significant association	Positive correlation WB folate & BW R^2 0.21, $p < 0.05$	No significant Association	Ferritin, folate, vitamin B12, and selected CBC data
Tamura et al., 1994	2nd: 38 ± 1	3511 ± 536 (2470 - 4790)	N/R	No significant association	N/R	N/A
Ackurt et al., 1995	2nd: 13.3 ± 5.9 3rd: 10.4 ± 6.5	3510 ± 560	N/R	$p > 0.05$	$p > 0.05$	N/A
Mathews et al., 2004	2nd: 19.7 (10.2, 30.6)	M 3402 ± 489 F 3285 ± 431	N/R	Marginally significant correlation β = 10.3, 95% CI (25.5, 20.4), p = 0.045	N/R	Maternal height, smoking
Relton et al., 2005	1st: 947.2*	3430 ± 470	β = 0.14, 95% CI (0.07, 0.23), R^2 0.021, $p < 0.001$	N/R	N/R	N/A
Muthayya et al., 2006b	1st : 518, 745, 1028* [2] 2nd : 634, 888, 1235* 3rd : 509, 824, 1228*	2400 ± 340 2850 ± 450	Adjusted OR (95% CI) 1.12 (0.66, 4.48) p = 0.25 N/A	Adjusted OR (95% CI) 1.17 (0.39, 3.50) p = 0.76 N/A	Adjusted OR (95% CI) 2.03 (0.46, 8.92) p = 0.36 N/A	Maternal age quartile, education, parity, baseline (1st trimester) weight quartile N/A

Values are mean or mean ± SD or [1]median [5th, 95th centile] or [2] median (interquartile range) or [3]median [25th , 75th centile] or [4]geometric means (95% CI), N/R = not relevant, (N/A, n/a) = not available, FA users = folic acid users, Non-users = non-folic acid users, † Whole blood folate, *RBC folate

Cont. Table 2.16

References	Folate levels (nmol/l) per trimester	BW (g)	Association under study and effect size (per trimester)			Variables controlled in multivariate analysis
			1st trimester	2nd trimester	3rd trimester	
Mamabolo et al., 2006	3rd :18.8 ± 11	3120 ± 550	N/R	N/R	No significant Association	N/A
Takimoto et al., 2007	1st : 23.2 ± 9.7 2nd : 19.3 ± 23.8 3rd :23.1 ± 69.3	3120 ± 411	Effect size - 2.60, p = 0.80	Effect size - 3.51, p = 0.36	Effect size - 0.12, p = 0.87	Maternal age, pre-pregnancy BMI and parity
Yajnik et al., 2008	2nd : 874 [687, 1.106]* [3] 3rd : 961 [736, 1.269]*	2665 ± 358 ‡	N/R	No significant association	No significant Association	N/A
Hogeveen et al., 2010	3rd : 9.1 (6.1 - 16.4)	3425 (3075 - 3855)	N/R	N/R	R = - 0.04, p > 0.15	Gestational age, smoking, and infant sex.
Hay et al., 2010	2nd : 7 (7, 8) [4]	3673 ± 455	N/R	No association between BW and cord folate	N/R	Maternal factors, newborn anthropometric data with P< 0.1 by univariate analyses and maternal plasma vitamin indexes
Veena et al., 2010	3rd : 34.7 ± 19.2	2873 ± 454	N/R	N/R	β = 3.89, 95% CI (0.28, 7.50), p < 0.05	Child's sex and current age
Takimoto et al., 2011	3rd : FA users 18.3 ± 7.5 Non-users 13.8 ± 7.7	2.894 ± 318 3.154 ± 230	N/R	N/R	β = - 37.1, 95% CI (- 56.8, - 17.4), p < 0.01	Maternal age, height, pre-pregnancy BMI, weight gain, gestational length, and infant sex.

Values are mean or mean ± SD or [1]median [5th, 95th centile] or [2] median (interquartile range) or [3]median [25th , 75th centile] or [4]geometric means (95% CI), N/R = not relevant, (N/A, n/a) = not available, FA users = folic acid users, Non-users = non-folic acid users, *RBC folate, ‡ BW obtained from (Rao et al. 2001).

2.4.2. Discussion

This is the first systematic review investigating the association between maternal vitamin B12 levels in blood and infant birth weight. Generally, the results did not support our hypothesis that the maternal vitamin B12 level independently predicts low birth weight. Although mixed results were found in the literature in the association between maternal vitamin B12 and BW: positive (Whiteside et al. 1968; Muthayya et al. 2006b) and negative association (Hay et al. 2010). The only evidence in favour of this came from the study by Muthayya et al with good quality on our assessment (see **Appendix V**). This study reported a relationship between low maternal vitamin B12 status and the incidence of IUGR and of high significance at the second trimester. In fact, no statistical association was made between normal BW and maternal vitamin B12 in this study. However, a sub-sample of 112 pregnant of the same population with mean BW (2900 ± 390 g) found that the maternal serum vitamin B12 concentration at each trimester increased with increasing BW. Though, these increases in maternal serum B12 were only significant during the second trimester ($p = 0.036$) (Muthayya et al. 2006a). In addition, Whiteside et al with low quality on our assessment (see **Appendix V**) found a positive association between maternal vitamin B12 and BW particularly at the second trimester. This was reported to be mostly from the contribution of serum iron taken together with the vitamin B12 and folate which made a significant improvement over the results obtained if the serum iron level only was fitted into the regression equation. The authors indicated that deficiency of 1 or all the 3 nutrient levels may affect the foetus rate of growth in a deficient mother. Although, another study showed that maternal serum iron, folate, and vitamin B12 concentrations had no significant correlation with foetal BW (Yusufji et al. 1973). In contrast to Muthayya et al. and Whiteside et al., Hay et al. with relatively good quality on our assessment (**see**

Appendix V) showed an association in the opposite direction to what we expected: birth weight was negatively associated with cord vitamin B12 after controlling of the confounding factor, i.e. the heavier babies had the lowest vitamin B12 status. Our expectation came from that vitamin B12 concentration in cord blood shown to be significantly higher than, but correlated to levels in the mother, at all trimesters of pregnancy (Monsen et al. 2001; Muthayya et al. 2006a). Furthermore, cord serum vitamin B12 levels were linearly associated with BW, such that increasing cord levels were associated with increasing BW (Muthayya 2009).

Our results support the belief that increased BW are associated with high folate concentrations (Goldenberg et al. 1992). The role of folate in DNA synthesis and cell replication suggests that folate can influence foetal growth (Scholl and Johnson 2000). The influence of dietary and circulating folate on infant low birth weight was studied in 832 women from the Camden Study (Scholl et al. 1996). It was reported that both low dietary and circulating folate at 28 weeks gestation was associated with a greater risk of low birth weight (Scholl et al. 1996). In our review, studies that showed the association between folate and BW did not clearly mention a folic acid containing supplement during pregnancy, except in the Takimoto et al. study where folic acid supplement users were compared with non-users in 33 healthy Japanese pregnancies at the third trimester. It was found that higher serum folate due to folic acid supplement with low vitamin B12 status may affect foetal growth. However, this study used a small sample size (n = 33) and the proportion of low birth weight in folic acid users (14.2%) was unexpectedly higher compared to their national data (about 9%) (Takimoto et al. 2011). The imbalance between maternal vitamin B12 and folate level has been reported in known vegetarian populations (Bondevik et al. 2001; Yajnik et al. 2008) and their possible contribution to higher risks for chronic diseases has been discussed (**Chapter 1**). Even

though the Yajnik et al. study, with good quality assessment in our review, showed no association between maternal vitamin B12 and neonatal size, it allows analysis of the relationship between maternal nutritional status and mid- and late pregnancy and childhood outcomes at 6 years of age. Children born to mothers with higher erythrocyte folate but low serum vitamin B12 during pregnancy had a higher rate of insulin resistance and adiposity (Yajnik et al. 2008).

The present review has several limitations: it included only published English-languages studies. It is possible that either non-published data or data in other language exist which we did not identify. In addition, it included studies that measured vitamin B12 at different trimesters during pregnancy. Although, we are aware of several other published studies which also measured vitamin B12 level at delivery time only and correlated these levels with BW. However, the pattern of vitamin B12 during pregnancy decreased before 27^{th} week gestation and after that the levels were almost constant until delivery (Carretti et al. 1994). Furthermore, early-mid pregnancy has been found to be a time for significant association between BW and both maternal vitamin B12 and folate, thus could be a critical period for the foetal programing for development of disease in later life (Yajnik et al. 2008).

In conclusion, the present review has highlighted much heterogeneity in the results of available observational studies of the association between maternal vitamin B12 and BW. Mixed results were identified. Studies included in the review differ substantially in study methodology and quality. Overall, the review did not support our hypothesis that low maternal vitamin B12 level independently predicts low birth weight.

Chapter 3

Low vitamin B12 level is associated with maternal obesity and higher birth weight in GDM

3.1 Introduction

As previously discussed on **Chapter 1**, the prevalence of DM is increasing dramatically worldwide (Shaw et al. 2010). More importantly, T2DM has been reported in adolescents and children, particularly in high prevalence populations (Alberti et al. 2004). One consequence of this is high incidence of GDM, which affects up to 14% of the pregnant population, approximately 135,000 women per year in the US (Jovanovic and Pettitt 2001). GDM seems to increase the risk of developing diabetes in the offspring of affected mothers (Pettitt et al. 1993). In addition, the intrauterine environment such as birth weight and maternal hyperglycaemia may possibly affect the development of T2DM among the young (Alberti et al. 2004). A prospective study found that the prevalence of IGT in the children of mothers with diabetes during pregnancy increased with time from 1.2% at < 5 years of age to 19.3% at 10 –16 years of age. This was compared with 2.5% at 10 –16 years of age in control subjects (Silverman et al. 1995). Furthermore, a U-shaped relationship between birth weight and risk of T2DM in later life was reported. A study on children aged 6 - 18 years in Taiwan showed that the risk of T2DM was lowest in those with a birth weight of 3 - 3.5 kg, and was significantly increased in those children who had either low (< 2.5 kg) or high (> 4.0 kg) birth weight, despite adjustment for other factors, including gestational diabetes, family history of diabetes, and socioeconomic status (Wei et al. 2003). In Pima India, a follow-up study of infants born during 1940 - 72 showed that the prevalence of DM was greatest in those with the lowest and highest birth weights. The age-adjusted prevalence for BW < 2500 g, 2500 - 4499 g, and ≥ 4500 g were 30%, 17% and 32%, respectively (McCance et al. 1994).

Thus, the intrauterine environment is a critical period in human life. The development of many organs, particularly brain, adipose and muscle tissue, occur during a limited time in this period. The concept is that the environmental exposures *in utero* during certain phases of development will result in permanent changes in the organism (Strauss 1997). Maternal nutrition during the intrauterine period is a factor that could affect foetal development and growth (Godfrey and Barker 2000). Infants exposed to malnutrition in early pregnancy are more likely to be obese in later life. Similarly, children exposed to hyperglycaemia *in utero* are also more likely to develop insulin resistance and obesity during childhood. The mechanisms underlying these changes are not understood (Strauss 1997).

The PMNS was the first study in India to investigate the relationship between maternal nutrition and offspring risk of T2DM. It is the first to report an association between maternal intake (green leafy vegetables, milk, and fruit) and biomarkers (erythrocyte folate and serum vitamin C) and infant size at birth (Rao et al. 2001). Also, it reported that low vitamin B12 is common while folate insufficiency is rare in the Indian population (Yajnik et al. 2006). Further investigation found that low maternal vitamin B12 is related to higher incidence of GDM (Krishnaveni et al. 2009), higher adiposity and insulin resistance in offspring (Yajnik et al. 2008) and future T2DM in mothers (Krishnaveni et al. 2009). The population in the above study is known to be lacto-vegetarian with infrequent meat consumption. It is not known whether similar phenomena exist in a non-vegetarian population such as the UK population. Therefore, we aimed to study the vitamin B12 status in pregnancy and investigate its relationship with maternal BMI, incidence of GDM and BW in women with and without GDM.

3.2 Materials and Methods

3.2.1 Study population

This is a retrospective study of mothers attending the antenatal diabetes and medical obstetrics clinics in a district general hospital in Warwickshire, UK between 2005 and 2010. Patients attending these clinics are mainly UK Caucasian population. As part of routine practice, maternal vitamin B12 and folate levels were checked for most women. Also, it was routine for general practitioners and obstetricians to prescribe a folic acid supplement of 400 µg/day to pregnant women. Any pregnant woman giving birth to live and singleton babies were eligible for the study. In addition, ethics approval from the hospital was obtained to conduct this study.

3.2.2 Data collection

A pre-designed questionnaire was used for data collection (**see Appendix VI**). Personal information such as BMI at booking, gravidity and parity, history of pre-eclampsia, history of GDM, other obstetrics and medical history, family history of chronic diseases, used medication, smoking status and use of alcohol was obtained from the medical notes for each woman. Information about folic acid supplements and vitamin B12 injections was also obtained, in addition to the delivery information, such as date of delivery; weeks of gestation; mode of delivery; sex of the baby and infant weight; length; and head circumferences (when available).

Maternal vitamin B12 and folate levels were collected from the medical notes and biochemistry laboratory. From 2005 to 2009, the haematology lab was using the immunoassay technique (Beckman Access analyser) to determine the level of serum vitamin B12 and folate. For the samples collected between 2009 and 2010 in the hospital,

electrochemiluminescence immunoassay and binding assay (Elecsys and cobas e immunoassay analysers, Roche Diagnostics, USA) were used to determine the level of vitamin B12 and folate respectively. As there is no specific cut-off level for vitamin B12 and folate during pregnancy, low vitamin B12 was defined as a concentration of < 150 pmol/l as widely used in many studies (Bondevik et al. 2001; Yajnik et al. 2008; Vanderjagt et al. 2009; Veena et al. 2010; Goedhart et al. 2011) and folate < 9 nmol/l based on the lower limit of the normal level used by the hospital lab.

3.2.3 Statistical analysis

All statistical analyses were performed using the SPSS programme version 18.0 (SPSS Inc., Chicago, IL, USA). The analysis focused on the assessment of serum vitamin B12, serum folate, incidence of GDM and infant BW for a pregnant born singleton, live baby. Sociodemographic factors considered in the analysis were age, BMI at booking, gravidity, parity, gestational age at delivery and smoking status. The data were expressed in mean ± SD or median [interquartile range] unless otherwise stated. Of the overall population, 61 did not have vitamin B12 and folate levels checked during the pregnancy, therefore 209 (77.4%) were included for purposes of vitamin B12 related analysis. To compare the quantitative variables between the groups, Anova table was used, while Chi square (χ^2) was used to compare the categorical data. The p-value of less than 0.05 was considered significant. Stepwise linear regression was used to identify factors that were independently associated with low vitamin B12 level.

3.3 Results

3.3.1 Study characteristics

The total number of pregnancies studied is 303. Of which, 17 cases were excluded because of incomplete notes, or twin pregnancy or stillbirth. From the 286 pregnancies, 16 mothers had pre-existing T1DM and T2DM. Of the 270 mothers who met the inclusion criteria, 60 of them had a diagnosis of GDM (22.2%). Vitamin B12 and folate levels were measured in 171/210 women with a normal pregnancy and 38/60 women with GDM at median 24 weeks of gestation (**Figure 3.1**).

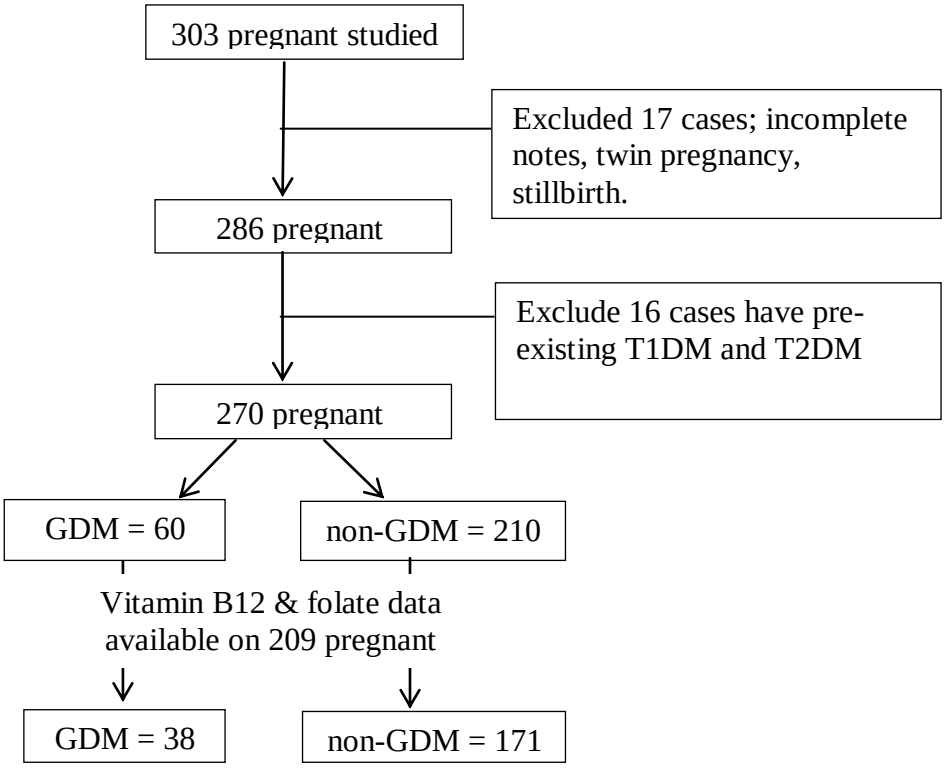


Figure 3.1: Gives an overview of the participants included in the study

As expected, GDM mothers were older than non-GDM (33.5 vs. 30.5 years, $p < 0.001$), had higher BMI (31.0 vs. 26.1, $p < 0.0001$) and lower gestational age at delivery (38.4 vs. 39.5, $p < 0.001$). Birth weight was not statistically different between the GDM and

the non-GDM group (3426.4 ± 426.3 vs. 3407.9 ± 568.8. p = 0.81). Only 14% of the total sample were smokers (**Table 3.1**).

Table 3.1: Characteristics of the pregnant subjects who participated in the study

	All (n = 270)	**GDM (n = 60)**	**Non-GDM (n = 210)**	**P-value**
Age (years)	31.2 ± 5.84	33.5 ± 4.98	30.5 ± 5.91	< 0.001
BMI at booking	27.2 ± 6.25	31.0 ± 6.21	26.10 ± 5.82	< 0.001
(kg/m^2)	*(n = 253)	*(n = 58)	*(n = 195)	
Gravidity	2.50 ± 1.41	2.25 ± 1.17	2.58 ± 1.49	0.11
Parity	1.04 ± 1.07	0.88 ± 0.88	1.09 ± 1.12	0.20
GA at delivery	39.2 ± 1.63	38.4 ± 1.15	39.5 ± 1.67	< 0.001
BW (g)	3412.1 ± 539.7	3426.4 ± 426.3	3407.9 ± 568.8	0.82
Smoking status				
non-smoker	231 (85.9%)	52 (86.7%)	179 (85.6%)	
smoker	38 (14.1%)	8 (13.3%)	30 (14.4%)	
Vitamin B12 (pmol/l)	143.9 [106.3,191.5]	129.9 [92.9,174]	143.9 [106.3,195.6]	0.26
< 150 pmol/l	115 (55%)	22 (57.9%)	93 (54.4%)	
	*(n = 209)	*(n = 38)	*(n = 171)	
Folate (nmol/l)	15.9 [10.0, 25.6]	15.2 [10.6, 25.1]	16.1 [10.0, 26.3]	0.77
< 9 nmol/l	45 (21.8%)	9 (24.3%)	36 (21.3%)	
	*(n = 207)	*(n = 37)	*(n = 169)	

Values presented are mean ± SD or median [IQR] or no. (%),
* available no. for specific data

3.3.2. Vitamin B12 and folate status during pregnancy

Of the total sample, the median [IQR] for vitamin B12 and folate levels were 143.9 [106.3, 191.5] pmol/l and 15.9 [10.0, 25.6] nmol/l respectively (**Table 3.1**). At a mean of 24 weeks gestation, the mean vitamin B12 level was 167.7 pmol/l (Data is not shown). The proportion of women with vitamin B12 levels < 150 pmol/l was 115/209 (55%). In addition,

approximately 58% of the GDM group had low vitamin B12 compared to the 54% of the non-GDM group (**Figure 3.2**). Moreover, low folate level was higher among the GDM group (24%) compared to the non-GDM group (21%) (**Table 3.1**).

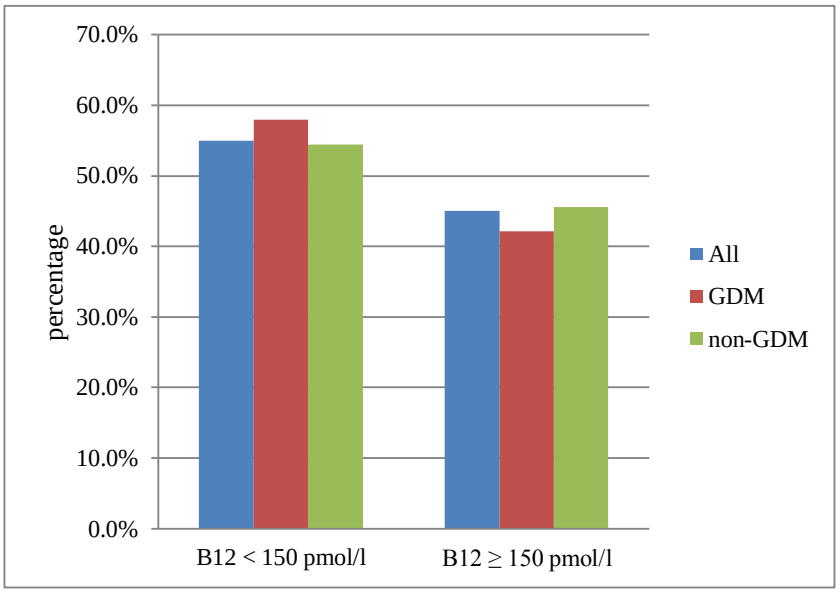


Figure 3.2: The proportion of vitamin B12 in the studied sample

The proportion of pregnant women with vitamin B12 < 150 pmol/l and folate < 9 nmol/l were 35/115 (30.4%), while the proportion of those pregnant with vitamin B12 < 150 pmol/l and folate > 9 nmol/l were 80/115 (69.6%) (**Figure 3.3**). A significant proportion of the women with vitamin B12 < 150 pmol/l had co-existent folate deficiency compared to those with vitamin B12 > 150 pmol/l (30.4% vs. 11.0%, OR 3.54 (95% CI 1.64 - 7.64), $p < 0.001$. Mothers with vitamin B12 insufficiency had a folate concentration 9.2 nmol/l less than those without insufficiency ($p < 0.001$).

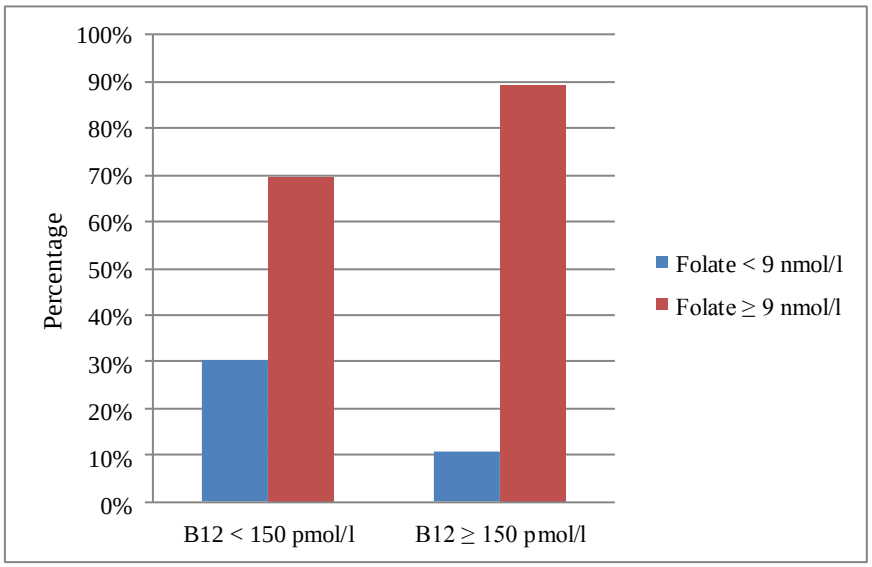


Figure 3.3: Folate status among vitamin B12 sufficient and insufficient mother

3.3.3 Maternal vitamin B12 and BW

GDM women with low vitamin B12 had an infant 37 g heavier than GDM women with normal vitamin B12 levels, although this difference did not reach the significant level (p = 0.80) (**Table 3.2**).

Table 3.2: Neonatal BW associated with maternal vitamin B12 levels

	GDM (n = 38)	**Non-GDM (n = 171)**
B12 < 150 pmol/l	3382 ± 543 (n = 22)	3396 ± 553 (n = 93)
B12 ≥ 150 pmol/l	3345 ± 231 (n = 16)	3369 ± 618 (n = 78)

Values presented are mean ± (SD) standard deviation

3.3.4 Maternal association with low vitamin B12

Compared to vitamin B12 levels ≥ 150 pmol/l, women with low vitamin B12 (< 150 pmol/l) were not significantly different in age (31.2 vs. 31.5, p = 0.71) but had a higher BMI at booking (28.0 vs. 25.7, β = - 0.171, p = 0.013); controlled for age, smoking, folate and presence of GDM (**Table 3.3**). Women who developed GDM had lower vitamin B12 level (129.9 vs. 143.9 pmol/L, p = 0.26) though this was not statistically significant (**Table 3.1**). Similarly, a non-significant difference was seen in the proportion of women with low vitamin B12 developing GDM (19.1 vs. 17%, p = 0.42) (Table 3). GDM mothers with vitamin B12 levels below the median had heavier babies (3522 vs. 3211g, p = 0.025). This difference persisted even after controlling for folate levels, gestation age at birth, maternal age, smoking and BMI at booking (β = - 0.39, p = 0.017). No such difference was seen for non-GDM mothers (3421.8 vs. 3345.8g, p = 0.395).

Table 3.3: Maternal association with low vitamin B12

	B12 < 150 pmol/l (n = 115)	**B12 ≥ 150 pmol/l (n = 94)**
Age (years)	31.2 ± 6.0	31.5 ± 5.6
Pre-pregnancy BMI (kg/m^2)	28.0 ± 6.0 † *(n = 103)	25.7 ± 6.7† *(n = 93)
Prevalence of GDM	22 (19.1%)	16 (17%)

Values presented are mean ± (SD) or no. (%), *available no. for BMI data,
†(β = - 0.171, p = 0.013) controlled for age, smoking, folate, and presence of GDM

3.4 Discussion

In this study, we observed a high prevalence of low vitamin B12 and a relatively lower prevalence of folate insufficiency in pregnant women attending one general hospital in Warwickshire. This result is consistent with the result from the UK population which showed higher low vitamin B12 and lower folate insufficiency in early pregnancy compared to age-matched control non-pregnant women (20% vs. 4% and 6% vs.13% respectively (Saravanan and Yajnik 2010). Earlier studies supported the presence of vitamin B12 insufficiency during pregnancy in a Caucasian population (Ball and Giles 1964; Frery et al. 1992; Koebnick et al. 2002; Garcia-Casal et al. 2005; Milman et al. 2006). However, the percentage of vitamin B12 insufficiency varied from one study to another. The median folate level in this study was within the normal limit. This is likely due to the routine folic acid supplement (400µg /day) with the intention of preventing NTDs. Furthermore, folate insufficiency was higher among the GDM group compared to the non-GDM group. In addition, GDM pregnant women had both lower vitamin B12 and folate levels compared to non-GDM pregnant women. However, the difference did not reach significant levels. This is consistent with other studies, where vitamin B12 and folate levels were lower in the GDM group compared to the control pregnant group (Seghieri et al. 2003; Hassan et al. 2008). The proportion of pregnant women with low vitamin B12 and high folate (67%) was higher compared to low vitamin B12 and normal/low folate (33.0%). A study in pregnant Greeks and Albanian immigrants observed a high prevalence of low vitamin B12 and a lower prevalence of folate insufficiency at delivery particularly among the Albanian pregnant women (Schulpis et al. 2004).

Low vitamin B12 concentration in our study appeared to be associated with maternal BMI and a possible higher incidence of GDM after controlling for age, smoking, folate and the presence of GDM. This is consistent with the other result, where lower vitamin B12 levels were associated with higher maternal adiposity and GDM was twice as frequent in women with low vitamin B12 compared with normal level (Krishnaveni et al. 2009). Moreover, GDM women with low vitamin B12 had heavier babies compared to GDM women with normal vitamin B12 levels. However, previous studies observed no significant association between maternal vitamin B12 insufficiency and birth size (Yajnik et al. 2005; Yajnik et al. 2008).

As this study has been done in an almost entirely Caucasian population, which is generally known to be non-vegetarian, we did not expect to find a high prevalence of maternal vitamin B12 insufficiency. However, one possibility could be the increase of vegetarianism within the UK, which has become more popular, with the current estimate of 3% of the population eating a vegetarian diet (The Vegetarian Society 2011). People tend to be vegetarian as a good option to be healthy and to avoid metabolic diseases. This diet, though, should be well planned, in order to be nutritionally adequate and provide health benefits in the prevention and treatment of some diseases (Messina and Burke 1997). Another possibility could be the poor of quality food intake, rich in energy but less so in micronutrients which could contribute to low vitamin B12 and obesity (Krishnaveni et al. 2009). High prevalence of low micronutrients among overweight and obese adults has previously been observed. This may be as a result of inadequate nutrient intake and/or alteration in nutrient absorption or metabolism (Kimmons et al. 2006). This could give some

explanation to what we observed in our study, association between maternal vitamin B12 insufficiency and adiposity.

In addition, the combination of low vitamin B12 and adequate/high folate concentration and its relationship to insulin resistance and prevalence of GDM has been previously reported (Krishnaveni et al. 2009). The plausible biochemical reasons why low vitamin B12 could increase adiposity and insulin resistance, particularly in the presence of adequate/high folate has been discussed in the PMNS study (Yajnik et al. 2008). Vitamin B12 insufficiency will trap cellular folate as 5-methyltetrahydrofolate (Scott 1992). This will prevent the formation of methionine from homocysteine and thus impair protein synthesis and reduce lean tissue deposition. In addition, MM-CoA mutase, for which vitamin B12 acts as a cofactor, is required for the degradation of odd-chain fatty acids and branched-chain-amino acids, in particular the conversion of MM-CoA to succinyl-CoA. In the presence of vitamin B12 insufficiency, an accumulation of MMA will occur and may increase lipogenesis and insulin resistance (Yajnik et al. 2008).

The limitation of this study is that it is retrospective and observational study done in a selected clinic population (medical obstetric clinic). Therefore, causality cannot be ascertained. However, this is the first study to show the possible interaction between low vitamin B12 levels, GDM, and their additive influence on BW. Finally, vitamin B12 and folate concentrations are difficult to interpret in pregnancy and there is no agreed cut-off level for deficiency, thus we assigned the cut-off level based on the previous published studies and the lower limit of the normal range from the hospital lab respectively.

In conclusion, this study showed that vitamin B12 insufficiency is not rare in the UK Caucasian population. It is associated with maternal obesity, possible higher incidence of

GDM and macrosomia in GDM. Therefore, adequately powered prospective studies are urgently needed on the influence of early pregnancy vitamin B12 levels on the risk of GDM and neonatal outcomes.

Chapter 4

Validation of a modified Semi-Food Frequency Questionnaire for use in Saudi Arabia

4.1 Introduction

The prevalence of obesity is increasing worldwide and the rate of increase is particularly rapid in Saudi Arabia (World Health Organization). Genetic and environmental factors such as diet and lifestyle have been the main factors that lead to obesity and related metabolic diseases such as DM, hypertension, and cardiovascular diseases. Environmental factors are likely to be the major contributors for such a rapid increase as genetic changes tend to take longer (Al-Othaimeen et al. 2007).

Lifestyle, physical activity, food consumption patterns and dietary habits have changed markedly during the past few decades. In fact, the Eastern Mediterranean region showed an increase in per capita energy and fat intake in all countries. This is probably due to the high consumption of foods rich in fats and calories and a sedentary lifestyle which may play an important role in the rise in the prevalence of obesity and chronic diseases in this region. It is particularly true that there has been a huge shift from traditional foods to more westernized foods, which are characterized by high fat, high cholesterol, high sodium and low fibre. For instance, the daily per capita fat showed an increase in most countries, ranging from 13.65% in the Sudan to 143.3% in Saudi Arabia (Musaiger 2002; Musaiger 2004). Studying the relationship between food consumption patterns and dietary habits in the Saudi population is likely to provide huge insights into understanding the recent rapid changes in food habits.

Food habits and dietary intakes can be studied using different dietary assessment methods. The common methods used to assess an individual's dietary intake are a food record, a 24 hours food recall, and a Food Frequency Questionnaire (FFQ). A 24 hours food recall or diet record methods are based on foods and amounts actually consumed by

an individual on one or more specific days, while an FFQ is based on an individual's perception of the usual intake over a less precisely defined period of time. Thus, a food record could estimate an absolute rather than relative intake compared to an FFQ. However, they are expensive, time- consuming, and not representative of the usual intake as only a few days are assessed. In addition, they need a skilled and trained interviewer and a cooperative participant to obtain accurate and complete information (Buzzard 1998; Willett 1998). The major strength of the 24 hours food recall method compared with a food record is that it does not require literacy, which is an important factor in obtaining a representative sample of a population (Buzzard 1998).

An FFQ is the most commonly used method to assess eating habits and to rank an individual's energy and nutrients intake. It is a preferable dietary assessment tool in many epidemiological studies to relate diet and chronic diseases because of its ability to estimate a long-term dietary intake. In addition, it is practical, easy to administer on a large sample and relatively inexpensive (Willett et al. 1985; Block et al. 1986). It is recommended that an FFQ should be validated in a specific population prior to its wider use in that population because of likely differences in diet between populations (Willett and Lenart 1998). However, it is impossible to evaluate the validity of an FFQ in the true sense due to lack of a "gold standard" method. As Willett and Lenart described, "The validity of one method can only be evaluated relative to another method of unknown but supposedly higher validity, i.e. only the 'relative validity' can be estimated" (Willett and Lenart 1998). Many studies have examined various food frequency methods by comparing the concordance of food frequency responses with reference instrument such as multiple 24 hours food recall

or diet record to estimate correlations between nutrient intakes measured by the FFQ and the actual intake (Thompson and Byers 1994).

The calculation of the daily intake from an FFQ is normally done in two ways: asking the participants to record how often on average they have eaten the food items each week/month and to report the usual portion sizes for each food item (known as FFQ);. (e.g. How often do you eat white fish? Then how many grams do you have per day?) or to assign pre-specified standard portion sizes (known as semi-quantitative FFQ- SFFQ) for each food item; (e.g. How often do you have jam/honey on bread?). While the information about portion sizes in an FFQ is important in measuring the differences in food intakes, the assignment of an overall portion size is recommended to facilitate data collection (Noethlings et al. 2003). Indeed, asking frequency alone provides a good estimate of relative consumption (Samet et al. 1984). Results from several studies (Samet et al. 1984; Hunter et al. 1988; Noethlings et al. 2003) have shown that omitting separate questions on portion size in an SFFQ is preferable, particularly in large epidemiological studies where an FFQ should be kept simple and easy to administer. Hunter et al. found that the day-to-day variation in portion size, for the same individual, was much larger than the variation in portion size between individuals. These results suggest that using standard portion sizes instead of an individual's own-estimate may not necessarily introduce a larger error (Hunter et al. 1988).

In Saudi Arabia, most of the studies conducted used either a one day 24 hours food recall (Al-Shoshan 2007a; Al-Shoshan 2007b) or an FFQ that was developed and validated in a different population (Abalkhail 1998; Alissa et al. 2007). To our knowledge, there is no precedent for developing an FFQ that suits a Saudi population, apart from one study which

used an SFFQ to specifically measure the intake of vitamin A with its relevant biomarker (Alissa et al. 2005) and hence was only validated for vitamin A.

This study has been aimed at modifying and developing an SFFQ that was validated in a different population to suit a Saudi population. The performance of the modified SFFQ is then validated in a pilot study by comparing daily nutrient intake obtained from the frequency questionnaires (SFFQ and FFQ) with those derived from three days of 24 hour food recall (24 h FR).

4.2 Materials and methods

4.2.1 Study population

This study was carried out in a group of volunteer women from members working in the UDC as well as living in Riyadh city in the Kingdom of Saudi Arabia (KSA) in 2008-2009. A total of 46 apparently healthy, aged between 18 and 45 years, non-smoking Saudi women, without any significant medical problems nor taking any medication, participated in this pilot study. Permission was obtained from the UDC to conduct this pilot study on their staff with institutional research ethics committee approval. Consent was obtained from all the participants.

In order to have a socio-demographic characteristics overview on the collected sample, information on education level, occupation, total family monthly income, use of dietary supplements, and level of physical activity during work and leisure time were collected by using a standardized self-administered questionnaire. Participants' height and weight were measured by a trained dietician to calculate participants' basal metabolic rate (BMR).

4.2.2 Dietary assessment tools

The dietary information was obtained from the participants by a qualified dietician in order to assess their intake of different macro- and micronutrients by using two main methods. All dietary methods were always obtained in the same order for all participants. The difference between the dietary assessment methods used in the study is shown in **Table 4.1.**

4.2.2.1 First: Food Frequency Questionnaire

The Food Frequency Questionnaire used is a validated food intake questionnaire - DIETQ000037 (Tunuviel Software 2001-2007) with a few modifications made to suit the Saudi food dietary intake. These modification were: additional food items added to the questionnaire from traditional Saudi dishes as well as other commonly used food items (**Appendix VII**). Food items not used in Saudi Arabia were deleted (such as pork, ham, alcoholic beverages, etc.). The final modified questionnaire included 112 questions about different food items, which is comparable to the usual size of the FFQs (ranging between 100 and 150 food items) (Willett 1994).

The modified Food Frequency Questionnaire then was used to ask the participant to record how often on average they had eaten the individual food items each week over the previous months. Nine possible answers were given for each item namely: once to seven times per week, every two to three weeks (fortnight (F)), or rarely– never (R) (**see example**). Each food item was assigned a portion size employing commonly used units where possible (for example, a slice of bread or a teaspoon of sugar or a slice of cheese) or by using average portion sizes from a previous study of weighed records, this is known as a

Semi-Food Frequency Questionnaire (SFFQ). In addition, questions on the types of cooking oils and fats used were included.

Example of types of questions asked

How often do you eat the following bread?	
White bread	7 6 5 4 3 2 1 F R
How many slices do you have per day?	.....
What is the usual size of slice?	
1. Large	
2. Medium	
3. Small	
How often do you eat the following meats and meat dishes?	
Beef: roast, steak, stewed, burgers, lasagna, Bolognese, curry	7 6 5 4 3 2 1 F R
Lamb: roast, chops, stewed, curry	7 6 5 4 3 2 1 F R
Liver, kidney or heart	7 6 5 4 3 2 1 F R
Do you usually eat the fat on meat?	Yes / No

To evaluate the portion sizes used in the SFFQ, participants were also asked to report the usual amount consumed for each food item; this is known as a Food Frequency Questionnaire (FFQ). A Food Photographic Atlas was used to standardize the data collection from the participants (Nelson et al. 1997). The Food Photographic Atlas contained coloured pictures of eight different portion sizes (**Figure 4.1**) of foods commonly eaten in Saudi Arabia. Also, food models and household measurements were used in order to assess the portion size of some of the local food used in Saudi Arabia.

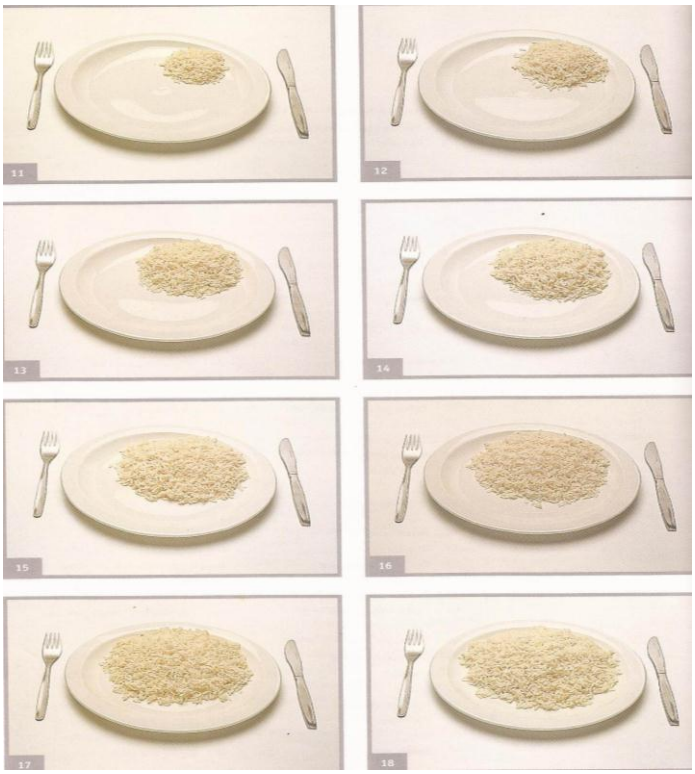


Figure 4.1: Shows an example of the different portion size for rice used in the Food Photographic Atlas

4.2.2.2 Second: 24 hours Food Recall (24 h FR)

This was assessed on three non-consecutive days (two weekdays and one weekend day) with a minimum gap of four days between them and within two weeks. The first 24 h FR was collected during the first visit and after the frequency questionnaire, while the second and third 24 h FR were collected through phone call on two separate days. All subjects were asked to provide telephone numbers and it was emphasised to them that they would be contacted after an appropriate period of time. The date was not specified; as such knowledge might have changed the eating habits of some of the participants. The foods were recalled chronologically from the previous day. The dietician asked the participants

what they had eaten in the previous 24 h from the first food eaten in the morning to the last food eaten before breakfast on the day of the interview. The Food Photographic Atlas and household measurements were also used to estimate the food portion sizes. At the end of each interview the foods were summarized for the respondent.

Table 4.1: Shows the difference between dietary assessment methods used in the study

	SFFQ	FFQ	24 h FR
Same Food Frequency Questionnaire	√	√	-
Assign a pre-specified standard portion size for each food item	√	-	-
Each participant reports the portion size consumed	-	√	√
Food Photographic Atlas & household measures used to report the portion size consumed	-	√	√
UK food composition table used to analyse the nutrients intake	√	√	√

4.2.3 Analysis of food consumption data

The daily intakes of energy and nutrients obtained from the SFFQ and FFQ were then analysed using the Q-Builder Software nutrition analysis program (Version 2). This includes an up-to-date food composition database with the nutrient contents of approximately 5000 foods and a database of portion sizes for 3800 foods. The nutritional analysis of Q-Builder is based on the UK food composition tables (McCance 1991). Information on food consumption collected by the SFFQ and FFQ are converted by Q-

Builder into a list of foods and weights, to generate mean daily food and nutrient intakes. The approximate daily intake was calculated by multiplying the weekly frequency of consumption of a food by the nutrient content of a portion size.

In addition, daily intake was estimated from the average of the three 24 h FR by using the Tunuviel Software WISP Food Intake Nutritional Analysis System (Version 3). Like the Q-Builder program, it is based on the UK food composition tables (see above). WISP is a nutritional analysis program that enables the user to conduct a broad nutritional analysis. A wide range of food items was included in the analysis program, but where an exact food was not found, a similar food was substituted or a recipe of that food was analysed taking cooking methods into account.

4.2.4 Statistical analysis

All statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Medians and 25 - 75th percentile of absolute intakes for energy, macro- and micronutrients were calculated and compared between the SFFQ, FFQ, and the 24 h FRs. The energy and nutrients were not normally distributed. Therefore, the Spearman correlation coefficient test across different methods (SFFQ vs. 24 h FR, SFFQ vs. FFQ, and FFQ vs. 24 h FR) was used to determine the strength of the relationship for nutrient intakes between the dietary assessment methods. In addition, all correlations were adjusted for total energy intake to remove variation due to energy and thus allow a reasonable estimate of a true correlation. As suggested by Willett et al. the adjustment was done by replacing nutrient intake values with their respective residuals from a regression model with nutrient intake as the dependent variable and total energy intake as the independent variable (Willett

et al. 1985). In addition, the GraphPad Prism5 software statistics program was used to assess agreement between the different dietary assessment methods. Agreement was expressed by 95% limits of agreement (LOAs), as recommended by Bland-Altman, where the difference between two methods was plotted against the average (known as bias) (Martin Bland and Altman 1986). Finally, to examine the degree of energy under- or over-reporting between different methods, we calculated the ratio of reported energy intake (EI) to calculate the basal metabolic rate (BMR). The EI/BMR ratio of 1.35 has been suggested as the lower cut-off for reasonable habitual energy intake (Goldberg et al. 1991). The BMR was calculated by using the equation for prediction of basal metabolic rate identified by the World Health Organization for women aged 30-60 years: [8.7 × weight (kg)] – [25 × height (m)] + 865 as previously used (Wirfält et al. 1998).

4.3 Results

4.3.1 Study characteristics

Of the 46 women who agreed to participate in this pilot study, 41 (89%) completed the SFFQ, FFQ and three days 24 h FR. Five participants were excluded from the analysis because they did not complete the three days 24 h FR. The characteristics of the participants are described in **Table 4.2**. The mean age (SD) was 28.2 (6.6) and the mean BMI (SD) was 24.2 (4.6). The majority of the participants had a university education (78.1%), are working (60.9%), and with a high annual income more than 10000 Saudi Riyals (~ 37500$) (78.0%). They are moderately active during their work (46.3%) and leisure time (46.3%).

Table 4.2: Shows the characteristics of women who participated in the study (n = 41)

Age (years)*	28.2	(6.6)
Height (cm)*	158.3	(6.05)
Weight (kg)*	60.7	(12.5)
BMI (kg/m²)*	24.2	(4.6)
Education level		
Illiterate	2	4.9%
Primary	1	2.4%
Secondary	6	14.6%
Graduate	30	73.2%
Postgraduate	2	4.9%
Occupation		
Not working	8	19.5%
Student	8	19.5%
Employee	24	58.5%
Others	1	2.4%
Monthly income (SR)		
1000-2999	3	7.3%
3000-5999	3	7.3%
6000-10000	3	7.3%
> 10000	32	78.0%
PAL during work		
Not-active (such as office worker)	12	29.3%
Moderately active (such as teacher)	19	46.3%
Very active (such as worker)	2	4.9%
PAL during leisure time		
Not-active (such as sitting, watching TV)	20	48.8%
Moderately active (such as walking)	19	46.3%
Very active (such as exercising)	2	4.9%

Data represent as N and % or *mean (SD)
SR- Saudi Riyals, PAL- Physical Activity Level

4.3.2 Comparison of medians

The results of the comparison of estimated energy and nutrients intake derived from the different dietary assessment methods are shown in **Table 4.3**. Of the daily median [25-75th percentile] energy intake, median fat intake were 34% [31-36.5], 34% [31-38], 35.4%

[29.8-41.3], median protein intake were 16% [15-18], 14% [12.5-15], 13% [11.4-16.5] and median CHO intake 49% [46-52.5], 52% [47.5-56], 51.5% [45.5-56.9] by SFFQ, FFQ & 24h FR respectively (data not shown). The SFFQ estimated a higher total energy intake compared to the 24 h FR and FFQ (2234 [1695-2699], 1570 [1243-1880], and1347 [1052-1771] kcal respectively).

Table 4.3: Compares the estimated energy and nutrients median intake of different dietary assessment methods

	SFFQ		FFQ		24 h FR	
Nutrient	**Median**	**25 - 75th percentile**	**Median**	**25 - 75th percentile**	**Median**	**25 - 75th percentile**
Energy (kcal)	2234	1695 - 2699	1347	1052 - 1771	1570	1243 - 1880
Protein (g)	85.4	67.6 - 107.8	46.0	35.2 - 58.7	52.5	42.7 - 61.7
Fat (g)	77.3	56.6 - 106.9	49.8	34.1 - 67.6	60.0	44.2 - 73.4
Saturated fat (g)	30.5	23.1 - 42.1	23.8	17.0 - 31.4	20.4	14.1 - 26.8
Monounsaturated fat (g)	26.9	20.7 - 33.6	15.2	10.5 - 21.6	19.8	12.2 - 24.3
Polyunsaturated fat (g)	10.2	8.0 - 15.2	5.7	3.6 - 8.2	7.8	6.2 - 10.7
Cholesterol (mg)	282	226 - 356	158	123.5 - 199	153	108.5 - 217
Carbohydrate (g)	292.5	205.7 - 359.9	188.6	137.8 - 246.5	201.2	156.2 - 258.5
Fibre (g)	17.7	14 - 23.9	9.3	6.6 - 13.4	12.5	9.2 - 15
Sodium (mg)	2123	1458 - 2453	1359	1008 - 1861	1664	1401 - 1994
Calcium (mg)	1007	800 - 1294	894	721 - 1277	593	428 - 807
Iron (mg)	9.2	7.2 - 11.5	5.9	4.2 - 6.9	7.8	6.3 - 10.3
Copper (mg)	1.25	1 - 1.7	0.56	0.4 - 0.75	0.90	0.7 - 1.3
Zinc (mg)	10.1	8.2 - 13	5.7	4.5 - 7.8	6.3	5.4 - 7.6
Selenium (µg)	64	52.5 - 78	26	17.5 - 34	33	25 - 41
Retinol (µg)	466	272 - 1137.5	237	180.5 - 323	197	160.5 - 260
Vitamin D (µg)	1.6	1.1 - 3	0.5	0.4 - 0.85	0.96	0.5 - 1.4
Vitamin E (mg)	5.8	4.3 - 7.4	3.1	2.1 - 4.9	5.8	4.2 - 7
Thiamin (mg)	1.3	0.9 - 1.5	0.8	0.5 - 1	0.86	0.7 - 1
Vitamin B6 (mg)	1.4	1.2 - 1.8	0.8	0.6 - 1	0.9	0.7 - 1.1
Vitamin B12 (µg)	6.5	4.7 - 8.2	4.0	3.2 - 5.1	2.2	1.4 - 3.8
Folate (µg)	251	202 - 305	149	109 - 212	137	121 - 172
Vitamin C (mg)	95	55 - 122	59	31 - 91	58	27 - 86

4.3.3 Comparison of correlation coefficients

The results of unadjusted and energy-adjusted correlations between energy, macro and micronutrients intake derived from the three assessment methods are shown in **Table 4.4**. In general, the correlations of individual nutrients between the two FFQ were strong. However, the correlation between the FFQs and 24 h FR were weak (SFFQ vs. FFQ: 0.84), (SFFQ vs. 24 h FR: 0.31), and (FFQ vs. 24 h FR: 0.24).

4.3.3.1 Correlation coefficients of macronutrients

The energy un-adjusted correlations between the SFFQ and FFQ for macronutrients were high; fat ($r = 0.87$, $P < 0.001$), carbohydrate ($r = 0.86$, $P < 0.001$) and protein ($r = 0.79$, $P < 0.001$). In contrast, they were weak between the SFFQ and 24 h FR, and FFQ and 24 h FR (**Table 4.4**).

4.3.3.2 Correlation coefficients of micronutrients

Similar to macronutrients, the correlations for the micronutrients intake were higher between the SFFQ and FFQ than to 24 h FR. They ranged between $r = 0.96$ ($P <0.001$) for calcium to $r = 0.40$ ($P = 0.01$) for retinol. However, some micronutrients showed stronger correlations between the SFFQ and 24 h FR (for example: vitamin C, vitamin D, sodium and calcium) and between the FFQ and 24 h FR (for example: vitamin C, vitamin E and calcium) (**Table 4.4**).

The energy-adjustment correlation coefficient showed similar if not stronger associations between the three assessment methods (**Table 4.4**).

Table 4.4: Shows the Spearman correlation coefficient between different dietary assessment methods

	Energy-unadjusted correlation			Energy-adjusted correlation		
Nutrient	**SFFQ vs. FFQ**	**SFFQ vs. 24 h FR**	**FFQ vs. 24 h FR**	**SFFQ vs. FFQ**	**SFFQ vs. 24 h FR**	**FFQ vs. 24 h FR**
Energy (kcal)	0.86**	0.38*	0.33*			
Protein (g)	0.79**	0.32*	0.24	0.86**	0.38*	0.34*
Fat (g)	0.87**	0.43**	0.33*	0.86**	0.38*	0.32*
Saturated fat (g)	0.86**	0.42**	0.35*	0.86**	0.38*	0.32*
Monounsaturated fat (g)	0.82**	0.40**	0.28	0.86**	0.37*	0.32
Polyunsaturated fat (g)	0.83**	0.31	0.13	0.87**	0.37*	0.33*
Cholesterol (mg)	0.86**	0.48**	0.33*	0.86**	0.33*	0.30
Carbohydrate (g)	0.86**	0.26	0.30	0.87**	0.37*	0.33*
Fibre (g)	0.81**	0.25	0.22	0.86**	0.40**	0.36*
Sodium (mg)	0.85**	0.40**	0.26	0.86**	0.36*	0.33*
Calcium (mg)	0.96**	0.33*	0.31*	0.86**	0.35*	0.31*
Iron (mg)	0.86**	0.08	0.08	0.86**	0.39*	0.34*
Copper (mg)	0.59**	0.25	-0.08	0.87**	0.41**	0.38*
Zinc (mg)	0.81**	0.18	0.15	0.86**	0.39*	0.34*
Selenium (µg)	0.66**	-0.04	-0.21	0.87**	-0.16	-0.10
Retinol (µg)	0.40**	0.10	-0.04	0.87**	0.48**	0.44**
Vitamin D (µg)	0.50**	0.52**	0.26	0.86**	0.43**	0.35*
Vitamin E (mg)	0.81**	0.40*	0.31*	0.86**	0.38*	0.34*
Thiamin (mg)	0.86**	0.29	0.17	0.86**	0.36*	0.33*
Vitamin B6 (mg)	0.84**	0.27	0.19	0.86**	0.37*	0.33*
Vitamin B12 (µg)	0.62**	0.22	0.13	0.86**	0.46**	0.43**
Folate (µg)	0.84**	0.28	0.17	0.86**	0.35*	0.31
Vitamin C (mg)	0.93**	0.55**	0.44**	0.84**	0.30*	0.25

* correlation is significant at 0.05 level (2 tailed)

** correlation is significant at 0.01 level (2 tailed)

4.3.4 Level of agreement

The level of agreement between methods was used to determine whether two methods agreed sufficiently well for them to be used interchangeably. The level of agreement is based on the median difference of two methods (bias) and plotted against the average (Martin Bland and Altman 1986). The level of agreement of the estimated energy and nutrient intakes derived from the three dietary assessment methods are shown in (**Table 4.5**). It shows that the SFFQ estimated a higher energy intake by 38.7% and 49.5% compared to the 24 h FR and FFQ, respectively. In contrast, the FFQ estimated a lower mean energy intake by 15.3% compared to the 24 h FR.

The overall agreement between nutrient intake measured by the SFFQ, 24 h FR and FFQ was assessed using Bland-Altman plots. The 95% limits of agreement, which is the median difference plus or minus 1.96 SD, would show how far the median by the two methods was likely to be for most individuals. The two methods will agree if the difference equals to zero. Our results from the Bland-Altman plots confirmed the higher intake estimation of the SFFQ across the range of intake except for vitamin E (vs. 24 h FR). **Figure 4.2** gives examples of the level of agreement on the Bland-Altman plots.

Table 4.5: Shows the level of agreement of estimated energy and nutrients median intake between different dietary assessment methods

	% bias †			Limits of Agreements (LOAs)					
				Low	High	Low	High	Low	High
Nutrient	SFFQ vs. 24 h FR	SFFQ vs. FFQ	FFQ vs. 24 h FR	SFFQ vs. 24 h FR		SFFQ vs. FFQ		FFQ vs. 24 h FR	
Energy (kcal)	38.7	49.5	-15.3	-808.1	2114	98.3	1416	-1435	1227
Protein (g)	53.7	59.9	-13.2	-17.4	87.4	10.8	64.6	-48.8	43.4
Fat (g)	27.7	43.3	-18.6	-42.4	87.9	-1.8	56.5	-62.2	53.1
Saturated fat (g)	40.5	24.7	15.4	-15.03	40.9	-5.4	20.03	-19.6	30.8
Monounsaturated fat (g)	34.4	55.6	-26.3	-12.6	30.6	-0.34	21.1	-22	19.3
Polyunsaturated fat (g)	30.4	56.6	-31.1	-10.8	18.3	-1.5	12.2	-14.6	11.4
Cholesterol (mg)	65.3	56.4	3.2	-96.9	344.2	6.4	239	-208.5	210.4
Carbohydrate (g)	40.1	43.2	-6.5	-137	310.6	-9.4	193	-219.7	209.6
Fiber (g)	39.5	62.2	-29.3	-6.95	20.8	1.9	14.85	-13.2	10.3
Sodium (mg)	26.7	43.9	-20.2	-1237	2171	-263.7	1513	-1801	1485
Calcium (mg)	49.8	11.9	40.5	-296.6	1098	-127	245.3	-376.2	1086
Iron (mg)	18.3	43.7	-27.7	-9.04	11.4	0.77	6.29	-11.8	7.1
Copper (mg)	38.7	76.2	-46.6	-3.86	3.99	-0.19	1.75	-4.5	3.1
Zinc (mg)	51.6	55.7	-10	-10.65	3.05	-6.38	6.65	-7.4	-0.5
Selenium (µg)	75.6	84.4	-23.7	-26.75	94.05	2.57	76.41	-51.2	39.5
Retinol (µg)	89.7	65.1	18.4	-5537	5470	-897.3	1967	-5978	4841
Vitamin D (µg)	62.7	104.8	-63	-1.45	3.89	-1.34	4.35	-1.6	0.97
Vitamin E (mg)	0	60.7	-60.7	-5.79	6.94	-1.3	6.4	-7.2	3.2
Thiamine (mg)	44.6	47.6	-7.2	-0.48	1.3	0.06	0.89	-0.9	0.8
Vitamin B6 (mg)	48.4	54.5	-11.7	-0.4	1.7	0.13	1.29	-1	0.9
Vitamin B12 (µg)	101.6	47.6	58.1	-20.9	25.2	-2.99	8.84	-23.7	22.2
Folate (µg)	63.7	51	8.4	-38.4	256.6	29.60	166.50	-140.2	162.2
Vitamin C (mg)	52.3	46.7	1.7	-143.8	177.5	-9.99	62.86	-180.3	161.1

% bias - difference of two medians estimated from the two assessment methods divided by the average

LOAs - the two methods will agree if the median difference is equal to zero

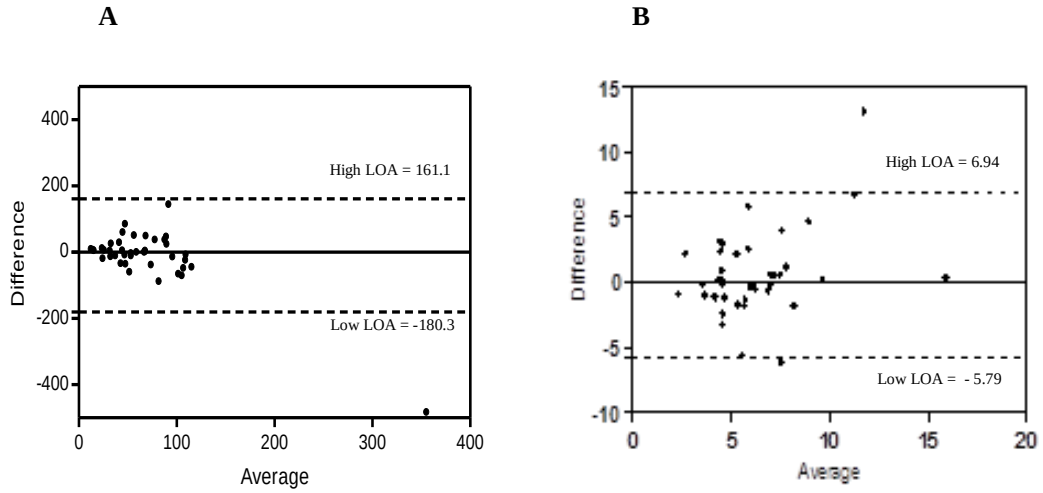


Figure 4.2: Bland-Altman plots (different of two dietary assessment methods against the average) and 95% limits of agreement - LOA (broken lines) (**A**) for vitamin C between the FFQ vs. the 24 h FR and (**B**) for vitamin E between the SFFQ vs. the 24 h FR.

4.3.5 Under-reporting of energy

To ensure that the dietary assessment methods used in this study estimated the habitual energy intake from the participants, we examined the EI/BMR ratios of the three dietary assessment methods. The mean ratios were 1.65, 1.16 and 1.00 for the SFFQ, 24 h FR, and FFQ respectively. The EI/BMR ratio of 1.35 is suggested to be the lower cut-off limit for reasonable energy intake. Thus, the FFQ and 24 h FR estimated lower energy and nutrients intake.

4.4 Discussion

In this study, we evaluated the performance of a modified SFFQ by comparing the daily nutrient intake obtained from the FFQs with those derived from the three days 24 h FR. As there is no approved nutritional database available in Saudi Arabia, we used a nutritional analysis software program that was developed in the UK. This was chosen for several reasons; firstly, it provides comprehensive information on the nutrient

content of approximately 5000 foods, including levels of fat, sodium, fibre, and carbohydrates, vitamins and minerals. Secondly, it includes foods used by many immigrants, such as South Asian and Arabs (for example Tabulla, Hummus, Falafel, Kebbab, Samosa, biryiani rice). Thirdly, a high level of agreement has been confirmed between the British and American food composition table (Kushi 1994; Garcia et al. 2003). Finally, it is well established and many studies have been published using the same nutrition analysis program to assess dietary intake (Hill et al. 2004; Magee et al. 2005; Broadfield et al. 2007; Harrington et al. 2008).

Our results showed firstly, that the SFFQ generally has a higher energy and nutrient estimate compared to the 24 h FR. The mean EI/BMR ratios indicated that the 24 h FR was under-reporting habitual energy intake compared to the SFFQ. For the EI to be representative of habitual or average dietary intake, it must be greater than 1.35 × BMR. The 1.35 indicates the lowest physical activity level for any healthy individuals and values less than 1.35 cannot represent habitual intake (Goldberg et al. 1991; Goldberg and Black 1998). For women between 30 - 60 years of age with light, moderate and heavy levels of physical activity, the EI/BMR ratio is estimated to be 1.4, 1.65 and 1.95, respectively (Goldberg and Black 1998). As our study participants were moderately active, an EI/BMR ratio of 1.65 estimated by an SFFQ seems more appropriate than a 24 h FR.

The recommended dietary allowance of energy intake for Saudi adult women with BMI 22.1 has been estimated to be 2100 kcal per day (Khan and Al-Kanhal 1998). Based on this estimate, the total energy intake for our sample with a mean BMI of 24.2 should be approximately 2299 kcal. This also supports that our modified SFFQ could reflect the right food consumption pattern among the Saudi population compared to 24 h FR. Furthermore, the SFFQ show a high consumption of fat (35% of total energy). This

probably reflects the current trend in Saudi Arabia where there is an increasing fat intake and a decreasing carbohydrate intake since the 1960s (Stephen et al. 1995). In addition, this is also supported by the recent national nutrition survey of the representative population of Saudi Arabia which showed that the percentage of energy from carbohydrates decreased from 75% to 43% and from fat increased from 10% to 42% between the years 1971 and 1992. Protein intake increased from 49 g to 114 g/day, while fat intake increased from 34 g to 144 g/day during the same period (Al-Othaimeen et al. 2004). All of the above points support that our modified SFFQ with a pre-specified standard portion size gives a good dietary intake estimation particularly for energy and macronutrients intake and its' results correspond to the national estimates.

The lower estimation of the FFQ and 24 h FR could be due to inaccurate self-reported portion size a deliberate underestimation of the actual dietary intake by the participants. Because the degree of under-reporting increases with intake, individuals tend to report intakes that are closer to perceived norms than to actual intake (Schoeller 1990). As previously reviewed (Macdiarmid and Blundell 1998), the under-reporting is higher among women than men. This has been linked to the increased prevalence of weight consciousness and thus dietary restraint in this group (Hill and Davies 2001). This could be true as our study participants were well-educated women. On the other hand, it could also be due to the limitation of the 24 h FR as only three days of 24 h FR were used in our study. It has been shown that the correlations for most nutrients increase with an increasing number of recalls (Block and Hartman 1989).

Secondly, the correlations between the two Food Frequency Questionnaires were overall more similar to each other than between the frequency questionnaires and recall. This is supported by other studies (Block et al. 1990; TjøNneland et al. 1992;

Noethlings et al. 2003), where the same questionnaires, asking about portion size (FFQ) or applying standard portion sizes (SFFQ) showed a good correlation.

Thirdly, despite the fact that the FFQ was not different from the 24 h FR in the median energy, macro- and some of the micronutrients intake, they generally had a weaker correlation compared to other methods. In addition, the FFQ tends to have a limited agreement with the 24 h FR for many nutrients except vitamin C and cholesterol, for which there was very good agreement with 1.7% and 3.2% bias, and LOA (-180.3, 161.1) and LOA (-208.5, 210.4) respectively. Interestingly, the SFFQ vs. 24 h FR had a good correlation in terms of total fat, saturated fat, monounsaturated fat, and cholesterol, as well as vitamin C and D intake, compared to the FFQ vs. 24 h FR. More importantly, energy-adjusted correlation tends to be better between the SFFQ vs. 24 h FR compared to the FFQ vs. 24 h FR, particularly for retinol, vitamin B12, copper, fibre, vitamin C, and folate. Vitamin E has 0% bias between the SFFQ vs. 24 h FR. This supports the use of our SFFQ when a particular nutrient intake is to be tested.

Finally, our pilot study has several limitations; first it uses < 50 years old women only. Thus the overall impression in dietary estimate is biased towards this younger female group. Secondly, eating patterns may differ from men to women, even though a prior study suggested that major eating patterns apply to both men and women (Slattery et al. 1998). Another limitation is the small sample size, which may have affected our results by having a wider confidence interval. However, this is a pilot study and the SFFQ needs to be replicated and validated on a large group of people from different parts of Saudi Arabia

In conclusion, the SFFQ that has been used in this study gave useful estimates of energy and some nutrient intakes and appears to be a valid tool in comparison to a 24 h FR. Further studies are needed to evaluate such frequency questionnaires through

comparing the dietary intake versus actual levels in the blood to give accurate validity, particularly for the relevant micronutrients, in order to understand the diet-disease relationship among this population.

Chapter 5

Does the estimated dietary intake of vitamin B12 and folate correlate with actual blood levels in the Saudi population?

5.1 Introduction

The Middle Eastern region is experiencing an epidemic of T2DM. It is ranked second in the world for the prevalence of T2DM (Shaw et al. 2010a). More specifically, Saudi Arabia has shown a rapid increase in the prevalence of DM from 2.5% in 1982 to 23.7% in 2000 (Al-Nozha et al. 2004; Elhadd et al. 2007). In addition, nutrition-related chronic diseases are a growing epidemic and of major concern in this region (Musaiger 2004; Al-Othaimeen et al. 2007; Elhadd et al. 2007). Rapid urbanization has shifted the population's dietary habits from traditional foods to more westernized foods, this is believed to contribute to the increasing prevalence of obesity and chronic diseases (Musaiger 2000; Musaiger 2004). The Saudi population has variable dietary patterns that are influenced mainly by cultural factors. However, urbanization and westernization have not only changed the lifestyle but also dietary behaviours. There are no recent studies that systematically studied the general dietary patterns or specific nutrients that may be contributing to the diabetes epidemic to understand the diet-disease relationship in this population. However, dietary intakes are complicated and difficult to measure even under optimal conditions (Willett 1998).

With the growing interest in understanding the role of vitamin B12 and folate in disease processes such as obesity and DM, particularly during pregnancy, it is important to be able to assess dietary intake of these vitamins in epidemiological studies. Accurate and objective estimates of the intake are necessary in order to assess any effects of nutritional status in those studies. As mentioned in the previous chapter (**Chapter 4**), FFQ are the method of choice for many epidemiological studies to relate diet and chronic diseases because of its ability to estimate long-term dietary intake (Willett et al. 1985; Block et al. 1986). FFQ has been validated and compared with different dietary assessment method (known as reference method), all of which may be susceptible to

measurement errors (Bolton-Smith et al. 1991), i.e. the same factors that affect the reference method may also affect the FFQ resulting in over- or under-reporting. It suggested that at least two additional measurements are necessary to determine the validity of the FFQ method, for instance, nutritional biomarkers and multiple 24 h FR (Kaaks 1997). The advantage of using nutritional biomarkers is that this method is not influenced by factors related to under or over reporting (Verkleij-Hagoort et al. 2006). In addition, the potential sources of random error occurring with biomarkers are different from those of dietary questionnaire measurement methods; therefore, errors are independent between the two measurements (Kaaks 1997). The triangular approach to such validation known as the 'method of triads' uses the correlations between each of the three measurement methods (FFQ, 24 h FR and nutrition biomarkers) to obtain a quantitative estimate of the questionnaire's validity coefficient (VC) (Kaaks 1997). Ocke and Kaaks described this method in further detail. The method of triads assumes that the correlations between the three methods are explained by the fact that all are positively linearly related to unknown true long term intake and that their random errors are mutually independent (Ocke and Kaaks 1997). Although the method of triads was introduced and described several years ago, few dietary validation studies have applied it in their method (McNaughton et al. 2004). The traditional method of validation using a dietary assessment method such as FFQ vs. biomarker has been widely applied by many studies (McNaughton et al. 2004) and only a few studies have interpreted their results with respect to the VC using the method of triads (Ocke and Kaaks 1997; Kabagambe et al. 2001; McNaughton et al. 2004; Andersen et al. 2005; Verkleij-Hagoort et al. 2006).

Although suitable markers are not available for many nutrients, serum values for several vitamins are reported to reflect dietary intake in controlled studies (Willett et al.

1983). A few biochemical markers are known to provide a valid reference measurement of absolute daily intake (Kaaks 1997) such as 24 h urinary nitrogen excretion; as a valid reference measurement for protein intake (Bingham and Cummings 1985). Unfortunately, the correlation between questionnaire measurements of nutrient intake and many biomarkers is relatively weak, with values < 0.40. This could be related to the variability in individuals' absorption, availability and metabolism of the vitamins, which can be important sources of random variations in the biomarker, unrelated to true dietary intake (Kaaks 1997). Among the Saudi population, a correlation of nutrient intake and its relevant biomarker was previously reported with correlation of ($r = 0.13$, $p < 0.05$) between dietary and serum vitamin A,($r = - 0.06$, $P > 0.05$) vitamin E (Alissa et al. 2005) and ($r = 0.136$, $P < 0.05$) iron (Alissa et al. 2007). No such correlation was reported between dietary and serum vitamin B12.

Therefore, the present study aimed to investigate the vitamin B12 and folate intake measured by different dietary assessment methods with corresponding nutritional biomarkers among the Saudi pregnant population. This is particularly useful as the bioavailability of both vitamins is likely to be influenced by cooking methods (e.g. over cooking can reduce the bioavailability of folate) and improving hygiene or refrigeration (e.g. hygiene, vacuum packing and refrigeration of meat products are likely to kill the microorganisms that are essential for the vitamin B12 synthesis in them).

5.2 Materials and methods

5.2.1 Study population

The study population consisted of Saudi pregnant women attending the antenatal clinics at University hospitals in Riyadh city, KSA. Women aged 20 to 40 years, non-smokers, with pre-pregnancy BMI > 18 and < 35 kg/m², without any significant medical

problems nor taking any medication were eligible for the study. Women were excluded if they were on multivitamins or known cases of anemia or had any nutrition related pre-existing medical condition such as coeliac disease, known DM or have any liver, cardiac or kidney disease. As a routine practice, general practitioners and obstetricians at the hospitals were prescribing a folic acid supplement of 500 µg/day in the first trimester and fefol tablets (iron and folic acid) 200 mg/day afterwards for all pregnant women attending the clinic.

5.2.2 Ethical approval

The College of Medicine Research Ethics Committee (CMRC) at the College of Medicine, and King Khalid University Hospital in Riyadh approved the study protocol. The written informed consent was obtained from every participant.

5.2.3 Data collection

The data collection was carried out between 2009 and 2010. Data was collected as follows: between 12^{th} - 14^{th}, between 24^{th} - 28^{th} and between 35^{th} - 37^{th} weeks of gestation. Pregnant women taking part in the data collection at the first and/or second trimesters were asked to be followed up at the next visit. Only 23 pregnant were seen at the three visits, 6 pregnant at first and second trimesters and 11 pregnant at second and third trimesters. Sociodemographic and anthropometric data was collected using a pre-designed questionnaire. The data collected included maternal age, education level, occupation, total family income, gravidity and parity. In addition, gestational age and maternal weight prior to or at the beginning of gestation were obtained from the patients' medical records. Height and weight were measured using an appropriate international standard scale (Digital Person Scale, ADAM Equipment Inc., USA). A

research nurse interviewed the participants, explained the study protocol, obtained the consent form and prepared for blood sampling.

5.2.4 Dietary assessment tools

The dietary information was obtained from the participants by a trained dietician in order to assess their intake of different macro- & micronutrients by using two main methods: FFQ and 24 h FR. All dietary methods were always obtained in the same order for all participants. The details of the dietary assessment methods used and analysis of food consumption data were described in **Chapter 4**.

5.2.5 Blood sampling and laboratory analysis

During the whole study period, blood samples were collected three times (at the first, second and third trimester) after an overnight fast for the measurement of serum vitamin B12 and folate. Serum samples were separated from whole blood collected in the plain tube after standing for 20 - 30 minutes, then centrifuged in 3500 rmp for 15 minutes Serum aliquots were then kept in the ice-box for about 4 - 6 hours, then later transferred and stored in - 80° fridge. At the end of the study, all samples were shipped in dry ice boxes from the research lab at the UDC, Riyadh, KSA to the Clinical Sciences Research labs at the University of Warwick, Coventry, UK after signing the academic human material transfer agreement between the parties and stored until measurements. Serum vitamin B12 and folate were measured by electrochemiluminescence immunoassay and binding assay (Elecsys and cobas e immunoassay analyzers, Roche Diagnostics, USA) respectively. In the laboratory, quality controls were tested once per day. Serum vitamin B12 inter-assay CVs were 6.0%, 2.3% and 4.4% at 142.4, 554.2 and 693.7 pmol/l respectively, while serum folate

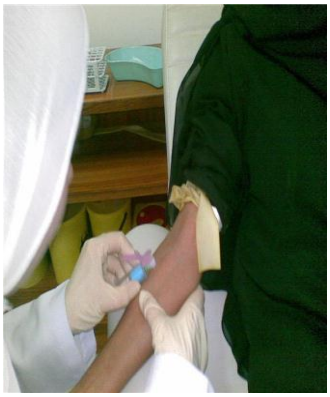
were 7.4% and 6.1% at 7.7 and 21.3 nmol/l respectively. From the total blood samples collected, 3 and 7 samples were insufficient for vitamin B12 and folate assays respectively. The overview of the study's procedure is shown in **Figure 5.1**.


Kingdom of Saudi Arabia
Ministry of Higher Education
King Saud University
College of Medicine
King Abdul Aziz Univ. Hospital

1. Consent form


Thiamine status in Saudi Gestational Diabetes Study

2. Data collection



3. Extracting the blood

4. Preparing the blood samples

5. Taking the diet history

Figure 5.1 Overview of the study's procedure

5.2.6 Statistical analysis

All statistical analyses were performed using the SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Medians and 25th - 75th percentile of absolute intakes for energy, macronutrients, vitamin B12 and folate from the different dietary assessment methods

(SFFQ, FFQ, and 24 h FR) were estimated and compared at different trimesters. The estimated vitamin B12 intake from SFFQ and 24 h FR and serum vitamin B12 were not normally distributed, thus log transformation was performed on those variables and used for further statistical analysis. Pearson correlation coefficient tests across different assessment methods (SFFQ vs. 24 h FR, SFFQ vs. FFQ, and FFQ vs. 24 h FR) were used to determine the strength of the relationship for nutrient intakes between the dietary assessment methods. Nutrients correlations were adjusted for total energy intake to remove variation due to energy and thus allow a reasonable estimate of a true correlation as described in **Chapter 4**. In addition, partial correlation coefficients adjusted for age, BMI and estimated energy intake were calculated for the association between estimated vitamin B12 and folate intake from different dietary method and its actual level in blood.

The method of triads was used to calculate the VC between those estimated from the SFFQ, FFQ, 24 h FR and the biomarker measures and the unknown true intake as shown in **Figure 5.2**. This was interpreted as the upper limit of the VC whereas the correlation between the biomarker and the dietary assessment method (SFFQ, FFQ and 24 h FR) was interpreted as the lower limit of the VC. The existence of negative correlation and high random variation in the sample correlation could cause VCs to be inestimable or > 1, a condition referred to as Heywood case. Heywood case occurs if the product of two correlation coefficients is much higher than the third correlation (Ocke and Kaaks 1997).

Vitamin B 12 insufficiency was defined as concentration of < 150 pmol/l as previously used (Bondevik et al. 2001; Yajnik et al. 2008; Vanderjagt et al. 2009; Veena et al. 2010; Goedhart et al. 2011). The proportional of pregnant women who had a serum vitamin B12 level below or above the cut-off value were compared with the

estimated vitamin B12 intake below and above the recommended level (2.6 µg/d) (Institute of Medicine 1998b; Bailey 2004).

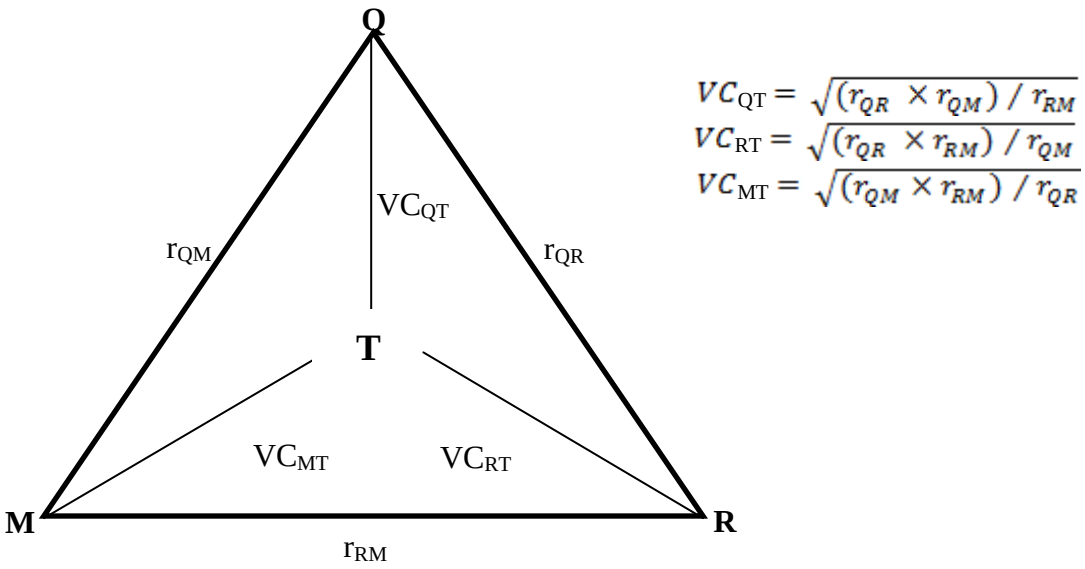


Figure 5.2: Graphical representation of the method of triads (Kaaks 1997; Andersen et al. 2005). T = unknown true dietary intake, Q = estimated intake from SFFQ or FFQ, R = estimated intake from 24 h FR, M = measurement of biomarker, r = correlation and VC = validity coefficient. In this study, the r_{QR} were estimated by Pearson correlation coefficient and r_{QM} and r_{RM} as Partial correlation coefficient.

5.3 Results

5.3.1 Study characteristics

A total of 72 randomly selected pregnant women participated in this study with a mean age ± SD of 30.3 ± 5.5, BMI 27.4 ± 5.5 and parity 2.68 ± 2.5. About 40% had high school and university level of education, only 8.4% were working and with a moderate monthly income; about 6289 ± 3907 Saudi Riyals (~ 23584$). The general characteristics and nutritional biomarkers of the pregnant participants at different trimesters are shown in **Table 5.1**. Serum vitamin B12 level was at lower levels, while serum folate was at higher levels in the pregnant at different trimesters (first trimester

162, second trimester 138.9 and third trimester 137.8) and (first trimester 30.1, second trimester 25.6, and third trimester 22.7) respectively (**Table 5.1**). On the 23 pregnant women who completed the 3 visits, a significant decline in serum vitamin B12 from the first trimester to the second and third trimester ($p < 0.001$) were observed, while the level was constant between second and third trimester. Furthermore, serum folate was constant from the first to second trimester then after the level decreased but it did not reach the significant level (**Table 5.2**).

5.3.2 Median intake of different dietary assessment methods during pregnancy

The estimated energy, macronutrients (protein, fat and carbohydrate), dietary fibre, vitamin B12 and folate derived from the different dietary assessment methods are shown in (**Table 5.3**, **Table 5.4** and **Table 5.5**). **Table 5.3** shows the median daily estimate derived from the SFFQ during pregnancy. There is a slight decrease in the total energy intake as pregnancy advances. Protein, fat, vitamin B12 and folate intake were increased from the first to second trimester, then decreased from the second to third trimester. While carbohydrate was slightly decreased from the first to second trimester, then increased from the second to third trimester. Fibre intake was almost the same throughout pregnancy. During pregnancy, vitamin B12 and folate median intake estimate were 5.8 µg and 232.5 µg respectively. **Table 5.4** shows the median daily estimate derived from the 24 h FR during pregnancy. Of the 40 pregnant women seen at the first trimester, 6 did not complete the 3 days FR and of the 60 at the second trimester, 4 did not complete the 3 days FR.

Table 5.1: General characteristics and nutritional biomarkers of the participants in the study

Nutrient	Pregnant (n = 134)		
	1st trimester (n = 40)	**2nd trimester (n = 60)**	**3rd trimester (n = 34)**
Age (years)	29.4 ± 5.2	30.6 ± 5.4	31.03 ± 5.3
Education level			
Primary	30.0 (12)	28.3 (17)	26.5 (9)
Secondary	30.0 (12)	30.0 (18)	29.4 (10)
High school	27.5 (11)	18.3 (11)	23.5 (8)
University	12.5 (5)	23.3 (14)	20.6 (7)
Occupation			
Not working	97.5 (39)	88.3 (53)	94.1 (32)
Student	-	1.7 (1)	-
Working	2.5 (1)	10.1 (6)	5.9 (2)
Monthly family income (SR)	5854 ± 3824	6544 ± 4176	6568 ± 4283
Parity	2.61 ± 2.4	2.76 ± 2.6	3.26 ± 2.7
BMI (kg/m^2)	27.4 ± 5.6	27.4 ± 5.5	28.9 ± 6.2
Gestational age (wks.)	12.6 ± 0.73	25.9 ± 1.5	35.5 ± 0.74
Pregnancy weight (kg)	67.9 ± 15.7	72.1 ± 13.4	75.6 ± 15.5
Serum vitamin B12 (pmol/l)	162 [129.1, 209.2] (n = 38)	138.9 [110.6, 168.8] (n=59)	137.8 [117.2, 172.9] † (n = 33)
Serum Folate (nmol/l)	30.1 [27.3, 33.3] (n = 38)	25.6 [19.7, 33.3] (n = 55)	22.7 [18.2, 33.1] † (n = 33)

Values present are mean ± SD, % (n) or median [25^{th}, 75^{th} centile], † significant decline ($p < 0.05$) from 1^{st} to 3^{rd} trimester

Table 5.2: The change in serum vitamin B12 and folate throughout pregnancy in the 23 pregnant completed the 3 visits

Characteristic	Pregnant (n = 23)		
	1st trimester	**2nd trimester**	**3rd trimester**
Gestational age (wks.)	12.6 ± 0.73	25.1 ± 1.1	35.3 ± 0.54
Pregnancy weight (kg)	68.4 ± 15.9	71.3 ± 13.4	73.8 ± 15.5
Serum vitamin B12 (pmol/l)	164 [129.1, 201.7]	130.2 [105.1, 159.4] ‡	131 [117.2, 172.9] †
Serum Folate (nmol/l)	28.94 [25.6, 32.6]	28.91 [23.04, 34.03]	23.4 [18.5, 33.9]

Values present are mean ± SD or median [25^{th}, 75^{th} centile], significant decline ‡ $p < 0.001$ between 1^{st} & 2^{nd} trimester, † $p < 0.001$ between 1^{st} & 3^{rd} trimester

Compared to the SFFQ, 24 h FR had similar total energy (1937 vs. 1837, p = 0.38), CHO (251 vs. 252, p = 0.85) and fat (71 vs. 70, p = 0.21) estimated intake. However, estimated protein intake was lower (76 vs. 64, p < 0.001). It also shows that there was a slight decrease in energy, carbohydrate and vitamin B12 intake from the first to second trimester, but increase from the second to third trimester. In addition, protein intake increased, while fat intake decreased during pregnancy. Fibre intake was almost the same throughout pregnancy. During pregnancy, vitamin B12 and folate median intake estimate was 2.4 µg and 162 µg respectively. **Table 5.5** shows the median daily estimate derived from the FFQ during pregnancy. Compared to the SFFQ and 24 h FR, FFQ estimated lower median intake for most nutrients, except for vitamin B12 intake where it was lowest by the 24 h FR.

5.3.3 Correlation coefficient between different dietary assessment methods

The results of unadjusted and energy-adjusted correlation between energy, macronutrients, vitamin B12 and folate intakes derived from the three dietary assessment methods in pregnancy are shown in **Table 5.6**. Similar to the result of the nutrients correlation of the pilot study (**see Chapter 4**), the correlations between the two frequency questionnaires (SFFQ vs. FFQ) were higher compared to the other dietary assessment methods. In the present study, only carbohydrate showed a weak correlation between SFFQ vs. 24 h FR; however, the energy-adjustment correlation improved the correlation between these methods. In addition, vitamin B12 intake showed weaker correlation between SFFQ vs. FFQ and FFQ vs. 24 h FR (r = 0.59, p < 0.001 and r = 0.31, p < 0.001 respectively), however the energy-adjustment correlation improved vitamin B12 correlation between those methods (r = 0.70, p < 0.001 and r = 0.37, p < 0.001 respectively).

Table 5.3: Comparison of estimated energy and nutrients intake by the SFFQ during pregnancy

Nutrient	Pregnancy (n = 134)						**Total pregnant (n = 134)**	
	1st trimester (n = 40)		**2nd trimester (n = 60)**		**3rd trimester (= 34)**			
Energy (kcal)	2013	[1560, 2463]	1925	[1592, 2275]	1915	[1505, 2077]	1937	[1574, 2321]
Protein (g)	77	[62.3, 96.1]	78	[60.8, 89]	76	[57.7, 84.1]	76.3	[61.4, 89.1]
Fat (g)	71	[54.3, 102.8]	74	[58.3, 90.2]	65	[52.9, 93.5]	71	[56.9, 93.2]
Carbohydrate (g)	269	[189.8, 318.9]	234	[201.4, 289]	248	[223.8, 277.3]	251	[200, 299]
Fibre (g)	18	[13.8, 21.9]	18	[13.7, 21.2]	17.4	[14.2, 20.4]	18	[13.9, 21.4]
Vitamin B12 (µg)	**2.1**	**[1.6, 5.6]**	**6.3**	**[4.4, 8.4]**	**4.9**	**[3.9, 7.0]**	**5.8**	**[4.2, 8.0]**
Folate (µg)	**134**	**[7.6, 230]**	**231**	**[188, 278.5]**	**228**	**[181, 263]**	**232.5**	**[188, 278]**

Values are median [25th, 75th centile]

Table 5.4 Comparison of estimated energy and nutrients intake by the 24 h FR during pregnancy

Nutrient	Pregnancy (n = 121)						**Total pregnant (n = 121)**	
	1st trimester (n = 34)		**2nd trimester (n = 56)**		**3rd trimester (n = 31)**			
Energy (kcal)	1851	[1580, 2067]	1778	[1472.5, 2252]	1873	[1540, 2230]	1837	[1534, 2154]
Protein (g)	57.3	[44.8, 80.2]	66.5	[49.9, 83]	69	[58.6, 84.5]	64.3	[50.9, 80.6]
Fat (g)	69	[54.1, 80.6]	67	[50.8, 90.7]	65	[49.3, 86.2]	70	[49.7, 85.4]
Carbohydrate (g)	245	[222, 269]	241	[196, 293]	265	[238, 292]	252	[210, 286.4]
Fibre (g)	12.5	[9.2, 15.7]	12.6	[9.9, 17.3]	13.1	[10.8, 20.5]	13	[9.9, 17.3]
Vitamin B12 (µg)	**2.6**	**[1.3, 3.3]**	**2.0**	**[1.2, 3.9]**	**2.5**	**[1.0, 3.2]**	2.4	[1.2, 3.3]
Folate (µg)	**153**	**[118, 192]**	**168.5**	**[128, 198.5]**	**167**	**[145, 222]**	162	[134, 204]

Values are median [25th, 75th centile]

Table 5.5: Comparison of estimated energy and nutrients intake by the FFQ during pregnancy

Nutrient	Pregnancy (n = 134)						Total pregnant (n = 134)	
	1st trimester (n = 40)		2nd trimester (n = 60)		3rd trimester (n = 34)			
Energy (kcal)	1302	[994, 1667]	1170	[1020, 1470]	1272	[862, 1533]	1264	[982, 1559]
Protein (g)	43	[32, 55]	40.4	[32, 51]	44	[34.5, 55.5]	42.7	[32, 53]
Fat (g)	47	[34, 58]	48	[33, 57]	43	[31.4, 57]	46.9	[33, 58]
Carbohydrate (g)	184	[139, 238]	169	[130, 207]	173	[123, 225]	174	[131, 220]
Fibre (g)	9.0	[6.4, 13]	9.1	[6.7, 12.6]	9.5	[7, 11.5]	9.1	[6.7, 12.6]
Vitamin B12 (µg)	**3.6**	**[3, 5]**	**3.6**	**[3.1, 4.4]**	**4.0**	**[2.7, 4.8]**	**3.7**	**[3.1, 4.5]**
Folate (µg)	**145**	**[103, 175.5]**	**128**	**[103, 152.5]**	**167**	**[145, 222]**	**130**	**[99, 164]**

Values present are median [25th, 75th centile]

Table 5.6: Pearson correlation coefficient between different dietary assessment methods

Nutrient	Energy-unadjusted correlation			Energy-adjusted correlation		
	SFFQ vs. FFQ	SFFQ vs. 24 h FR	FFQ vs. 24 h FR	SFFQ vs. FFQ	SFFQ vs. 24 h FR	FFQ vs. 24 h FR
Energy (kcal)	0.81**	0.27**	0.42**			
Protein (g)	0.73**	0.30**	0.39**	0.81**	0.26**	0.41**
Fat (g)	0.76**	0.33**	0.40**	0.81**	0.26**	0.41**
Carbohydrate (g)	0.73**	0.15	0.34**	0.81**	0.27**	0.42**
Fibre (g)	0.71**	0.25**	0.37**	0.81**	0.27**	0.41**
Vitamin B12 (µg)	0.59**	0.31**	0.31**	0.70**	0.24**	0.37**
Folate (µg)	0.77**	0.32**	0.43**	0.81**	0.26**	0.41**

** correlation is significant at 0.01 level (2 tailed)

5.3.4 The validity coefficient (VC) for vitamin B12 and folate using the method of triads

The correlation between different dietary assessment methods and biomarkers were used to calculate the validity coefficient for SFFQ and FFQ using the method of triads approach and the results are shown in (**Table 5.7)** and (**Table 5.8**) respectively along with the 95% CIs. **Table 5.7** shows that the correlation between both vitamin B12 and folate estimated intake from SFFQ and 24 h FR vs. biomarker level were weak (vitamin B12: r_{QM} = 0.12, r_{RM} = 0.16 and folate: r_{QM} = 0.19, r_{RM} = 0.03 respectively) adjusted for age, BMI and estimated energy intake. However, vitamin B12 VC was higher between biomarker and the unknown true intake (MT = 0.49) followed by SFFQ (QT = 0.40), and 24 h FR (RT = 0.35). In addition, **Table 5.8** shows that the correlation between vitamin B12 intake estimated from FFQ vs. serum level was negative, thus the VC cannot be estimated. In contrast, relatively good correlation between folate intake estimated from FFQ vs. biomarker level was obtained (r_{QM} = 0.31, $p < 0.01$) compared to the 24 h FR. Moreover, folate VC was higher between biomarker and the unknown true intake (MT = 0.64) followed by 24 h FR (RT = 0.56), and FFQ (QT = 0.17).

5.3.5 Serum vitamin B12 insufficiency and estimated intake below and above the recommended dietary intake (RDI) level during pregnancy

From the total pregnant sample (n=133), 50.8% (n= 67) had vitamin B12 < 150 pmol/l. The proportion of pregnant women who had serum vitamin B12 level < 150 pmol/l versus estimated vitamin B12 intake below and above the recommended dietary (RDI) level (2.6 µg/d) is presented in (**Figure 5.2**). The proportion of pregnant women with serum vitamin B12 < 150 pmol/l and dietary vitamin B12 intake < 2.6 µg/day were 6/66 (9%), 10/66 (15.2%) and 39/60 (65%) in SFFQ, FFQ and 24 h FR respectively. In

contrast, the proportion of those pregnant with serum vitamin B12 < 150 pmol/l and dietary vitamin B12 intake ≥ 2.6 µg/d were 60/66 (91%), 56/66 (84.8%) and 21/60 (35%) in SFFQ, FFQ and 24 h FR respectively.

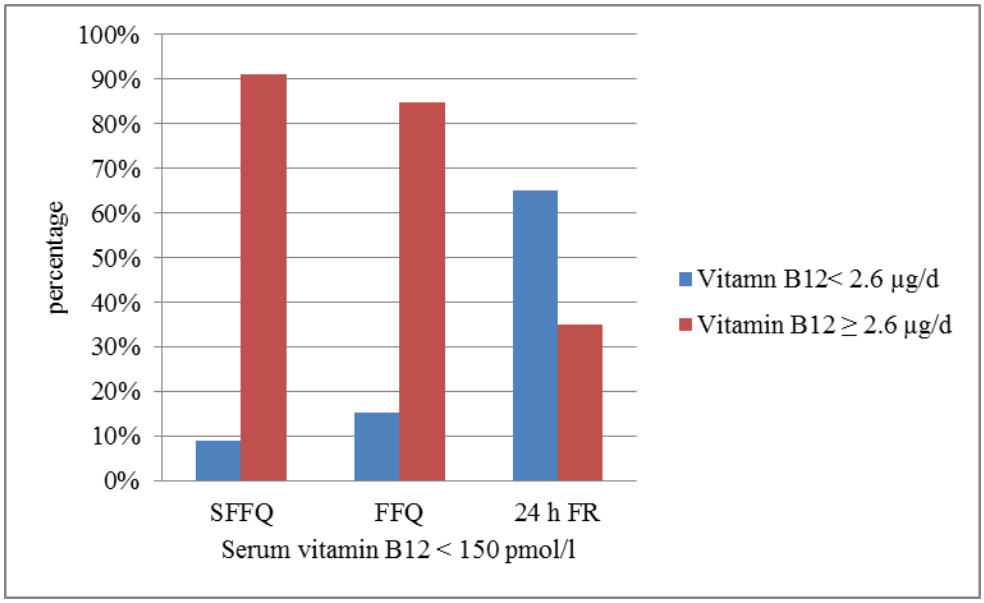


Figure 5.3: Proportion of pregnant women had serum vitamin B12 level < 150 pmol/l vs. estimated intake below and above the RDI during pregnancy.

Table 5.7 Validity coefficients (VC) for vitamin B12 and folate estimated by SFFQ, 24 h FR and serum vitamin B12 and folate levels using the method of triads

	Correlation coefficient (r)			Validity coefficient (VC)			Range for the VC §		
				SFFQ	24 h FR	Biomarker			
	r_{QM}†	r_{RM}†	r_{QR} ‡	QT	RT	MT	QT	RT	MT
Vitamin B12	0.12	0.16	0.24**	0.40	0.35	0.49	0.12 - 0.40	0.16 - 0.35	0.12 - 0.49
Folate	0.19*	0.03	0.26**	0.17	0.44	0.51	0.17 - 0.19	0.03 - 0.44	0.19 - 0.51

T = unknown true dietary intake, Q = SFFQ, R = 24 h FR, M = biomarker (serum level), † Partial correlation adjusted for age, BMI and estimated energy intake, ‡ energy-adjusted correlation, *correlation is significant at 0.05 level, **correlation is significant at 0.01 level, § The lower limit is r_{QM} for the SFFQ and the biomarker and r_{RM} for the 24 h FR and the upper limit is calculated by the method of triads.

Table 5.8 : Validity coefficients (VC) for vitamin B12 and folate estimated by FFQ, 24 h FR and serum vitamin B12 and folate levels during pregnancy using the method of triads

	Correlation coefficient (r)			Validity coefficient (VC)			Range for the VC §		
				FFQ	24 h FR	Biomarker			
	r_{QM}†	r_{RM}†	r_{QR} ‡	QT	RT	MT	QT	RT	MT
Vitamin B12	- 0.03	0.16	0.37**	-	-	-	-	-	-
Folate	0.31**	0.03	0.41**	0.17	0.56	0.64	0.17 - 0.31	0.03 - 0.56	0.31 - 0.64

T = unknown true dietary intake, Q = FFQ, R = 24 h FR, M = biomarker (serum level), † Partial correlation adjusted for age, BMI and estimated energy intake, ‡ energy-adjusted correlation, **correlation is significant at 0.01 level, § The lower limit is r_{QM} for the FFQ and the biomarker and r_{RM} for the 24 h FR and the upper limit is calculated by the method of triads.
VC for vitamin B12 cannot be estimated because of the presence of negative correlation.

5.4 Discussion

In this study, we compared the dietary vitamin B12 and folate intake estimated by the FFQs and 24 h FR with the corresponding nutritional biomarker among the Saudi pregnant population. Firstly, our results on dietary assessment were comparable to what has been shown in the pilot study (**Chapter 4**), where SFFQ generally has a higher energy and nutrient estimate compared to the 24 h FR and FFQ. In fact, the present study showed that FFQ estimated lower mean energy intake by 42% and 37% compared to the SFFQ and 24 h FR respectively, whereas the SFFQ and 24 h FR provided a relatively similar energy estimate with a mean difference of 5.3% among the pregnant women. Furthermore, the energy and carbohydrate intakes estimated by both SFFQ and 24 h FR were similar to what has been reported on 1771 Saudi pregnant women, with a mean intake of 1811 kcal and 251 g respectively (Al-Shoshan 2007a). A higher mean vitamin B12 intake level in the SFFQ versus the 24 h FR were also previously observed (Forsythe and Gage 1994) (4.7 vs. 2.8, $p < 0.05$). The fluctuation on the intake during pregnancy could be related to the change in appetite/food habit accompanying the pregnancy state.

Secondly, The correlation coefficients between the SFFQ and 24 h FR for energy, fat and (energy-adjusted correlation) carbohydrate were comparable to a previous study (Mouratidou et al. 2006). Despite the differences in estimated protein intake between the SFFQ and 24 h FR, they had a relatively good correlation ($r = 0.34$, $p < 0.001$) compared to the other study (Mouratidou et al. 2006). The energy-adjusted correlation coefficient for all macronutrients were higher than the other study (Baer et al. 2005). In addition, our results of the correlation coefficient between the SFFQ and 24 h FR for vitamin B12 was higher compared to the other studies (Wei et al. 1999; Mouratidou et al. 2006) ($r = 0.03$ and $– 0.09$ respectively), while for folate, it was

higher to the other (Mouratidou et al. 2006) (r = 0.29). Moreover, the validity of different dietary assessment methods for micronutrient intake in pregnant women were reviewed and found that the correlation coefficient between SFFQ with a reference method of short-term or long-term intake for vitamin B12 ranged between 0.03 to 0.44, while for folate ranged between 0.29 to 0.57 (Ortiz-Andrellucchi et al. 2009), which supported our results.

Thirdly, the results did not show any correlation between dietary intake and serum levels in both vitamin B12 and folate. Only weak correlation has been shown between FFQs and serum folate. As previously reviewed (Ortiz-Andrellucchi et al. 2009), no study correlated vitamin B12 intake and serum level during pregnancy. However, in women of reproductive age, a slightly comparable result for vitamin B12 correlation between SFFQ and serum was reported (Green et al. 1998; Verkleij-Hagoort et al. 2006) (r = 0.19 and 0.21 respectively). In studies examining folate levels, only one study showed a weak correlation between folate intake and serum level (Brantsæter et al. 2007). In the latter study, folate intake was estimated from both food and supplementation. In general, it was observed that, micronutrient biomarker did not add any more certainty in terms of dietary intake method reliability during pregnancy (Ortiz-Andrellucchi et al. 2009). Unlike most validation studies, in which data from FFQ and one reference method are available, the present study also estimated vitamin B12 and folate intake by using three assessment measures (FFQs, 24 h FR and biomarker) known as the method of triads. Even though the correlations between the methods were low, vitamin B12 VC for the SFFQ was relatively good and similar to the biomarker compared to folate VC, suggesting that the SFFQ is a valid tool particularly when assessing vitamin B12 intake. Unlike the previous study (Verkleij-Hagoort et al. 2006), no Heywood case (VC > 1) was shown for vitamin B12. Heywood case reflects

either the presence of random sampling fluctuation between sample correlations or one or more of the model assumptions was violated, i.e. the VCs are biased (overestimation for the questionnaire and reference method and underestimation for the biomarker) (Ocke and Kaaks 1997). However, a negative correlation was obtained between FFQ and biomarker, thus VC could not be estimated, which is also a type of Heywood case. This could support the underestimation of the FFQ compared to the SFFQ method.

Fourthly, serum vitamin B12 during pregnancy was at the lower level. In addition, it decreased from the first to the second trimester. Unlike other studies (Knight et al. 1991; Ackurt et al. 1995; Miliman et al. 2006; Takimoto et al. 2007), serum vitamin B12 showed a constant level between the second and third trimester. This is supported by Carretti et al. were the pattern of vitamin B12 decreased before the 27th week gestation and after that the levels were almost constant (Carretti et al. 1994).

Fifthly, even though adequate or a high estimated vitamin B12 intake was observed compared to the RDI level, particularly from FFQs, low serum vitamin B12 was present in about half of the sample. Furthermore, the proportion of pregnant women with adequate estimated intake and low serum vitamin B12 (< 150 pmol/l) was high compared to those with high serum vitamin B12 (91 vs. 9% and 84.8 vs. 15.2% for SFFQ and FFQ respectively) and vice versa for 24 h FR (35 vs. 65%). The difference in the FFQs and 24 h FR estimation of vitamin B12 can be explained by under-reporting in the 24 h FR for this vitamin (Harrison et al. 2000; Johansson et al. 2001). The possible factors that could contribute to this are (1) the improving hygiene, refrigeration and prolong cooking of meat products among the general population which is likely to kill the microbes that are essential for the vitamin B12 synthesis in the meat and (2) the methods used to estimate the dietary vitamin B12 (from the food composition tables) could be inaccurate and overestimate the intakes, as they are old and developed in the

1970s (Church 2006). As the hygiene is inversely linked to vitamin B12 bioavailability (Ball 2004b) this could very well be the reason for such over estimation. Studies looking at the bioavailability and nutrient content of different food products are therefore urgently required. In addition, the present results support the need to revise the RDI levels for micronutrients. The current RDI for micronutrients have been derived from levels known to prevent deficiency diseases such as anaemia (Fenech 2001). However, micronutrients, particularly vitamin B12 and folate, play a crucial role in the genomic stability of human cells by preventing chromosomal breakage and hypomethylation of DNA (Fenech 2001), as well as being involved in several other critical pathways during development and adult life (Saravanan and Yajnik 2010). Vitamin B12 and folate supplementation in controlled studies showed that DNA damage can be minimized when plasma vitamin B12 is > 300 pmol/l and folate is > 34 nmol/l. These concentration can only be achieved at intake levels in excess of the current RDI (Fenech et al. 1998; Fenech 2002).

In conclusion, this is the first study to examine the correlation between dietary vitamin B12 and folate intake and its nutritional biomarker among Saudi pregnant women using the method of triads. This study supported our pilot study that the modified SFFQ used in the study gave useful estimates of energy and some nutrients intake in pregnancy, and could be suitable for the investigation of associations between nutrition, health and disease in the future studies among Saudi population. However, further studies on a larger scale population are also needed to assess dietary vitamin B12 and its actual level in blood.

Chapter 6

Maternal vitamin B12 and folate status among Saudi population

6.1 Introduction

Vitamin B12 is an essential nutrient in cell metabolism; particularly in DNA synthesis as well as for homocysteine (Hcy) metabolism. It is an important co-factor for 2 enzymatic reactions: methionine synthase (MS) which converts Hcy to methionine in the presence of vitamin B12 and folic acid and methylmalonyl-CoA mutase (MCM) which is required for the conversion of MM-CoA to Succinyl-CoA in the presence of vitamin B12 (Saravanan and Yajnik 2010). Vitamin B12 in nature is produced by bacteria and other microorganisms and it is only found in foods of animal sources and vegetables contaminated with vitamin B12-synthesized bacteria (Ball 2004b). Nutritional insufficiency of vitamin B12 may cause a variety of disorders including anaemia, megaloblastosis and neurological disease (Baik and Russell 1999; Bender 2003), all of which could theoretically be linked to the block in Hcy re-methylation or the resulting hyperhomocysteinemia (Scott 1999; Selhub et al. 2007).

During pregnancy, women have increased an physiological need for this vitamin (Milman et al. 2006). This is to meet the increased demand for rapid cell proliferation that occurs in the developing embryo, foetus, neonate and pregnant women (Hussein et al. 2009). An average of 20 - 30% of pregnant women have been reported to suffer from a vitamin deficiency (Baker et al. 2002). Furthermore, frequencies of low birth weight infants, still births, and babies born with congenital disorders are high among mothers suffering from malnutrition (Leader et al. 1981; Pitkin 1981). As nutritional requirements are high, the effects of malnutrition can be severe and long-lasting in pregnant mothers (Ackurt et al. 1995). Thus, adequate dietary intake of vitamin B12 is required. As previously discussed in **Chapter 1**, inadequate vitamin B12 status has been reported to be associated with a significantly increased risk for NTDs (Suarez et al. 2003; Molloy et al. 2008). Even after the era of folic acid fortification started, the risk

for NTDs tripled in the presence of low maternal vitamin B12 (Ray and Blom 2003; Ray et al. 2007). So, the recent concern is the potential role of folic acid to mask or exacerbate vitamin B12 insufficiency (Miller et al. 2009). Furthermore, Hcy and related B-vitamins like vitamin B12 and folate have been shown to play a crucial role in foetal nutrition, growth and development (Hoet and Hanson 1999; Molloy et al. 2009). It has been found that high total Hcy and low B-vitamin concentrations are associated with birth defects and common pregnancy complications, such as preeclampsia and recurrent early pregnancy loss (Aubard et al. 2000; Nelen et al. 2000; Vollset et al. 2000; Refsum 2001). Recently, the imbalance between the two vitamins (vitamin B12 and folate) has been one of the main areas of interest by researchers furthering understanding of the effect of these vitamins on human health. It has been reported that the combination of low maternal vitamin B12 and adequate/high folate concentration could increase adiposity and insulin resistance in the offspring (Yajnik et al. 2008) as well as insulin resistance and prevalence of GDM in the mother (Krishnaveni et al. 2009). In addition the interaction between serum folate and vitamin B12 status was examined in older people from US National Health and Nutrition Examination Survey (NHANES) between 1999 - 2002. It was reported that cognitive impairment and anaemia were higher among those with low vitamin B12 and high folate compared with low vitamin B12 and adequate folate levels (Morris et al. 2007). In a follow-up study using the NHANES (before and after folic acid fortification) data, the combination of low serum vitamin B12 and high serum folate was also associated with higher Hcy and MMA (Selhub et al. 2007). More recently, a cross-sectional study from the Sacramento Area Latino Study on Ageing (SALSA) provided an important confirmation of the metabolic association, but not the worse cognitive impairment (Carmel 2009; Miller et al. 2009). A separate study in an elderly UK population reported no evidence of metabolic,

cognitive, or anaemia association of such combination (low vitamin B12 and high folate levels) in the setting of voluntary fortification (Clarke et al. 2008). The interaction between folate and vitamin B12 status imply that in vitamin B12 insufficiency, high folate status is associated with the impaired activity of the 2 vitamin B12 dependent enzymes, MS and MCM (Selhub et al. 2007; Selhub et al. 2009).

As maternal vitamin B12 stores in women eating a mixed diet are approximately 3000 µg and the vitamin requirement of the foetus is approximately 50 µg, it may be assumed that the event of a single pregnancy has minimal impact on maternal stores (Koebnick et al. 2002; Schulpis et al. 2004). However, vitamin B12 insufficiency occurs in 10 - 28% of women with uncomplicated pregnancies (Pardo et al. 2000; Schulpis et al. 2004; Takimoto et al. 2007). As previously reviewed in **Chapter 2,** vitamin B12 insufficiency is not rare during pregnancy and it varied between different countries. In a vegetarian population, such as in India and Nepal, vitamin B12 insufficiency was common with an average prevalence of 50 - 60% (Bondevik et al. 2001; Pathak et al. 2007; Yajnik et al. 2008; Katre et al. 2010). In the Middle East (the area of our interest), it showed an average prevalence of 49 and 51% at the second and third trimester respectively (Ackurt et al. 1995; Pardo et al. 2000). Vitamin B12 status has not previously been studied in pregnant Saudi women, particularly in the presence of mandatory wheat flour fortification with folic acid (150µg/100g) in the KSA (Food Safety Authority of Ireland 2006; Safdar et al. 2007). Therefore, we aimed to investigate the vitamin B12 and folate status among Saudi pregnancies and estimate the frequency of vitamin B12 and folate insufficiency at the second trimester compared to a control group of healthy non-pregnant Saudi women. Furthermore, identify the factors that independently predict vitamin B12 insufficiency during pregnancy. We also examined the interaction between serum vitamin B12, folate and Hcy levels.

6.2 Materials and methods

6.2.1 Study population

A total of 394 healthy women: 186 pregnant and 208 non-pregnant control women living in Riyadh, KSA participated in the present study. Subjects were randomly selected from women attending the antenatal clinics and other primary health clinics from 4 primary health care centres and the Prince Salman Bin Abdulaziz Hospital in the western health sector in Riyadh city. Subjects who agreed to participate were asked to fast before their visit to be enrolled in the study. Women aged 18 - 40 years, non-smokers, with pre-pregnancy BMI > 17 and < 37 kg/m², without any significant medical problems and not taking any medication were eligible for the study. Women were excluded if they were anaemic, on multivitamins or had any nutrition related pre-existing medical condition such as coeliac disease, known DM or had any liver, cardiac or kidney disease. As a routine practice, general practitioners and obstetricians at the hospitals were prescribing a folic acid supplement of 500 µg/day in the first trimester and fefol (iron and folic acid) tablets 200 mg/day afterwards for all pregnant women attending the clinic.

6.2.2 Ethical approval

The Research Ethics Committee at Prince Salman Bin Abdulaziz Hospital, and the General Directorate of Health Affairs at the Ministry of Health in Riyadh approved the study protocol. The written informed consent was obtained from every participant.

6.2.3 Data collection

The data collection was carried out from May 2009 to December 2010. Data from pregnant women was collected at the second trimester; between 16 - 28 weeks gestation.

Sociodemographic and anthropometric data was collected. The gestational age and maternal weight prior to or at the beginning of gestation were obtained from the patient's medical record. Height and pregnancy weight were measured using the appropriate international standard scale (Digital Person Scale, ADAM Equipment Inc., USA). Blood pressure was determined with a mercury Sphygmomanometer while participants were in a sitting position after being allowed 15 minutes' rest. A research nurse interviewed the participants, explained the study protocol, obtained the consent form and prepared them for blood sampling.

According to the Ministry of Health Protocol for GDM screening, pregnant women with a high risk of developing GDM and between 24 - 28 weeks of gestation should have a 75-gm OGTT (Oral Glucose Tolerance Test) to confirm the presence of DM during pregnancy. Pregnant women who meet WHO criteria for DM (fasting glucose ≥ 7.0 mmol/l or 2 h glucose ≥ 11.1 mmol/l) or impaired glucose tolerance (IGT) (2 h glucose ≥ 7.8 mmol/l and non-diabetes) are classified as having GDM (WHO Consultation 1999; Clarke et al. 2008). From a total of 82 pregnant women who had this test, only 10 (12.2%) pregnant women were diagnosed as having GDM. In the present study, GDM women were used to evaluate and compare the different biomarker levels with the normal pregnant and non-pregnant control women.

6.2.4 Blood samples and laboratory analysis

Fasting blood samples were collected around 8:00 am from the pregnant and non-pregnant control women for the measurement of serum vitamin B12, folate, and homocystiene. Serum samples were separated from whole blood collected in the plain tube after standing for 20 - 30 minutes, then centrifuged in 3500 rmp for 15 minutes. Serum aliquots were then kept in the ice-box for about 4 - 6 hours, then later transferred

and stored in a - 80° fridge. At the end of the study, all samples were shipped in dry ice boxes from the research lab at the UDC, Riyadh, KSA to the Clinical Sciences Research labs at the University of Warwick, Coventry, UK after signing the academic human material transfer agreement between the parties, and stored until measurements. Serum vitamin B12 and folate were measured using the same techniques as mentioned in the previous chapter (see **Chapter 5**). Serum Hcy was measured by ion-paired reversed-phase HPLC (high-performance liquid chromatography) with electrochemical detection as described in Martin et al. (Martin et al. 1999). Hcy analyses were performed in a serum sample. Hcy in serum or plasma is shown to be stable for a few days at room temperature (Ueland et al. 1993); but our samples were prepared in less than an hour and immediately kept on ice. In addition, no significant difference was found comparing Hcy in the serum and plasma samples using the HPLC analyser (Church 2006). Imprecision data indicated intra-assay coefficients variations (CVs) were 2.2% at mean concentration 7 µM (n = 20) and 2.4% at 20 µM (n = 20), while the inter-assay CVs were 8.6% at mean concentration 8.5 µM (n = 8) and 6.8% at 25 µM (n = 8). The reference intervals (for individuals aged 20 - 74 years) was 4.6 - 14.2 µmol/L.

6.2.4 Statistical analysis

All statistical analyses were performed using the SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). The data was expressed as mean ± SD or median [interquartile range]. Means were compared using independent t-test. The p-value of less than 0.05 was considered significant. As there is no specific serum cut-off for pregnancy, the cut-off levels used to define vitamin B12 and folate insufficiency were similar to what has been illustrated in the previous chapters (see **Chapter 3** and **5**). The cut-off levels for defining elevated homocysteine level during pregnancy was varied and ranged between

> 6.71(Hogeveen et al. 2010) to ≥ 15.42 (Park et al. 2004). However, the cut-off level of > 10 umol/l was used in the present study as previously defined (Yajnik et al. 2008; Katre et al. 2010). Pearson correlation coefficient was used to determine the strength of the relationship between serum vitamin B12, folate and Hcy among pregnant and non-pregnant control women. Scatter plots were used to present this association through the regression line after removing the extreme outliers as described in (Williams 2011). Stepwise linear regression analysis was used to identify factors that were independently associated with serum vitamin B12 levels during pregnancy. Maternal age, BMI, gestational age, weight gain during pregnancy and serum folate and Hcy levels were used as independent variables. In addition, logistic regression analysis was used to assess the influence of maternal serum vitamin B12 (as a dichotomous variable) on birth weight. The proportion of women who had a serum vitamin B12 below or above the cut-off level value was compared with the normal folate level. Geometric mean (95% CI) circulating Hcy for serum folate categories (tertiles) was estimated according to serum vitamin B12 cut-off levels.

6.3 Results

6.3.1 Study characteristics

A total of 394 randomly selected women participated in this study: 186 pregnant (10 had GDM and 176 normal pregnancy) and 208 control non-pregnant women. As expected, GDM pregnant women were older with a mean age of 35.5 ± 5.0 years and heavier with a mean BMI of 29.8 ± 6.7 kg/m2 compared with normal pregnant and non-pregnant women with mean age of 28.45 ± 5.4 and 27.3 ± 5.4 years and mean BMI of 26.4 ± 5.23 and 25.01 ± 3.4 kg/m2 respectively. The mean gestational age and weight

gain during pregnancy in GDM and normal pregnancy were 25.8 ± 1.6 weeks and 3.9 ± 3.7 kg and 22.8 ± 4.1 weeks and 4.8 ± 5.1 kg respectively. The general characteristics of the pregnant and non-pregnant control women are shown in (**Table 6.1**).

6.3.2 Study biomarkers results

The biomarkers results of the pregnant and non-pregnant control participants in the study are shown in **Table 6.2.** This shows that GDM women had lower serum vitamin B12 and higher serum folate compared to the normal pregnant (median vitamin B12 125.5 vs. 144.4, $p = 0.45$ and folate 24.9 vs. 21.4, $p = 0.256$) and non-pregnant control women (median vitamin B12 125.5 vs. 237.7, $p < 0.001$ and folate 24.9 vs. 17.9, $p = 0.001$).

Similarly, normal pregnant women had significant lower serum vitamin B12 (median 144.4 vs. 237.7, $p < 0.001$) and higher serum folate (median 21.4 vs. 17.9, $p < 0.001$) compared to the non-pregnant control women. Of the GDM, normal pregnant and non-pregnant women, 70%, 54% and 12.5% had a vitamin B12 concentration below the cut-off level (150 pmol/l) while 0%, 3% and 4% had folate concentration below the cut-off level (9 nmol/l) respectively. Interestingly, normal pregnant women had lower serum Hcy compared to the GDM and non-pregnant women (median 4.45 vs. 5.4, $p = 0.099$ and 7.2, $p < 0.001$ respectively). About 13% of non-pregnant women had a Hcy level more than 10 umol/l compared to 0% and 1.7% in GDM and pregnant women respectively.

Table 6.1 General characteristics of the participants in the study

	GDM (n = 10)	**Normal pregnant (n = 176)**	**Control (n = 208)**
Age (years)	35.5 ± 5.0	28.45 ± 5.4 (156) *	27.3 ± 5.4 ††
Weight (kg)	69.0 ± 13.5	64.0 ± 13.2 (156)	62.1 ± 9.6 †
Height (cm)	153.6 ± 5.3	156.2 ± 6.97 (162)	157.6 ± 6.4
BMI (Kg/m^2)	29.8 ± 6.7	26.4 ± 5.23 (156)	25.01 ± 3.4 ‡
Systolic BP	107.8 ± 11.0	106.7 ± 10.2 (160)	111.4 ± 9.6 (195) ‡‡
Diastolic BP	67.9 ± 6.6	67.5 ± 8.9 (160)	72.4 ± 6.1 (195) † ‡‡
Weight gain in pregnancy (kg)	3.9 ± 3.7	4.8 ± 5.1 (151)	-
Gestational age (wks.)	25.8 ± 1.6 (9)	22.8 ± 4.1 (153) *	-

Values present are mean ± SD, (n), BP = blood pressure, * $p < 0.001$ between GDM vs. normal pregnant, † $p < 0.05$ or †† $p < 0.001$ between GDM vs. control, ‡ $p < 0.05$ or ‡‡ $p < 0.001$ between normal pregnancy vs. control.

Table 6.2 Biomarkers results of the participants in the study

	GDM (n = 10)	**Pregnant (n = 176)**	**Control (n = 208)**
Serum Hcy (umol/l)	(n = 10)	(n = 176)	(n = 207)
Median [25th, 75th centile]	5.4 [4.9, 5.7]	4.45 [3.5, 5.4]	7.2 [6.0, 8.8]
Mean ± SD	5.3 ± 1.07 †	4.6 ± 1.65 *	7.6 ± 2.53
Range	3.6 - 7.3	1.5 - 10.96	2.9 - 18.2
> 10 umol/l, % (n)	0 (0)	1.7 (3)	13 (27)
Serum vitamin B12 (pmol/l)	(n = 10)	(n = 174)	(n = 201)
Median [25th, 75th centile]	125.5 [94.6, 181.5]	144.4 [112.8, 182.5]	237.7 [183.2, 312.9]
Mean ± SD	148.1 ± 72.2 †	162 ± 117.6 *	264.2 ± 122.2
Range	74.7 - 313.8	54.2 - 1476	87.4 - 814
< 150 pmol/l, % (n)	70 (7)	54 (94)	12.4 (25)
Serum folate (nmol/l)	(n = 9)	(n = 171)	(n = 197)
Median [25th, 75th centile]	24.9 [22.5, 26.1]	21.4 [16.1, 29.7]	17.9 [14.1, 20.8]
Mean ± SD	25.0 ± 3.8 †	23.2 ± 9.3 *	18.7 ± 6.75
Range	20.4 - 32.6	7.6 - 45.3	6.8 - 45.3
< 9 nmol/l, % (n)	0 (0)	1.8 (3)	2.0 (4)

Values present are mean ± SD or median [25th, 75th centile], * $p < 0.001$ between normal pregnant vs. control, † $p \leq 0.001$ between GDM vs. control.

The distribution of serum vitamin B12, folate and Hcy levels among normal pregnant and non-pregnant women are shown in a histogram in **Figure 6.1, 6.2** and **6.3** respectively.

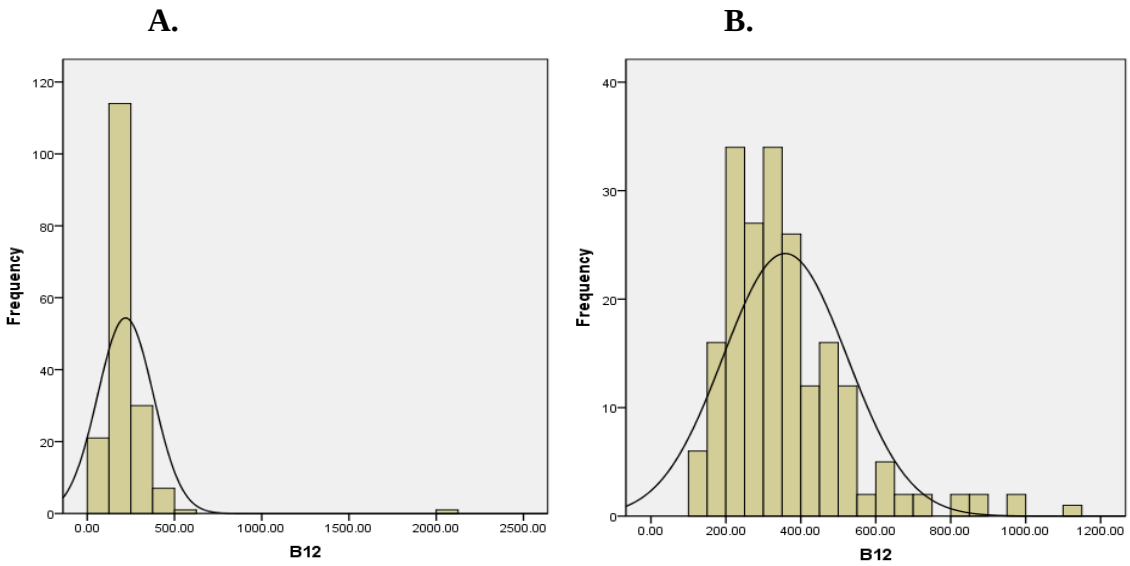


Figure 6.1: Frequency distribution of serum vitamin B12 levels among **A.** pregnant (n = 174) and **B.** non-pregnant control (n = 201) women.

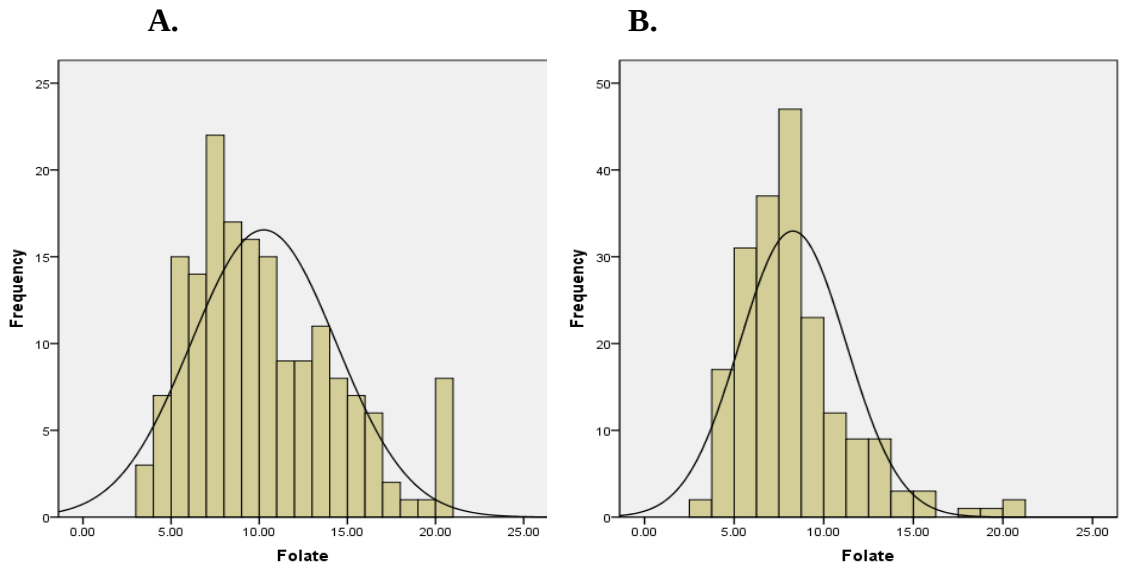


Figure 6.2: Frequency distribution of serum folate levels among **A.** pregnant (n = 171) and **B.** non-pregnant control (n = 197) women.

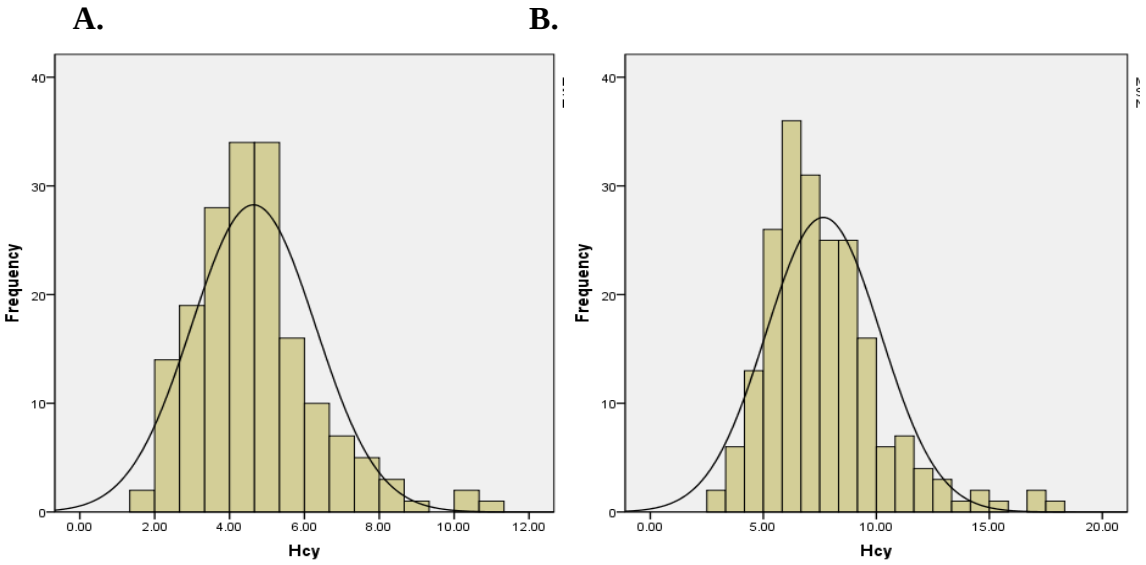


Figure 6.3: Frequency distribution of serum Hcy levels among **A.** pregnant (n = 176) and **B.** non-pregnant control (n = 207) women.

6.3.3 Variables associated with serum vitamin B12 and folate insufficiency

A Pearson correlation coefficient and scatter plots were used to describe the variables associated with vitamin B12 and folate insufficiency. A Significant negative association with Hcy was obtained for both serum vitamin B12; $r = -0.27$, $p < 0.001$, and folate; $r = -0.19$, $p = 0.007$ in the non-pregnant control women. No association was found between serum vitamin B12 or folate and Hcy levels in normal pregnant women (see **Figure 6.4** and **6.5**).

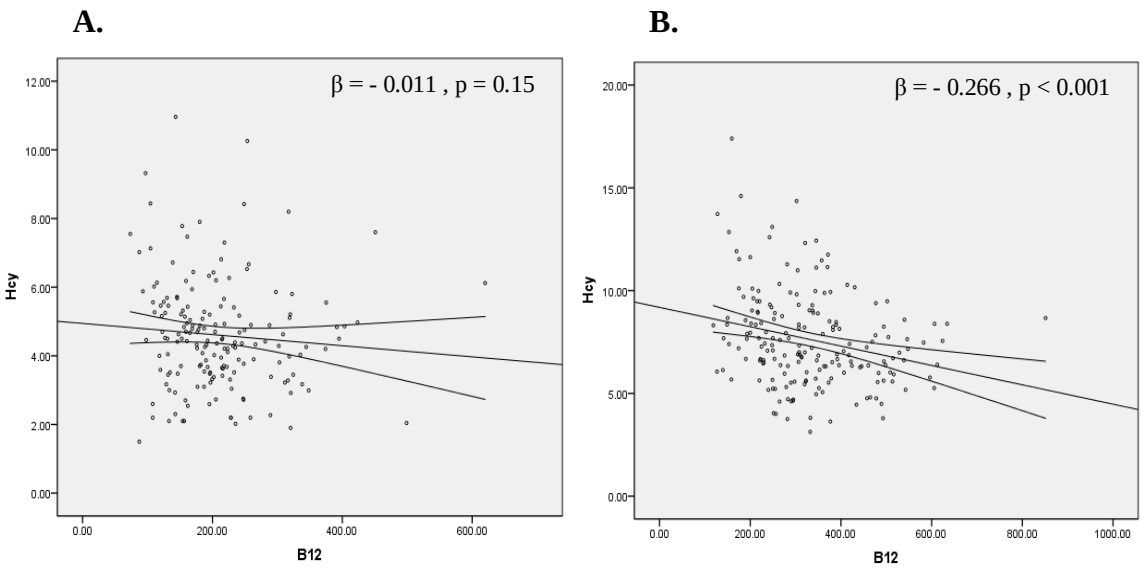


Figure 6.4: Scatter plots show regression – serum vitamin B12 vs. Hcy levels **A.** pregnant **B.** non-pregnant control women after removing extreme outlier. The med line shows the mean value and outer lines the 95% CI.

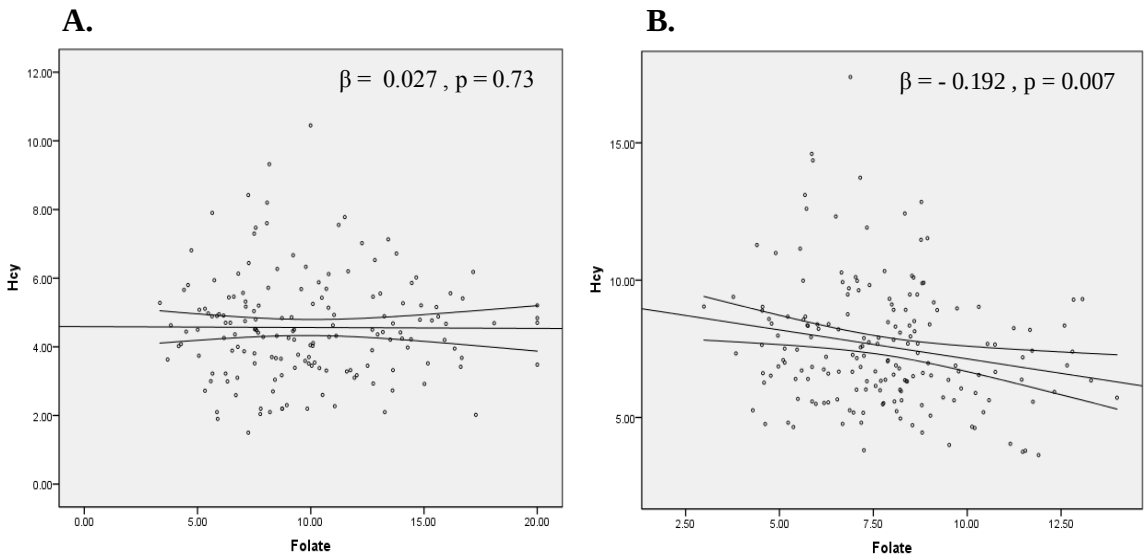


Figure 6.5: Scatter plots show regression – serum folate vs. Hcy levels **A.** pregnant **B.** non-pregnant control women after removing extreme outlier. The med line shows the mean value and outer lines the 95% CI.

In addition, stepwise linear regression analysis was used to predict factors that were independently associated with serum vitamin B12 insufficiency in pregnancy**.** It

was found that BMI was the strong negative predictor of serum vitamin B12 during normal pregnancy (standardized β (95% CI) - 0.210 (- 13.03, - 1.24), p = 0.02) using maternal age, gravidity, parity, BMI, gestational age, weight gain during pregnancy, serum Hcy and folate levels as independent variables. Pregnant women with low vitamin B12 levels had a mean BMI of 27.2 ± 5.3 compared to 25.4 ± 5.0 in those with normal levels. **Figure 6.6** shows the comparison scatter plots of the association between serum vitamin B12 and BMI in both pregnant and non-pregnant women.

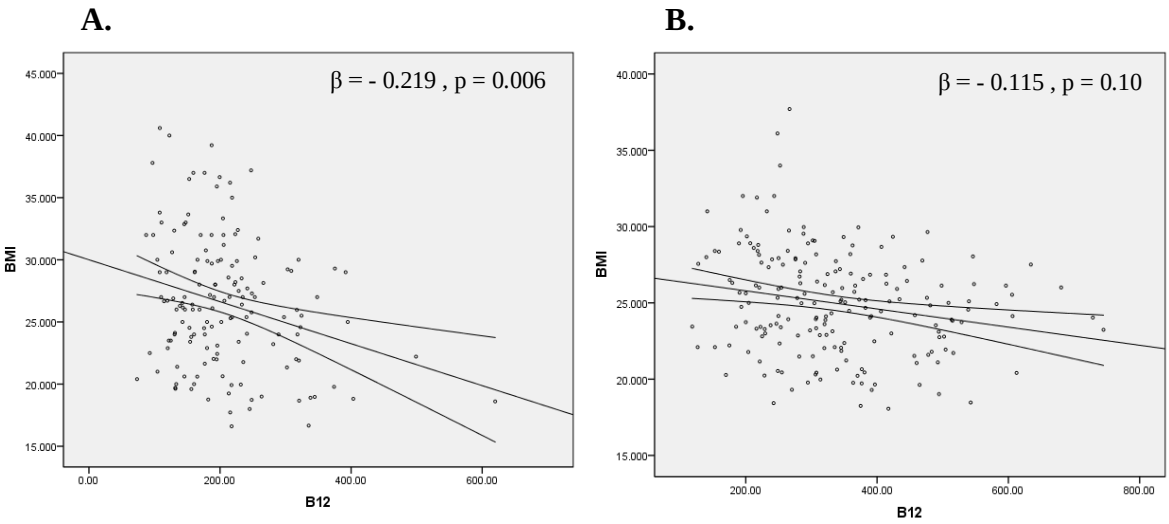


Figure 6.6: Scatter plots show regression – serum vitamin B12 vs. BMI **A.** pregnant **B.** non-pregnant control women after removing extreme outliers. The med line shows the mean value and outer lines the 95% CI.

Furthermore, a birth weight measure was available for only 45 normal pregnant women; mean 3.02 ± 0.5 (range 1.3 - 3.8) kg. So we studied the possible association between maternal serum vitamin B12 and birth weight. No significant association between low vitamin B12 and birth weight was found in normal pregnant women (OR 0.288, 95% CI (0.072 - 1.146), β = - 1.245, p = 0.07). However, pregnant women with low vitamin B12 levels had a slightly higher mean birth weight 3.20 ± 0.3 compared to those with normal levels 2.90 ± 0.6.

6.3.4 Serum vitamin B12, folate and Hcy

As shown in **Table 6.2**, about half of the Saudi pregnant women had low serum vitamin B12 compared to 12% in the non-pregnant control women. Furthermore, it was observed that the proportion of pregnant women with low vitamin B12 and normal folate levels was higher (55.4%) compared to the control women (13%) (**Figure 6.7**).

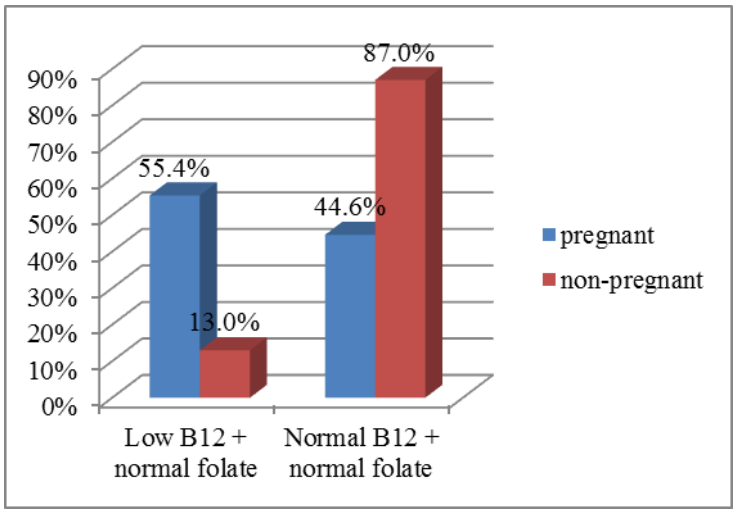


Figure 6.7: The proportion of pregnant and non-pregnant control women with (low vitamin B12 + normal folate levels) and (normal vitamin B12 + normal folate levels).

To understand the interaction between serum vitamin B12, folate and their functional biomarker Hcy during the pregnancy and non-pregnancy state, serum Hcy levels were compared against different folate categories and vitamin B12 cut-off levels (**Table 6.4**). Hcy levels were higher in all categories in vitamin B12 insufficient control women compared to those with normal vitamin B12 levels. However, such differences were not seen in pregnant women. In addition, we did not observe any increase in Hcy levels with increasing folate categories in the vitamin B12 insufficient group in both pregnant and non-pregnant women.

Table 6.3 Hcy concentration by serum vitamin B12 and serum folate categories.

	Pregnant			**Control**		
	Geometric mean Hcy			Geometric mean Hcy		
Serum folate	Serum vitamin B12 pmol/l			Serum vitamin B12 pmol/l		
nmol/l	< 150	≥ 150	p-value	< 150	≥ 150	p-value
5.9	4.4 (3.9 - 5.1) (24)	4.3 (3.8 - 5.0) (20)	0.82	10.2 (7.8 - 13.3) (9)	7.4 (6.9 - 7.9) (68)	0.002
8.3	4.3 (3.6 - 5.2) (22)	4.1 (3.4 - 5.0) (23)	0.75	9.02 (7.6 - 10.8) (9)	7.2 (6.7 - 7.7) (69)	0.03
13.3	4.6 (4.1 - 5.1) (48)	4.2 (3.7 - 4.7) (34)	0.21	7.0 (5.4 - 9.1) (7)	5.9 (5.3 - 6.6) (34)	0.19

Values presented are geometric mean (95% CI) (n). Serum folate values are category medians

6.4 Discussion

This study has examined the vitamin B12 and folate status among Saudi pregnant and non-pregnant control women. It has observed firstly that vitamin B12 insufficiency is more common than folate insufficiency in Saudi women, particularly during pregnancy. This is consistent with the results from other studies, from both known vegetarian and non-vegetarian populations which examined serum vitamin B12 and folate levels at the Second trimester, using approximately similar cut-off levels such as the studies from India: 60% vs. 0.2%, n = 638 (Yajnik et al. 2008) and 73% vs. 1%, n = 163 (Katre et al. 2010), Nepal: 49% vs. 4.5%, n = 328 (Bondevik et al. 2001), Newfoundland: ~ 44% vs. 3%, n = 1424 (House et al. 2000) and UK: 20% vs. 6%, n = 200 (Saravanan et al. 2010b). The median folate level at 16 - 28 wks. gestation was significantly higher compared to non-pregnant control women. Furthermore, more pregnant women had a serum folate above the level of 43 nmol/l compared to non-pregnant women (4.7% vs. 1.5%, p = 0.08). This is likely due to the folic acid supplementation practice during pregnancy. The median level of vitamin B12 in our study population was similar to what was observed in a recent study of a Caucasian population where the median vitamin B12 level in pregnant women (between 16 - 18 wks. gestation, mean age 27 yrs.) was significantly lower compared to age-matched control non-pregnant women (193.4 vs. 267.9 pmol/l, $p \leq 0.001$) respectively. The prevalence of vitamin B12 insufficiency (< 141 pmol/l) in that population was 20 vs. 4% respectively (Saravanan et al. 2010b). In addition, our results in non-pregnant control women were slightly comparable to a previous study on 784 Saudi healthy non-pregnant women (between 20 - 69 yrs.) which showed that mean vitamin B12 and folate levels were 292 ± 102 pmol/l and 14.4 ± 5.26 nmol/l respectively (Ardawi et al. 2002). However, the serum folate level was higher in another study with a mean of 33.1 ± 13.6 nmol/l (Al-Assaf et

al. 2007). In a country such as Saudi Arabia, a wide range of food is easily available to women during pregnancy (Al-Shoshan 2007a); thus it is expected that women have an adequate nutrient status during this period. However, despite the adequate vitamin B12 intake that has previously been observed in (Al-Shoshan 2007a) and (**Chapter 5**) compared to the recommended level, more than half of the Saudi pregnant women were found to be vitamin B12 insufficient compared to 12% non-pregnant women. Furthermore, the combination of low serum vitamin B12 and adequate serum folate levels was higher in the pregnant compared to the non-pregnant women. This could be owing to the increased metabolic utilization of vitamin B12 during pregnancy, especially in the presence of higher folate levels (Saravanan et al. 2010b). The results have also found that GDM women had even lower vitamin B12 and higher folate. This result is consistent with a result from previous studies (Seghieri et al. 2003; Guven et al. 2006).

Secondly, Hcy levels can be increased as a result of either inadequate vitamin B12 or folate status during pregnancy (Molloy et al. 2008). However, in the present study, we did not find any association between Hcy and vitamin B12 or folate during normal pregnancy. In fact, the Hcy level was lower in the pregnant compared to the non-pregnant control women. The presence of low Hcy concentrations during normal pregnancy has been confirmed by many investigations (Kang et al. 1986; Andersson et al. 1992; Holmes 2003). In addition, 30 - 60% lower in Hcy concentrations were found in pregnant compared to non-pregnant women (Ueland and Vollset 2004), and the lowest values were observed in the Second trimester (Andersson et al. 1992; Walker et al. 1999; Murphy et al. 2002; Ueland and Vollset 2004). In contrast, elevated Hcy was present in 13% of non-pregnant control women. The Hcy level was slightly comparable to a previous study on Saudi women with a mean of 8.5 ± 2.7 umol/l (Ardawi et al.

2002). Note that their study sample were older compared to the present study sample. Furthermore, Hcy concentration was inversely associated with vitamin B12 and folate in non-pregnant control women. Thus, it is suggested that folic acid supplementation could have an impact in Hcy concentrations during pregnancy, resulting in higher folate and lower Hcy concentrations in pregnancy compared to the non-pregnant control women (Walker et al. 1999). However, in a longitudinal study, Hcy reduction was not explained by folic acid supplementation during pregnancy (Murphy et al. 2002). Other studies in non-pregnant subjects demonstrated the relationship between vitamin supplementation - containing folic acid and vitamin B12 - and Hcy reduction (Ubbink et al. 1993; Ubbink et al. 1994). Other possible factors that could contribute to a low Hcy concentration during pregnancy are the physiological effect of pregnancy (Murphy et al. 2002; Holmes 2003) as well as foetus uptake of the Hcy (Andersson et al. 1992; Malinow et al. 1998; Holmes 2003).

Thirdly, vitamin B12 insufficiency in our study appeared to be associated with BMI among pregnant women. This is consistent with other results from Mysore in India, where lower serum vitamin B12 levels during pregnancy were associated with maternal adiposity compared to normal levels (Krishnaveni et al. 2009). Similarly, the results from the Caucasian population (see **Chapter 3**) showed a similar association between vitamin B12 insufficiency and maternal BMI during pregnancy. This in turn leads to 2 possible interpretations for this association: one could be that vitamin B12 insufficiency promotes adiposity or adiposity/obesity lowers serum vitamin B12 (Krishnaveni et al. 2009). A high prevalence of low micronutrients levels in overweight and obese individuals has previously been reported (Kimmons et al. 2006; Mahabir et al. 2008; Krishnaveni et al. 2009). This may be as a result of inadequate nutrient intake and/or an alteration in nutrient absorption or metabolism (Kimmons et al. 2006;

Krishnaveni et al. 2009). Both low serum vitamin B12 and adiposity may be as a result of poor quality dietary intake (food rich in energy but less so in micronutrients) (Krishnaveni et al. 2009). However, if this explained the association between vitamin B12 insufficiency and adiposity, we would expect to have a similar association in the non-pregnant control women.

Fourthly, as previously mentioned the effect of the combination of both low vitamin B12 and elevated folate on the increased Hcy level before and after the mandatory folic acid fortification (NHANES III and NHANES 1999-2002 respectively) in the US (Selhub et al. 2007) and in Mexico (SALSA) (Miller et al. 2009) has been reported. However, the present study was first to demonstrate such interaction among both Saudi pregnant and non-pregnant women. The results showed a different pattern between the 2 groups. The non-pregnant women had a similar trend to what was presented in the NHANES III survey, where geometric mean Hcy decreased across increasing serum folate categories reaching the significant level among women with high vitamin B12 levels. Unlike the NHANES 1999-2002 and SALSA surveys, non-pregnant women did not have higher Hcy with higher folate and low vitamin B12 categories. On the contrary, geometric mean Hcy was not different across different folate categories and vitamin B12 cut-off levels in the pregnant women. However, we observed a slight increase in geometric mean Hcy among the highest folate category and low vitamin B12 compared to high vitamin B12 levels. The difference in our results compared to the NHANES 1999 - 2002 and SALSA studies could be explained by the fact that the present study used younger people and a female sample only and the folate levels were ranged within normal reference intervals with a maximum level of 45.3 nmol/l.

Finally, this is the first investigation to study the vitamin B12 and folate status during pregnancy among the Saudi population. However, it is cross-sectional in design and so it is not possible to describe a cause and effect relation between vitamin B12 insufficiency and adequate/high serum folate. Moreover, vitamin B12, folate and Hcy concentrations are difficult to interpret in pregnancy and there is no agreed cut-off levels for deficiency, so we assigned the cut-off level based on previous published studies.

In conclusion, this study showed that at least half of the Saudi pregnant women had vitamin B12 insufficiency and more pronounced compared to folate insufficiency. Furthermore, vitamin B12 insufficiency was associated with maternal obesity during pregnancy. Therefore, well-planned prospective studies are needed to investigate the influence of vitamin B12 insufficiency in early pregnancy and the risk of diabetes in mother and offspring.

Chapter 7

Discussion

7.1 Discussion

It is well known that influences linked to early growth and development have an important effect on the risk of T2DM and CVD. The intrauterine environment, particularly maternal nutrition, can alter the vital development process *in utero*. Therefore, if the foetus is exposed to an adverse intrauterine environment, it can be "programmed" to develop diseases in childhood and adult life (Godfrey and Barker 2000; Barker 2004; Mcmillen and Robinson 2005), including metabolic disorders. Thus, such "gene-diet and gene-environment" interactions, particularly through DNA methylation, during foetal development could be an important contributor to the epidemic of T2DM (Fenech 2001). However, the exact mechanism of such an interaction is not clear. There is some evidence that 1-C metabolism involving key vitamins such as vitamin B12 and folate could play an important role in pregnancy and the metabolic risk of the offspring (Yajnik et al. 2008). In particular, the intrauterine imbalance between these two vitamins (low vitamin B12 and high folate) might play a crucial role in foetal programming, which seriously overlap with the growing global concern about obesity and adiposity (Rosenberg 2008). In a separate study from India, low maternal vitamin B12 in the presence of a high folate level was also related to a higher incidence of GDM, higher adiposity and future T2DM in mothers (Krishnaveni et al. 2009). Whether such imbalance is common in other parts of the world, in particular in the region of our interest where metabolic disorders are the highest in the world (Saudi Arabia) is not known. This thesis therefore focused on the prevalence of vitamin B12 insufficiency during pregnancy, across the world and in particular in Saudi Arabia, in order to identify the potential contribution of maternal vitamin B12 status during pregnancy and the associated metabolic risk.

In the first part of the thesis, we aimed to understand the worldwide pattern of vitamin B12 insufficiency, particularly in the non-vegetarian population. This was done by doing a systematic review to identify all relevant observational studies in the literature investigating the prevalence of vitamin B12 insufficiency during pregnancy. Vitamin B12 insufficiency was demonstrated in the different continents and countries, and the emerging data clearly identified that, despite the fact that the prevalence of vitamin B12 insufficiency varied between different countries, vitamin B12 insufficiency is not rare during pregnancy, even in non-vegetarian populations. Generally the Middle Eastern region, which ranked second in the world for the prevalence of T2DM (Shaw et al. 2010), showed an average prevalence of about 50% during pregnancy which could support the possible role of vitamin B12 insufficiency and increased risk of T2DM in this region. No previous studies on a prevalence of vitamin B12 insufficiency from Saudi Arabia, the area of our interest, were identified in our review. This reflects the need to understand the pattern of maternal vitamin B12 insufficiency in Saudi Arabia.

As expected, the maternal imbalance between vitamin B12 and folate levels were observed in vegetarian populations; however, a similar pattern was also observed in some studies from known non-vegetarian populations as well. The maternal imbalance between the two related nutrients, as previously discussed, is becoming the main concern (Glew et al. 2006), particularly after the mandatory folic acid fortification era started in 1997. In Canada, folic acid insufficiency is increasingly rare, while the prevalence of vitamin B12 insufficiency has increased. In addition, the NTDs related to vitamin B12 insufficiency have tripled in the same period (Ray and Blom 2003; Ray et al. 2007). Thus, an average prevalence of vitamin B12 insufficiency from the included studies in the review were plotted on a world map in order to understand the pattern of

maternal vitamin B12 status before and after the initiation of folic acid fortification in 1997. The trend of change in America can be clearly observed.

In addition, children from PMNS were short and thin but relatively adipose, and those born to mothers with low vitamin B12 but high folate concentrations were the most insulin resistant (Yajnik et al. 2008). Therefore, the thesis also investigated the possible role of maternal vitamin B12 insufficiency, as an independent risk factor, in predicting low birth weight in the offspring. Our results from the systematic review and the UK retrospective study were mixed and did not provide clear-cut conclusions. However, the data supported our hypothesis that vitamin B12 insufficiency is not rare in the UK population and other non-vegetarian populations worldwide as well as maternal obesity and a higher incidence of GDM. This provides independent support to the Indian data (Krishnaveni et al. 2009). The plausible biochemical reasons why vitamin B12 insufficiency could increase adiposity and insulin resistance, particularly in the presence of adequate/high folate, has been discussed in the PMNS study (Yajnik et al. 2008). On the one hand, vitamin B12 insufficiency will trap cellular folate as 5-methyltetrahydrofolate (Scott 1992). This will prevent the formation of methionine from homocysteine and thus impair protein synthesis and reduce lean tissue deposition. On the other hand, MM-CoA mutase, for which vitamin B12 acts as a cofactor, is required for the degradation of odd-chain fatty acids and branched-chain-amino acids, in particular the conversion of MM-CoA to succinyl-CoA. In the presence of vitamin B12 insufficiency, an accumulation of MMA will occur and may increase lipogenesis and insulin resistance (Yajnik et al. 2008). Moreover, GDM women with vitamin B12 insufficiency had heavier babies compared to GDM women with normal levels. It is conceivable that higher glucose levels in the presence of low vitamin B12 may particularly be adipogenic resulting in a higher birth weight. This observation needs

urgent replication in a prospective study along with the measurement of other 1-C metabolites in addition to the vitamin B12, folate and homocysteine levels.

The second part of the thesis focused on replicating this data in the Saudi population, where T2DM has already reached epidemic proportions and is of a major concern in the Middle Eastern region. In addition, changes in the life style and dietary pattern in Saudi are believed to contribute to the increasing prevalence of obesity and chronic diseases (Musaiger 2000; Musaiger 2004). As biomarker measurements are expensive to estimate in the general population, we conducted a validation study of an estimated intake of these 2 vitamins along with the actual blood levels. Thus, we aimed first to evaluate the performance of the modified SFFQ and compare the energy, macro- and micronutrient estimates to the different dietary assessment methods in a pilot study. This in turn helped us to use the SFFQ as a dietary assessment tool along with a 24 h FR to answer the question: "Does the estimated dietary intake of vitamin B12 and folate correlate with actual blood levels in the Saudi population?". The thesis showed no correlation between dietary intake and serum levels of both vitamins in the Saudi population. To our knowledge, this is the first to report a correlation between maternal serum and vitamin B12 intake among the Saudi population. This is similar to data from one study of a population during pregnancy (Ortiz-Andrellucchi et al. 2009) but slightly different to another which showed a weak correlation between serum levels and folate intake (Brantsæter et al. 2007). This could be related to the variability in dietary intake measurement, individuals' absorption, availability and metabolism of the vitamins, which can be important sources of random variations in the biomarker, unrelated to true dietary intake (Kaaks 1997).

The thesis also demonstrated that Saudi pregnant women in general had vitamin B12 at a lower limit and this is relatively similar to what was reported in both a

vegetarian population (Yajnik et al. 2008) and that of a non-vegetarian population (Saravanan et al. 2010c). This further strengthens the argument that vitamin B12 insufficiency is not rare during pregnancy even in populations with a high intake of animal proteins. The dietary estimate of vitamin B12 was adequate or high (in deed higher than the recommended; 5.8 µg by SFFQ) among the Saudi population compared to the RDI level. Despite this good intake, vitamin B12 insufficiency was present in about half of the pregnant sample. One of the possible factors that could contribute to this is the improving hygiene, refrigeration and prolong cooking of meat products among the general population which is likely to kill the microbes that are essential for vitamin B12 synthesis in meat. It is also possible that the methods used to estimate the dietary vitamin B12 intake are inaccurate and over-estimate, as they are old and were developed in the 1970s (Church 2006). As hygiene is inversely linked to vitamin B12 bioavailability (Ball 2004b) this could very well be the reason for such over estimation. Functional food studies looking at the bioavailability and nutrient content of different food products are therefore urgently required. This may mean the need to revise the RDI levels for micronutrients. Perhaps the current RDI derived from levels to prevent deficiency disease, which is rare in developed countries, and more attention should be given to defining an optimal micronutrients requirement for genomic stability to prevent degenerative and developmental disease (Fenech 2001; Fenech 2002).

Finally, the thesis presented the vitamin B12 and folate status among Saudi pregnant and non-pregnant healthy control women. Vitamin B12 insufficiency was more pronounced than folate insufficiency in Saudi women, particularly during pregnancy. This is consistent with the results from other studies, from both known vegetarian and non-vegetarian populations. Serum folate levels ranged within normal reference levels in both groups. However, more pregnant women had folate levels

above 43 nmol/l compared to the controls. This could be related to the mandatory folic acid fortification and the preconception folic acid supplementation practice in Saudi Arabia. In addition, the combination of low serum vitamin B12 and adequate serum folate levels was higher in the pregnant compared to the non-pregnant women. This could be due to the increased metabolic utilization of vitamin B12 during pregnancy, especially in the presence of higher folate levels (Saravanan et al. 2010b). Furthermore, the previous studies (Selhub et al. 2007; Miller et al. 2009) have provided us with new information which would need replicating. In individuals with vitamin B12 insufficiency, vitamin B12's enzymatic functions worsen with increasing levels of folate. Therefore, the thesis has studied the interaction between low vitamin B12/adequate folate on the increased Hcy level during pregnancy and the non-pregnancy state. The results showed a different pattern between the 2 groups. In non-pregnant women, Hcy levels were higher in all categories in women with low vitamin B12 compared to those with normal vitamin B12 levels. However, such differences were not seen in pregnant women. Unlike the previous mentioned studies, we did not observe any increase in Hcy levels with increasing folate categories in the vitamin B12-insufficient group in both pregnant and non-pregnant women. This could very well be due to the small sample size in that particular sub-group.

7.2 Conclusion and future research

The current thesis highlights the potential role of vitamin B12 and folate in foetal programming and the possible contribution to T2DM epidemics. It provides us with an overview of the worldwide vitamin B12 insufficiency during pregnancy. In fact, vitamin B12 insufficiency is not rare during pregnancy, even in non-vegetarian populations such as the Saudi population. Vitamin B12 insufficiency was observed even

in the presence of adequate or high levels of estimated vitamin B12 intake. Introducing mandatory vitamin B12 fortification is under consideration in the US (Food and Nutrition Program of the Pan American Health Organization; March of Dimes; Centers for Disease Control and Prevention 2004; Green 2009). However, such a recommendation should only be taken after careful evaluation (Saravanan and Yajnik 2010). These studies should also be designed to look at the adverse effects of inadvertent high-dose vitamin B12 supplementation (such as excess accumulation of cyanocobalamin) (Carmel 2008). Despite the new knowledge provided by this thesis, a number of questions are unanswered in the Saudi population. Further studies should be designed to look at:

a) Causative role of early pregnancy vitamin B12, folate and homocysteine levels on maternal adiposity and GDM.
b) Pre-pregnancy imbalance of vitamin B12/folate levels and the impact on birth weight and metabolic risk of offspring.
c) Functional food studies in traditional animal foods and the bioavailability of vitamin B12 in Saudi Arabia.
d) Mechanistic studies including epigenetic mechanisms contributing to the epidemic of metabolic diseases including T2DM and CVD.

List of Publications, papers and abstracts

List of publications (during the study period)

Published papers

1. Chittari MV, McTernan P, **Bawazeer N,** Constantinides K, Ciotola M, O'Hare JP, Kumar S, Ceriello A. (2011). Impact of acute hyperglycaemia on endothelial function and retinal vascular reactivity in patients with Type 2 diabetes. *Diabetes Medicine* 28, 450–454. http://onlinelibrary.wiley.com/doi/10.1111/j.1464-5491.2010.03223.x/abstract

2. **Bawazeer N,** Al-Daghri N, Valsamakis G, Al-Rubeaan K, Sabico SL, Huang T, Mastorakos G, Kumar S. (2009) Sleep duration and quality Associated with obesity among Arab children. *Obesity* 17 (12): 2251–2253. http://www.nature.com/oby/journal/v17/n12/full/oby2009169a.html

List of abstracts

Poster presentations:

1. **Bawazeer N**, Rafnsson S, Saravanan P. (2011). Does vitamin B12 insufficiency during pregnancy happen in non-vegetarian? 7th world congress of developmental origins of health and disease (DOHAD), Portland, USA on 18 - 20 September 2011.

Aims: In most population, both low and high birth weight is associated with onset of diabetes in later life. This is called "nutrient" and "fuel" mediated teratogenesis, respectively. Studies from India showed children born to mothers with low B12, especially in association with high folate levels, have higher adiposity at birth and higher insulin resistance at 6 years of age. In addition, mothers with low maternal B12 had higher incidence of gestational diabetes and future type 2 diabetes. The B12 insufficiency rate in these studies was high between 40 & 70%, presumably due to vegetarianism. It is not known whether similar phenomenon exists in non-vegetarian population across the world. We did a systematic review to study the prevalence of B12 insufficiency during pregnancy.

Methods: A comprehensive literature search in six electronic databases for publications on the prevalence of B12 insufficiency during pregnancy, screening of reference lists, and citation search was conducted. All databases were searched using a combination of keywords from inception to February 2011. Publications of all studies (longitudinal or cross sectional studies) on normal adult pregnancy were further inspected and studies mentioned the prevalence of B12 insufficiency were included.

Results: Thirty-three studies (Americas – 8, Europe & middle east – 4, Africa – 7 & Australasia – 11) have reported the prevalence of B12 insufficiency. Studies differed in

the population studied, period of blood collection and the cut-off used to define B12 insufficiency. Number of studies reporting the B12 insufficiency according to the trimesters were: 1st – 7, 2nd – 19 and 3rd – 22. The overall B12 insufficiency was between 42 - 74% in the vegetarian population (6 from India and 1 from Nepal – all 2nd & 3rd trimester) apart from 1 study in Nepal (28.3%; 1st trimester; cut-off 221 pmol/L). In non-vegetarian population, it ranged from 4.5 – 80.9%. The deficiency rates according to the trimesters were: 1st (n = 6) – 4.3-44.2%, 2nd (n = 17) – 4.5-60% and 3rd (n = 15) – 5.7-80%. Studies with rates below 10% used lower cut-off values to define B12 insufficiency.

Conclusions: This systematic review showed that B12 insufficiency during pregnancy is not uncommon in non-vegetarian population worldwide and can be high similar to the levels seen in vegetarian population. Studies correlating the B12 levels and the metabolic risk of mothers and offspring in non-vegetarian population are urgently warranted.

2. Sukumar N, **Bawazeer N**, Patel V, Saravanan P. (2011) Low B12 level is associated with maternal obesity and higher Birth weight in Gestational Diabetes. 7th world congress of developmental origins of health and disease (DOHAD), Portland, USA on 18 - 20 September 2011.

Background: In most populations, both low and high birth weights are associated with onset of T2DM in later life. Studies from India showed low maternal vitamin B12 is related to higher incidence of GDM, higher adiposity and insulin resistance in offspring and future T2DM in mothers. It is not known whether similar phenomenon exists in UK population. We aim to study vitamin B12 status in pregnancy and investigate its

relationship with maternal BMI, incidence of GDM and birth weight in women with and without GDM.

Methods: Retrospective study of mothers attending the antenatal diabetes and medical clinics (2005-10) in Warwickshire, UK (mainly Caucasian population). Maternal B12 and folate levels are checked routinely in most women. The inclusion criteria were women without pre-gestational diabetes delivering live, singleton babies.

Results: Of the 270 mothers who met the inclusion criteria, B12 and folate were measured in 209 and in 38 of the 60 women with GDM at median 24 weeks. As expected, GDM mothers were older than non-GDM (33.5 vs. 30.5 years, $p < 0.001$) and had higher BMI (31.0 vs. 26.1, $p < 0.001$). The median (IQR) B12 and folate levels were 143.9 (106.3, 194.5) pmol/L and 15.9 (10.0, 25.8) nmol/L respectively. Women with B12 insufficiency($\leq$ 150 pmol/L) had higher BMI at booking (28.0 vs. 25.7, $p = 0.013$; controlled for age, smoking, folate and presence of GDM). Women who developed GDM had slightly lower B12 (146.8 vs. 172.4 pmol/L, $p = 0.259$) though this was not statistically significant. Similarly, a non-significant difference was seen in the proportion of B12-insufficient women developing GDM (19.1 vs. 17%). GDM mothers with B12 levels below the median had heavier babies (3522.1 vs. 3211.1 g, $p = 0.025$). This difference persisted even after controlling for folate levels, gestation at birth, maternal age and booking BMI (β -0.39, $p = 0.017$). No such difference was seen for non-GDM mothers (3421.8 vs. 3345.8g, $p = 0.395$)

Conclusions: Our study showed that B12 insufficiency is not rare in UK Caucasian population; is associated with maternal obesity; possible higher incidence of GDM and macrosomia in GDM. As it is retrospective and observational study, causality cannot be ascertained. However, this is the first study to show the possible interaction between B12 levels, GDM, and their additive influence on birth weight. Adequately powered

prospective studies are urgently needed on the influence of early pregnancy B12 levels on the risk of GDM and neonatal outcomes.

Oral presentations

1. **Bawazeer N**, Rafnsson S, Saravanan P. (2011). "B12 insufficiency doesn't happen in non-vegetarians: Systematic review of B12 levels in pregnancy". (2011). WISDEM/IAS/CSRI Annual Symposium, University of Warwick, Coventry on 11 March 2011.

2. **Bawazeer N**, Al-Daghri N, Al-Rubeaan K, Sabico SL, Kumar S. (2010). Sleep duration strongly predicts childhood obesity among Arabs. 4th Saudi International Conference "Saudi minds in action", University of Manchester, Manchester on 30 - 31 July 2010.

Appendices

Appendix I : Worldwide mandatory folic acid food fortification

Worldwide mandatory folic acid food fortification has been introduced in more than 40 countries in order to reduce and/or prevent the NTDs pregnancies (**Figure**). In addition to the USA and Canada, many South American, African and Middle Eastern countries also implement a mandatory folic acid food fortification policy. Australia and New Zealand are currently exploring the option of mandatory fortification for the prevention of birth defects for the first time. Some European countries permit a range of foods to be fortified on a voluntary basis such as Belgium, France, Germany, Greece, Hungary, Iceland and Ireland. UK is currently re-examining their policy in this area. On the other hand, national legislation in the Netherlands prohibits both mandatory and voluntary folic acid food fortification (Food Safety Authority of Ireland 2006).

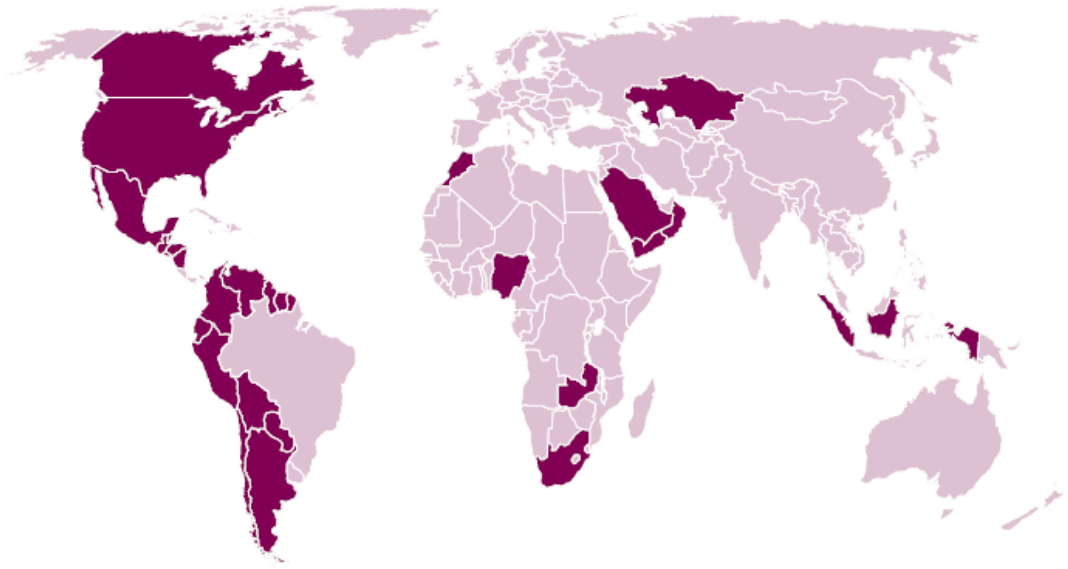


Figure illustrates the 42 countries that have mandatory folic acid food fortification program. It includes USA, Canada, South America and Caribbean, Israel, Saudi Arabia, Bahrin, Qatar, Oman, Yemen, Morroco, Nigeria, South Africa, Zambia, Malawi, Indonesia and Kazakhstan. Figure obtained from (Food Safety Authority of Ireland 2006).

Appendix II : The worldwide trend in wheat flour fortification from 2004 to 2007.

Fortification of wheat flour is an effective, simple and inexpensive method for providing folic acid to large segments of the world population. The number of countries with documented national regulations for mandatory wheat flour fortification increased from 2004 to 2007. Therefore, the worldwide percentage of wheat flour fortified increased from 18% in 2004 to 27% in 2007 (**Figure**). Approximately, 540 million additional persons, including 167 million additional women aged 15 - 60 years had access to fortified wheat flour and the annual number of newborns whose mothers had access to fortified wheat flour during pregnancy increased by approximately 14 million. By region, the greatest increase was in the Eastern Mediterranean Region: from 5% to 44%, while the region with the highest percentage of fortified wheat flour, America, increased from 90% to 97% , Africa increased from 26% to 31%, South-East Asia from 16% to 21%, Europe from 3% to 6%, and the Western Pacific region from 2% to 4% (Center for Disease Control and Prevention 2008).

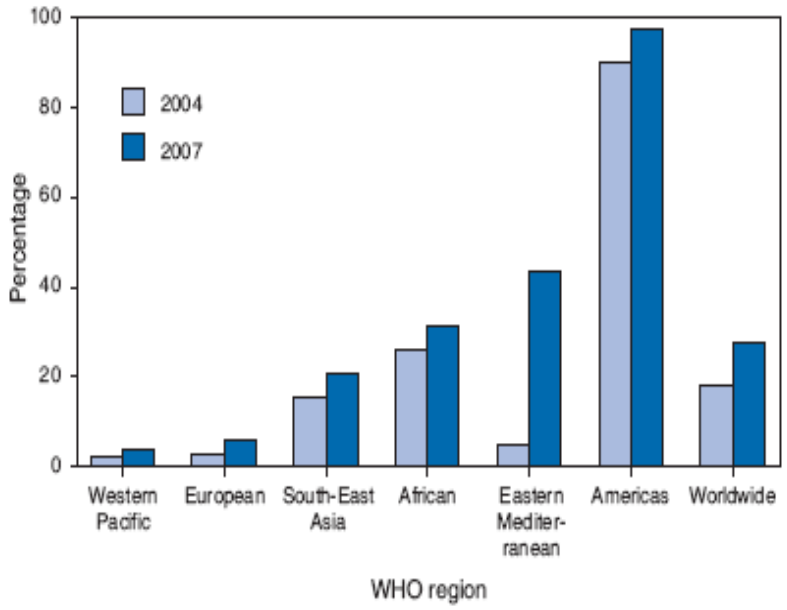


Figure illustrates the worldwide percentage change of wheat flour processed in roller mills that was fortified by World Health Organizations (WHO) region - 2004 and 2007. Figure obtained from (Center for Disease Control and Prevention 2008).

Appendix III: The quality assessment of the longitudinal studies included in the systematic review (A)

Table 1 The quality assessment of studies included in the review

Quality assessment criteria	Lowenstein et al. 1960	Whiteside et al. 1968	Robert et al. 1973	Barbin et al. 1986	Knight et al. 1991	Bruinse et al. 1995	Ackurt et al. 1995
1. Research question/ hypothesis clearly defined and stated?	√	√	√	√	√	√	√
2. Is the targeted population defined?	×	×	√	√	√	√	√
3. Is the sampling frame defined?	×	√	√	√	√	√	√
4. Is the study population defined?	×	√	√	√	√	√	√
5. Are the study setting and/or geographic location stated?	×	×	√	√	√	√	√
6. Are the dates between which the study was conducted stated or implicit?	×	×	√	√	√	×	√
7. Are eligibility criteria stated?	×	√	×	P	√	√	√
8. Are issue of "selection in" to the study mentioned?	×	×	√	√	√	√	√
9. Is the no. of participants justified?	×	×	×	×	×	×	×
10. Are no. meeting and not meeting the eligibility criteria stated?	×	×	×	×	×	√	√
11. For those not eligible, are the reasons why stated?	×	×	×	×	×	√	√
12. Are the numbers of people who did/did not consent to participate stated?	×	×	×	×	√	√	×
13. Are the reasons that people refused to consent stated?	×	×	×	×	×	×	×
14. Were consenters compared with non-consenters?	×	×	×	×	×	×	×
15. Was the number of participants at the beginning of the study stated?	×	√	√	√	√	√	√
16. Were methods of data collection stated?	×	√	√	√	√	√	√
17. Was the reliability (repeatability) of measurement methods mentioned?	√	×	×	√	√	×	×
18. Was the validity (against a 'gold standard') of measurement methods mentioned?	√	×	×	√	×	×	×
19. Were any confounders mentioned?	P	×	√	×	√	√	√

Based on NICE guidance for public health 2009 which was adapted from the Tooth et al. 2005 checklist criteria.
(n/a) was indicating, when the question was not applicable to the study design, P = poorly addressed.

Cont. Table 1

Quality assessment criteria	Lowenstein et al. 1960	Whiteside et al. 1968	Robert et al. 1973	Barbin et al. 1986	Knight et al. 1991	Bruinse et al. 1995	Ackurt et al. 1995
20. Was the number of participants at each stage/wave specified?	√	×	√	√	√	×	√
21. Were reasons for loss to follow-up quantified?	×	×	×	×	×	√	√
22. Was the missingness of data items at each wave mentioned?	×	×	×	×	×	×	×
23. Was the type of analyses conducted stated?	P	√	×	√	√	√	√
24. Were "longitudinal" analysis methods stated?	P	√	√	√	√	√	√
25. Were absolute effect sizes reported?	√	√	√	√	√	P	√
26. Were relative effect sizes reported?	×	×	×	×	×	×	×
27. Was loss to follow up taken into account in the analysis?	×	×	×	×	×	×	P
28. Were confounders accounted for the analyses?	×	×	√	×	×	√	×
29. Were missing data accounted for the analyses?	×	×	×	×	×	×	×
30. Was the impact of biases assessed qualitatively?	×	×	×	×	×	×	×
31. Was the impact of biases estimated quantitatively?	×	×	×	×	×	×	×
32. Did authors relate results back to a target population?	√	√	√	√	√	√	√
33. Was there any other discussion of generalizability?	×	×	P	×	×	×	√
Overall assessment	Few criteria fulfilled-unfulfilled criteria are unlikely/very likely to alter the conclusion	Few criteria fulfilled-unfulfilled criteria are unlikely/very likely to alter the conclusion	Some criteria fulfilled-unfulfilled criteria unlikely to alter the conclusion	Some criteria fulfilled-unfulfilled criteria unlikely to alter the conclusion	Some criteria fulfilled-unfulfilled criteria unlikely to alter the conclusion	Some criteria fulfilled-unfulfilled criteria unlikely to alter the conclusion	Some criteria fulfilled-unfulfilled criteria unlikely to alter the conclusion

Based on NICE guidance for public health 2009 which was adapted from the Tooth et al. 2005 checklist criteria.
(n/a) was indicating, when the question was not applicable to the study design, P = poorly addressed.

Table 2 The quality assessment of studies included in the review

Quality assessment criteria	Pagan et al. 2002	Milman et al. 2006	Murphy et al. 2007	Takimoto et al. 2007	Yajnik et al. 2008	Li et al. 2008	Katre et al. 2010	Haliloglu et al. 2010
1. Research question/ hypothesis clearly defined and stated?	√	√	√	√	√	√	√	√
2. Is the targeted population defined?	√	√	√	√	√	√	√	√
3. Is the sampling frame defined?	√	×	√	√	√	√	√	√
4. Is the study population defined?	√	√	√	√	√	√	√	√
5. Are the study setting and/or geographic location stated?	√	√	√	√	√	√	√	√
6. Are the dates between which the study was conducted stated or implicit?	√	√	×	√	√	√	√	×
7. Are eligibility criteria stated?	×	√	√	√	√	×	√	√
8. Are issue of "selection in" to the study mentioned?	√	√	√	√	√	√	√	√
9. Is the no. of participants justified?	×	×	×	×	×	×	×	×
10. Are no. meeting and not meeting the eligibility criteria stated?	×	√	×	√	√	√	√	√
11. For those not eligible, are the reasons why stated?	×	√	×	√	√	√	√	√
12. Are the numbers of people who did/did not consent to participate stated?	×	√	√	√	√	√	√	√
13. Are the reasons that people refused to consent stated?	×	×	×	×	×	×	×	×
14. Were consenters compared with non-consenters?	×	×	×	×	×	×	×	×
15. Was the number of participants at the beginning of the study stated?	√	√	√	√	√	√	√	√
16. Were methods of data collection stated?	√	√	√	√	√	√	√	√
17. Was the reliability (repeatability) of measurement methods mentioned?	×	×	√	×	×	×	√	×
18. Was the validity (against a 'gold standard') of measurement methods mentioned?	√	×	√	√	×	×	×	×
19. Were any confounders mentioned?	√	×	√	√	√	√	√	×

Based on NICE guidance for public health 2009 which was adapted from the Tooth et al. 2005 checklist criteria.
(n/a) was indicating, when the question was not applicable to the study design, P = poorly addressed.

Cont. Table 2

Quality assessment criteria	Pagan et al. 2002	Milman et al. 2006	Murphy et al. 2007	Takimoto et al. 2007	Yajnik et al. 2008	Li et al. 2008	Katre et al. 2010	Haliloglu et al. 2010
20. Was the number of participants at each stage/wave specified?	×	×	√	√	√	√	√	×
21. Were reasons for loss to follow-up quantified?	×	×	×	√	√	√	√	√
22. Was the missingness of data items at each wave mentioned?	×	×	√	√	×	√	√	×
23. Was the type of analyses conducted stated?	√	√	√	√	√	√	√	√
24. Were "longitudinal" analysis methods stated?	√	√	√	√	√	√	√	√
25. Were absolute effect sizes reported?	√	√	√	√	√	√	√	√
26. Were relative effect sizes reported?	√	×	×	√	×	×	√	×
27. Was loss to follow up taken into account in the analysis?	×	×	×	×	×	×	×	×
28. Were confounders accounted for the analyses?	√	×	√	√	√	√	√	×
29. Were missing data accounted for the analyses?	×	×	×	×	√	×	×	×
30. Was the impact of biases assessed qualitatively?	×	×	×	√	√	√	√	×
31. Was the impact of biases estimated quantitatively?	×	×	×	×	×	×	×	×
32. Did authors relate results back to a target population?	√	√	√	√	√	√	√	√
33. Was there any other discussion of generalizability?	×	√	×	×	√	×	×	×
Overall assessment	Some criteria fulfilled-unfulfilled criteria unlikely to alter the conclusion	Some criteria fulfilled-unfulfilled criteria unlikely to alter the conclusion	Some criteria fulfilled-unfulfilled criteria unlikely to alter the conclusion	Most criteria fulfilled-unfulfilled criteria very unlikely to alter the conclusion	Most criteria fulfilled-unfulfilled criteria very unlikely to alter the conclusion	Some criteria fulfilled-unfulfilled criteria unlikely to alter the conclusion	Most criteria fulfilled-unfulfilled criteria very unlikely to alter the conclusion	Some criteria fulfilled-unfulfilled criteria unlikely to alter the conclusion

Based on NICE guidance for public health 2009 which was adapted from the Tooth et al. 2005 checklist criteria.
(n/a) was indicating, when the question was not applicable to the study design, P = poorly addressed.

Appendix IV: The quality assessment of cross-sectional studies included in the systematic review (A)

Table 1 The quality assessment of cross-sectional studies included in the review

Quality assessment criteria	Zachau-Christiansen et al. 1962	Ball et al. 1964	Cook et al. 1971	Marzan et al. 1971	Yusufji et al. 1973	Cole et al. 1974
Study objective						
Clearly stated objective is described	1	0	1	1	1	0
Study population						
Were valid selection criteria used for the study population?	1	0	1	1	1	0
Did more than 80% of the eligible subjects participate in the study?	1	1	1	1	1	0
Exposure assessment						
Was the exposure measured with a valid and reproducible method?	n/a	n/a	n/a	n/a	n/a	n/a
Outcome assessment						
Was the outcome measured with a valid and reproducible method?	1	1	1	1	1	1
Were only new and incident patients used?	1	0	1	1	1	1
Analysis						
Were the results adjusted for possible confounders?	0	0	0	0	0	0
Were more than 100 subjects included in the study*	1	1	1	0	1	1
Total score	6/7	3/7	6/7	5/7	6/7	3/7
Overall assessment	86%	43%	86%	71%	86%	43%

Based on checklist from Ariens et al., 2000 and van der Windt et al., 2000.
Yes = 1 point, No or unclear = 0 points, Total score are ≥ 6 or ≥ 75% = high quality study and < 6 or < 75% = low quality study, n/a = not applicable.
*more than 50 participants was a requirement for inclusion in the review. Studies with more than 100 participants were rewarded with an additional point.

Table 2 The quality assessment of cross-sectional studies included in the review

Quality assessment criteria	Baker et al. 1975	Colman et al. 1975	Areekul et al. 1976	Jacob et al. 1976	Osifo et al. 1976	Srisupandit et al. 1983
Study objective						
Clearly stated objective is described	1	0	1	0	1	1
Study population						
Were valid selection criteria used for the study population?	1	1	1	1	0	1
Did more than 80% of the eligible subjects participate in the study?	1	1	1	1	1	1
Exposure assessment						
Was the exposure measured with a valid and reproducible method?	n/a	n/a	n/a	n/a	n/a	n/a
Outcome assessment						
Was the outcome measured with a valid and reproducible method?	1	1	1	1	1	1
Were only new and incident patients used?	1	1	1	1	1	1
Analysis						
Were the results adjusted for possible confounders?	1	0	0	0	0	0
Were more than 100 subjects included in the study*	1	1	1	1	0	1
Total score	7/7	5/7	6/7	5/7	4/7	6/7
Overall assessment	100%	71%	86%	71%	57%	86%

Based on checklist from Ariens et al., 2000 and van der Windt et al., 2000.
Yes = 1 point, No or unclear = 0 points, Total score are ≥ 6 or ≥ 75% = high quality study and < 6 or < 75% = low quality study, n/a = not applicable.
*more than 50 participants was a requirement for inclusion in the review. Studies with more than 100 participants were rewarded with an additional point.

Table 3 The quality assessment of cross-sectional studies included in the review

Quality assessment criteria	Giugliani et al. 1984	Ho Chao-hung et al . 1987	Frey et al. 1992	Black et al. 1994	House et al. 2000	Bjorke-Monsen et al 2001
Study objective						
Clearly stated objective is described	1	1	1	1	1	1
Study population						
Were valid selection criteria used for the study population?	1	1	1	1	1	1
Did more than 80% of the eligible subjects participate in the study?	1	1	1	1	1	1
Exposure assessment						
Was the exposure measured with a valid and reproducible method?	n/a	n/a	n/a	n/a	n/a	n/a
Outcome assessment						
Was the outcome measured with a valid and reproducible method?	1	1	1	1	1	1
Were only new and incident patients used?	1	1	1	1	0	1
Analysis						
Were the results adjusted for possible confounders?	0	0	1	0	0	1
Were more than 100 subjects included in the study*	0	1	1	0	1	1
Total score	5/7	6/7	7/7	5/7	5/7	7/7
Overall assessment	71%	86%	100%	71%	71%	100%

Based on checklist from Ariens et al., 2000 and van der Windt et al., 2000.
Yes = 1 point, No or unclear = 0 points, Total score are ≥ 6 or ≥ 75% = high quality study and < 6 or < 75% = low quality study, n/a = not applicable.
*more than 50 participants was a requirement for inclusion in the review. Studies with more than 100 participants were rewarded with an additional point.

Table 4 The quality assessment of cross-sectional studies included in the review

Quality assessment criteria	Bondevik et al. 2001	Hinderaker et al. 2002	Guerra-Shinohara et al. 2004	Ma et al. 2004	Park et al. 2004	Schulpis et al. 2004
Study objective						
Clearly stated objective is described	1	1	1	1	1	1
Study population						
Were valid selection criteria used for the study population?	1	1	1	1	1	1
Did more than 80% of the eligible subjects participate in the study?	1	1	1	1	1	1
Exposure assessment						
Was the exposure measured with a valid and reproducible method?	n/a	n/a	n/a	n/a	n/a	n/a
Outcome assessment						
Was the outcome measured with a valid and reproducible method?	1	1	1	1	1	1
Were only new and incident patients used?	1	1	1	1	1	0
Analysis						
Were the results adjusted for possible confounders?	1	0	0	0	0	0
Were more than 100 subjects included in the study*	1	1	1	1	0	1
Total score	7/7	6/7	6/7	6/7	5/7	5/7
Overall assessment	100%	86%	86%	86%	71%	71%

Based on checklist from Ariens et al., 2000 and van der Windt et al., 2000.
Yes = 1 point, No or unclear = 0 points, Total score are ≥ 6 or ≥ 75% = high quality study and < 6 or < 75% = low quality study, n/a = not applicable.
*more than 50 participants was a requirement for inclusion in the review. Studies with more than 100 participants were rewarded with an additional point.

Table 5 The quality assessment of cross-sectional studies included in the review

Quality assessment criteria	Garcia-Casal et al. 2005	Jiang et al. 2005	Koc et al 2006	Hall et al. 2007	Ipciglu et al. 2007	Pathak et al. 2007
Study objective						
Clearly stated objective is described	1	1	1	1	1	1
Study population						
Were valid selection criteria used for the study population?	1	1	1	0	1	1
Did more than 80% of the eligible subjects participate in the study?	1	1	1	1	1	1
Exposure assessment						
Was the exposure measured with a valid and reproducible method?	n/a	n/a	n/a	n/a	n/a	n/a
Outcome assessment						
Was the outcome measured with a valid and reproducible method?	1	1	1	1	1	1
Were only new and incident patients used?	0	1	1	0	1	1
Analysis						
Were the results adjusted for possible confounders?	0	1	0	1	0	0
Were more than 100 subjects included in the study*	1	1	1	1	0	1
Total score	5/7	7/7	6/7	5/7	5/7	6/7
Overall assessment	71%	100%	86%	71%	71%	86%

Based on checklist from Ariens et al., 2000 and van der Windt et al., 2000.
Yes = 1 point, No or unclear = 0 points, Total score are ≥ 6 or ≥ 75% = high quality study and < 6 or < 75% = low quality study, n/a = not applicable.
*more than 50 participants was a requirement for inclusion in the review. Studies with more than 100 participants were rewarded with an additional point.

Table 6 The quality assessment of cross-sectional studies included in the review

Quality assessment criteria	Vanderjagat et al. 2007	Barbosa et al. 2008	Gibson et al. 2008	Ray et al. 2008	Abdelrahim et al. 2009	Hussein et al. 2009
Study objective						
Clearly stated objective is described	1	1	1	1	1	1
Study population						
Were valid selection criteria used for the study population?	1	1	1	1	0	1
Did more than 80% of the eligible subjects participate in the study?	1	0	1	1	1	1
Exposure assessment						
Was the exposure measured with a valid and reproducible method?	n/a	n/a	n/a	n/a	n/a	n/a
Outcome assessment						
Was the outcome measured with a valid and reproducible method?	1	1	1	1	1	1
Were only new and incident patients used?	1	1	1	0	1	1
Analysis						
Were the results adjusted for possible confounders?	0	1	1	0	1	1
Were more than 100 subjects included in the study*	1	1	0	1	1	0
Total score	6/7	6/7	6/7	5/7	6/7	6/7
Overall assessment	86%	86%	86%	71%	86%	86%

Based on checklist from Ariens et al., 2000 and van der Windt et al., 2000.
Yes = 1 point, No or unclear = 0 points, Total score are ≥ 6 or ≥ 75% = high quality study and < 6 or < 75% = low quality study, n/a = not applicable.
*more than 50 participants was a requirement for inclusion in the review. Studies with more than 100 participants were rewarded with an additional point.

Table 7 The quality assessment of cross-sectional studies included in the review

Quality assessment criteria	Krishnaveni et al. 2009	Vanderjagat et al. 2009	Veena et al. 2010	Goedhart et al. 2011	Lindstrom et al. 2011	Shield et al. 2011
Study objective						
Clearly stated objective is described	1	1	1	1	1	1
Study population						
Were valid selection criteria used for the study population?	1	1	1	1	1	1
Did more than 80% of the eligible subjects participate in the study?	1	1	1	1	1	1
Exposure assessment						
Was the exposure measured with a valid and reproducible method?	n/a	n/a	n/a	n/a	n/a	n/a
Outcome assessment						
Was the outcome measured with a valid and reproducible method?	1	1	1	1	1	1
Were only new and incident patients used?	1	1	1	1	1	1
Analysis						
Were the results adjusted for possible confounders?	1	0	1	1	1	0
Were more than 100 subjects included in the study*	1	0	1	1	1	1
Total score	7/7	5/7	7/7	7/7	7/7	6/7
Overall assessment	100%	71%	86%	100%	100%	86%

Appendix V : The quality assessment of studies included in the systematic review (B)

Table 1 The quality assessment of studies included in the review

Quality assessment criteria	Whiteside et al. 1968	Knight et al. 1991	Tamura et al. 1994	Ackurt et al. 1995	Mathews et al 2004	Relton et al. 2005	Muthayya et al. 2006
1. Research question/ hypothesis clearly defined and stated?	√	√	√	√	√	√	√
2. Is the targeted population defined?	×	√	√	√	√	√	√
3. Is the sampling frame defined?	√	√	√	√	√	√	√
4. Is the study population defined?	√	√	√	√	√	√	√
5. Are the study setting and/or geographic location stated?	×	√	√	√	√	√	√
6. Are the dates between which the study was conducted stated or implicit?	×	√	×	√	√	√	√
7. Are eligibility criteria stated?	√	√	×	√	√	√	√
8. Are issue of "selection in" to the study mentioned?	×	√	×	√	√	√	√
9. Is the no. of participants justified?	×	×	×	×	√	×	×
10. Are no. meeting and not meeting the eligibility criteria stated?	×	×	×	√	√	×	√
11. For those not eligible, are the reasons why stated?	×	×	×	√	√	√	√
12. Are the numbers of people who did/did not consent to participate stated?	×	√	×	×	√	×	√
13. Are the reasons that people refused to consent stated?	×	×	×	×	×	×	×
14. Were consenters compared with non-consenters?	×	×	×	×	×	×	×
15. Was the number of participants at the beginning of the study stated?	√	√	√	√	√	√	√
16. Were methods of data collection stated?	√	√	√	√	√	√	√
17. Was the reliability (repeatability) of measurement methods mentioned?	×	√	×	×	√	×	√
18. Was the validity (against a 'gold standard') of measurement methods mentioned?	×	×	×	×	√	√	√
19. Were any confounders mentioned?	×	√	×	√	√	√	√

Based on NICE guidance for public health 2009 which was adapted from the Tooth et al. 2005 checklist criteria.

Cont. Table 1

Quality assessment criteria	Whiteside et al. 1968	Knight et al. 1991	Tamura et al. 1994	Ackurt et al. 1995	Mathews et al 2004	Relton et al. 2005	Muthayya et al. 2006
20. Was the number of participants at each stage/wave specified?	×	√	n/a	√	√	×	×
21. Were reasons for loss to follow-up quantified?	×	×	n/a	√	√	×	√
22. Was the missingness of data items at each wave mentioned?	×	×	n/a	×	√	×	×
23. Was the type of analyses conducted stated?	√	√	√	√	√	√	√
24. Were "longitudinal" analysis methods stated?	√	√	n/a	√	√	√	√
25. Were absolute effect sizes reported?	√	√	√	√	√	√	√
26. Were relative effect sizes reported?	×	×	×	×	×	×	√
27. Was loss to follow up taken into account in the analysis?	×	×	n/a	×	×	×	√
28. Were confounders accounted for the analyses?	×	×	×	√	√	√	√
29. Were missing data accounted for the analyses?	×	×	n/a	×	×	×	×
30. Was the impact of biases assessed qualitatively?	×	×	×	×	√	×	×
31. Was the impact of biases estimated quantitatively?	×	×	×	×	×	×	×
32. Did authors relate results back to a target population?	√	√	×	√	√	√	√
33. Was there any other discussion of generalizability?	×	×	×	√	×	×	×
Overall assessment	Few criteria fulfilled-unfulfilled criteria are likely/very likely to alter the conclusions	Some criteria fulfilled-unfulfilled criteria unlikely to alter the conclusions	Few criteria fulfilled-unfulfilled criteria are likely/very likely to alter the conclusions	Some criteria fulfilled-unfulfilled criteria unlikely to alter the conclusions	Most criteria fulfilled-unfulfilled criteria very unlikely to alter the conclusions	Some criteria fulfilled-unfulfilled criteria unlikely to alter the conclusions	Most criteria fulfilled-unfulfilled criteria very unlikely to alter the conclusions

(n/a) was indicating, when the question was not applicable to the study design

Table 2 The quality assessment of the studies included in the review

Quality assessment criteria	Mamabolo et al. 2006	Takimoto et al. 2007	Yajnik et al. 2008	Hogeveen et al. 2010	Hay et al. 2010	Veena et al. 2010	Takimoto et al. 2011
1. Research question/ hypothesis clearly defined and stated?	√	√	√	√	√	√	√
2. Is the targeted population defined?	√	√	√	√	√	√	√
3. Is the sampling frame defined?	√	√	×	√	×	√	√
4. Is the study population defined?	√	√	√	√	√	√	√
5. Are the study setting and/or geographic location stated?	√	√	√	√	√	√	√
6. Are the dates between which the study was conducted stated or implicit?	√	√	√	√	√	√	√
7. Are eligibility criteria stated?	√	√	√	√	√	√	√
8. Are issue of "selection in" to the study mentioned?	√	√	√	√	√	√	√
9. Is the no. of participants justified?	×	×	×	×	×	×	×
10. Are no. meeting and not meeting the eligibility criteria stated?	√	√	√	√	×	√	√
11. For those not eligible, are the reasons why stated?	√	√	√	√	×	√	√
12. Are the numbers of people who did/did not consent to participate stated?	√	√	√	×	√	×	√
13. Are the reasons that people refused to consent stated?	×	×	×	×	×	×	×
14. Were consenters compared with nonconsenters?	×	×	×	×	×	×	×
15. Was the number of participants at the beginning of the study stated?	√	√	√	√	√	√	√
16. Were methods of data collection stated?	√	√	√	√	√	√	√
17. Was the reliability (repeatability) of measurement methods mentioned?	×	×	×	√	×	√	×
18. Was the validity (against a 'gold standard') of measurement methods mentioned?	×	√	×	√	√	√	×
19. Were any confounders mentioned?	√	√	√	√	√	√	√

Based on NICE guidance for public health 2009 which was adapted from the Tooth et al. 2005 checklist criteria.

Cont. Table 2

Quality assessment criteria	Mamabolo et al. 2006	Takimoto et al. 2007	Yajnik et al. 2008	Hogeveen et al. 2010	Hay et al. 2009	Veena et al. 2010	Takimoto et al. 2011
20. Was the number of participants at each stage/wave specified?	√	√	√	√	√	√	√
21. Were reasons for loss to follow-up quantified?	n/a	√	√	n/a	×	√	×
22. Was the missingness of data items at each wave mentioned?	n/a	√	×	n/a	×	√	×
23. Was the type of analyses conducted stated?	√	√	√	√	√	√	√
24. Were "longitudinal" analysis methods stated?	√	√	√	√	√	√	√
25. Were absolute effect sizes reported?	√	√	√	√	√	√	√
26. Were relative effect sizes reported?	×	√	×	×	×	×	×
27. Was loss to follow up taken into account in the analysis?	n/a	×	×	n/a	×	×	×
28. Were confounders accounted for the analyses?	√	√	√	√	√	√	√
29. Were missing data accounted for the analyses?	n/a	×	√	n/a	×	×	×
30. Was the impact of biases assessed qualitatively?	×	√	√	√	√	√	√
31. Was the impact of biases estimated quantitatively?	×	×	×	×	×	×	×
32. Did authors relate results back to a target population?	√	√	√	√	√	√	√
33. Was there any other discussion of generalizability?	×	×	√	×	×	×	×
Overall assessment	Some criteria fulfilled-unfulfilled criteria unlikely to alter the conclusions	Most criteria fulfilled-unfulfilled criteria very unlikely to alter the conclusions	Most criteria fulfilled-unfulfilled criteria very unlikely to alter the conclusions	Most criteria fulfilled-unfulfilled criteria very unlikely to alter the conclusions	Some criteria fulfilled-unfulfilled criteria very unlikely to alter the conclusions	Most criteria fulfilled-unfulfilled criteria very unlikely to alter the conclusions	Some criteria fulfilled-unfulfilled criteria very unlikely to alter the conclusions

(n/a) was indicating, when the question was not applicable i.e. no loss to follow up nor missing data in the study.

Appendix VI

Vitamin B12 insufficiency in pregnancy

Data collection form 2010

Banda label here

MOTHER	
Delivery Date	
Gestation	
Mode of delivery	Normal vaginal delivery / assisted / C-section
Gravida / Para	
Pre-pregnancy BMI	
Pre-eclampsia	Yes / No
GDM	Yes / No
Other Obstetric History Past medical history	
Smoking	Yes / No
Ex-smoker	Yes / No
Alcohol	Yes / No
Family History	
Drugs	

BABY	
Sex	
Length	
Weight (kg / percentile)	
Head Circumference	
Congenital abnormalities	
Shoulder dystocia	Yes /No
Neonatal hypoglycaemia	Yes / No
APGAR scores:	
1 minute	
5 minutes	
Other information (infection / jaundice etc.)	
SCBU	Yes / No

BIOCHEMISTRY						
	First result / level	Weeks' gestation of first result	Deficient (Yes/No)	Supplementation (Yes/No)	Repeat result / level	Weeks' gestation of repeat result
Vit B12						
Folate						

Appendix VII : The food that has been added to the modified DIETQ SFFQ – used in the Pilot study.

Table shows the list of food that has been added to the modified DIETQ SFFQ

Rice: Kabsa, biryani, bukhari	Arabic sweets
Qursan, Marqook, Mataziz	Laban
Jareesh	Dates
Saleeq	Foul (broad beans)
Harees	Falafel
Marasssea	Bread such as tames
Mehala	Muttabeq
Henini	

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