CHAPTER 3
Maternal Micronutrients (Folic Acid, Vitamin B_{12}) and Homocysteine across Gestation in Preeclampsia
3.1 Introduction

Both human and animal studies carried out in our department have adequately demonstrated a link between DHA, micronutrients (folate and vitamin $B_{12}$) and homocysteine in the one carbon cycle. In Chapter 2, we have elaborately discussed altered LCPUFA levels from early pregnancy in preeclampsia. Therefore, this study aimed at examining the levels of maternal plasma folate, vitamin $B_{12}$ and homocysteine across gestation and also to examine their association with maternal fatty acid levels in normotensive control and women with preeclampsia. This will help in understanding the temporal relationship of micronutrients with the pathophysiology of preeclampsia.

3.1.1 Micronutrients

Pregnant women have to meet their own nutritional requirements and also supply nutrients to the growing fetus and the infant (reviewed by Ramachandran, 2002). Both macro- and micro-nutrients are essential during this critical period as it has now been recognized that poor growth results not only from a deficiency of macronutrients but also due to inadequate intake of micronutrients (reviewed by Abu-Saad and Fraser, 2010). Micronutrient deficiencies among pregnant women are associated with poor outcomes for both the mother (preeclampsia) and the baby (fetal growth restriction, preterm delivery and low neonatal micronutrient stores) (reviewed by Owens and Fall, 2008; Pathak et al., 2007).

Micronutrients such as folate and vitamin $B_{12}$ are crucial during pregnancy. These two vitamins play a critical role in nucleic acid synthesis and one carbon metabolism (Yajnik et al., 2008). The next section therefore describes micronutrients like folate and vitamin $B_{12}$ and their association with pregnancy outcome.
3.1.2 Folate

Structure

Folate is the generic term used for vitamin B_9 (Furness et al., 2012). Folates are a group of heterocyclic compounds based on the 4-[(pteridin-6-ylmethyl) amino] benzoic acid skeleton conjugated with one or more L-glutamate units. Folate is a naturally occurring form of the vitamin found in food, while folic acid is synthetically produced and used in fortified foods and supplements. Folate is considered an essential nutrient, since it cannot be synthesized in the human body (reviewed by Abu-Saad and Fraser, 2010). Most naturally occurring folates in food contain one to six additional glutamate molecules that are linked through a peptide bond to the gamma-carboxyl group of glutamine. Practically all tissue folates are polyglutamate forms in which the glutamate tail is extended via the gamma-carboxyl of glutamate and these glutamate chain lengths can vary from about 4 to 10 in human tissues. Metabolism of folates to polyglutamate forms is essential for their biological activity and for effective retention of folate by tissues (reviewed by Shane, 2008). They comprise a family of chemically related compounds based on the folic acid structure (reviewed by Shane, 2008). Folic acid on the other hand consists of a p-aminobenzoic acid molecule joined at one end to a pteridine ring and at the other to a single glutamic acid molecule (Fig. 21). It is the parent compound of the folate family which is not found in nature and is prepared by chemical synthesis and is the form which is used for supplements and fortified foods (reviewed by Selhub and Rosenberg, 2008).
**Sources**

Green leafy vegetables like spinach and broccoli, orange juice, legumes (e.g., black beans, kidney beans), nuts, asparagus and strawberries are among the rich sources of folate (reviewed by Simpson et al., 2010). Food folates are relatively unstable to oxidation and heat and therefore large losses are known to occur during food preparation and cooking (reviewed by Allen, 2008).

**Absorption, Transport and Bioavailability**

Folic acid and dietary folate lack the ability to act as a substrate until they have been absorbed from the gastrointestinal tract and hepatically converted to the metabolically active form (reviewed by Pietrzik et al., 2010). Before absorption, they are cleaved to their monoglutamyl forms by a brush border glutamyl hydrolase, sometimes called intestinal folate conjugase (Devlin et al., 2000). Folates are generally reduced to dihydrofolate or to tetrahydrofolate and they have a one carbon unit (methyl, methylene, methenyl, formyl or formimino) at the 5 or 10 positions, or both (Kim et al., 2012). They are then absorbed in the proximal small intestine by a saturable, pH-sensitive transporter that transports oxidized and reduced folates (Qiu et
al., 2006b). It is known that folic acid is absorbed two-fold better than folates (Milman, 2012).

The reduced folate is transported across the apical brush border and the basolateral membranes of the enterocytes by the reduced folate carrier (reviewed by Halsted, 2013). A distinct high affinity folate transporter known as folate-binding protein or the folate receptor are highly expressed in the choroid plexus, kidney proximal tubes and placenta while lower levels have been found in a variety of other tissues (reviewed by Shane, 2008). These receptors are responsible for reabsorption of folate in the kidney by a receptor-mediated endocytotic process and are believed to play a similar role in folate transport in other tissues (Stabler et al., 1991).

The bioavailability of natural folate is only half that of folic acid ingested through supplementation or fortification (reviewed by Hertrampf and Cortés, 2008). Many factors including chemical form of folate, food matrix and the chemical environment in the intestinal tract influence its bioavailability (reviewed by Simpson et al., 2010). The bioavailability of folic acid is close to 100% when consumed on an empty stomach (reviewed by Caudill, 2010). Although information about bioavailability of food folate and folic acid consumed with food is limited, the current best estimates are 50% and 85% for food folate and folic acid respectively (reviewed by Shane, 2008).

**Function**

Folic acid is essential for nucleic acid synthesis, cell multiplication and differentiation processes (Fox and Stover, 2008) and is therefore essential for growth (Antony, 2007). It plays a major role as a coenzyme in the one carbon metabolism (reviewed by Hovdenak and Haram, 2012) and also for many essential cellular reactions (reviewed by Scholl and Johnson, 2000). Folate coenzymes are known to be
involved in amino acid metabolism that involves the donation of a methyl group to homocysteine to form methionine, an essential amino acid that is converted to SAM which participates in the methylation of over 100 different compounds (reviewed by Simpson et al., 2010) (Fig. 22). It is also known to influence antioxidant defences through its role as a superoxide scavenger (Doshi et al., 2001) and can protect bio-constituents such as cellular membranes or DNA from free radical damage (Joshi et al., 2001). It also has a role in promoting immune function (Troen et al., 2006) and has beneficial effects on the endothelial function by improving nitric oxide availability (Antoniades et al., 2006).

**Figure 22: Functions of Folate**

DHF: Dihydrofolate; THF: Tetrahydrofolate; SAM: S-adenosylmethionine; SAH: S-deoxysylhomocysteine

Role of Folate in Pregnancy

Pregnancy is marked with an increase in the rate of DNA synthesis, methylation and chromatin modifications necessary for maternal and fetal tissue growth and development during this period (Gadgil et al., 2014). Hence, folate requirements increase during pregnancy, necessitated by demands to allow synthesis of DNA, RNA, amino acids and other biological compounds (reviewed by Simpson et al., 2010). It helps in increasing red cell mass, enlargement of uterus and growth of placenta and fetus during pregnancy (reviewed by Scholl and Johnson, 2000). Further, it is also known to contribute to oocyte maturation and placentation (Jongbloet et al., 2008). It has a key role in normal development of the fetal spine, brain and skull during the first four weeks of pregnancy (reviewed by Morse, 2012) and an adequate folate supply during the first trimester is important to ensure proper formation, development and closing of the neural tube in humans (reviewed by Imdad and Bhutta, 2012).

It is widely known that periconceptional folic acid supplementation reduces the risk of NTD and other congenital malformations (Furness et al., 2013). The developing embryo obtains folic acid from the maternal diet, in the form of naturally occurring folate found in foods and from maternal intake of synthetic folic acid from vitamins and fortified foods (Zhao et al., 2006). Folate is taken up into the fetal compartment against a concentration gradient and the plasma level in neonates at delivery is about twice the maternal concentration (reviewed by Molloy et al., 2008). It has also been postulated that folate levels in human pregnancies are associated with endothelial function in the neonate possibly through oxidative inactivation and reduced synthesis of nitric oxide (Martin et al., 2007). However, maternal plasma folate concentration is known to decline over the course of pregnancy to about 50% of
nonpregnant levels (Cikot et al., 2001). Maternal folate status is also known to attenuate some of the adverse effects of protein restriction (reviewed by Christian and Stewart, 2010). Thus, it is essential to have an adequate supply of folate during pregnancy (reviewed by Xu et al., 2009).

During pregnancy the recommended dietary allowance (RDA) for folate is almost 50% higher than that of the nonpregnant woman which is considered sufficient to maintain red blood cell folate concentrations (tissue stores) in the normal range (reviewed by Simpson et al., 2010). The National Anaemia Prophylaxis Programme in India recommends folic acid and iron (0.5 mg and 60 mg, respectively) supplementation to all pregnant mothers (reviewed by Kalaivani, 2009; ICMR, 2000).

**Folate Deficiency**

Folate deficiency is known to occur due to various reasons such as inadequate dietary intake, intestinal malabsorption, altered hepatic uptake and metabolism and reduced reabsorption by renal tubular cells (reviewed by Halsted, 2013). Inadequate folate availability results in profound adverse effects in rapidly dividing cells such as those of the developing conceptus thereby contributing to restricted fetal growth (Xiao et al., 2005). Low folate status is also known to cause hyperhomocysteinemia, hypercoagulability and venous thrombosis (reviewed by Hovdenak and Haram, 2012).

Dietary folate deficiency is most common in developing countries with about 25% of pregnant women in India being folate deficient (reviewed by Hovdenak and Haram, 2012). Compromised folate intake or status in mothers is shown to be associated with LBW, abruptio placentae and risk for spontaneous abortions, NTD, preeclampsia, stillbirth and preterm delivery (reviewed by Molloy et al., 2008). The major clinical concern for folate deficiency is increased incidence of NTD (reviewed by Simpson et al., 2010).
Widespread acknowledgement of the negative effects of folate deficiency has resulted in worldwide folate fortification of foods in order to prevent deficiency in pregnant women (reviewed by Berry et al., 2010). Studies have reported a reduced risk of NTD which includes spina bifida (malformation of the spinal column) and anencephaly (absence of major portion of the brain, skull and scalp) being associated with both increased maternal folate intake and higher maternal red blood cell folate concentration (reviewed by Morse, 2012).

**Folate in Pregnancy Complications**

Pregnancy disorders such as preeclampsia, small for gestational age and preterm birth are known to be associated with folate deficiency (Timmermans et al., 2009; Bodnar et al., 2006; reviewed by Scholl and Johnson, 2000). Folate deficiency along with hyperhomocysteinemia has been implicated as risk factors for the subsequent development of preeclampsia and other placenta-mediated diseases (reviewed by Ray and Laskin, 1999). Emerging evidence suggests that folic acid-containing multivitamins reduces the risk of gestational hypertension or preeclampsia (Hernández-Díaz et al., 2002). It has also been suggested that during the periconceptional period, folic acid from food intake and routine supplementation may be adequate, however larger doses must be provided in early gestation, particularly for women with a higher risk of adverse pregnancy outcomes (e.g., preeclampsia) (reviewed by Xu et al., 2009). Folic acid supplementation at 1 mg or higher daily is reported to be associated with a reduced risk of preeclampsia (Wen et al., 2008).

Folic acid is known to reduce the risk of preeclampsia in the following ways (Fig. 23): The first is related to placental implantation and development which is crucial for the health and wellbeing of the mother and the fetus. Therefore, higher folate intakes may be required to support appropriate placental implantation, growth
and development in early pregnancy (Wen et al., 2013). The second is related to the effect of folic acid on lowering blood homocysteine levels (Bernasconi et al., 2006; Chuang et al., 2006), as hyperhomocysteinemia is a risk factor for preeclampsia (Fayed et al., 2004). The third is related to the effect of folic acid supplementation in improving the function of endothelial cells (Antoniades et al., 2006) and therefore reducing the risk of preeclampsia.

**Figure 23: Schematic Representation of Different Proposed Mechanisms through which Folic Acid Reduces the Risk of Developing Preeclampsia**

![Diagram of folic acid mechanisms](source: reviewed by Wen et al., 2013, J Pregnancy. 2013:294312).

### 3.1.3 Vitamin B₁₂

**Structure**

Vitamin B₁₂ is a large organometallic molecule, ~1300-1500 Da in size and is the most chemically complex vitamin known (reviewed by Froese and Gravel, 2010). A more specific name for vitamin B₁₂ is cobalamin and is the largest of the B complex
vitamins stored in the liver. Vitamin B\textsubscript{12} consists of a central cobalt atom surrounded by a heme-like planar corrin ring structure, with the four pyrrole nitrogens coordinated to the cobalt. It contains a phosphoribo-5,6-dimethylbenzimidazolyl side group, with one of the nitrogens linked to the cobalt by coordination at the “bottom” position. The “upper” axial position can be occupied by a number of different ligands such as methyl, cyano, hydroxyl and 5′-deoxyadenosyl groups (reviewed by Shane, 2008) (Fig. 24).

**Figure 24: Structure of Vitamin B\textsubscript{12}**

![Structure of Vitamin B\textsubscript{12}](image)

*Source: reviewed by Shane, 2008, J Pregnancy. 29:S5-16.*

The most stable pharmacological form of the vitamin is cyanocobalamin (reviewed by Simpson et al., 2010). To be biologically active, all parts of the cobalamin molecule must be present (reviewed by Allen, 2008). Derivatives of vitamin B\textsubscript{12} of physiological importance are coenzyme B\textsubscript{12} or adenosylcobalamin (AdoCbl) and methylcobalamin, which have a 5′-deoxy-5′-adenosine or methyl group as the upper axial ligand, respectively (reviewed by Takahashi-Iñiguez et al., 2012). The predominant form in serum is methylcobalamin and the predominant form in the
cytosol is deoxyadenosylcobalamin (reviewed by Klee, 2000). There are three important, interrelated factors contributing to cobalamin reactivity and function: i) the oxidation state of cobalt; ii) whether the 5,6-dimethylbenzimidazole is coordinated to cobalt in the lower axial position; and iii) the identity of the R-group bound in the upper axial position (reviewed by Froese and Gravel, 2010).

Sources

Vitamin B<sub>12</sub> is generally found only in foods of animal origin (reviewed by Simpson et al., 2010). Major dietary sources include animal products such as liver, beef, kidney, chicken, fish such as salmon, halibut and tuna, yogurt, milk, cheese and eggs (reviewed by Anyanwu and Kanu, 2007). Synthetic crystalline vitamin B<sub>12</sub> is used as a fortificant in cereals or as supplements (reviewed by Dror and Allen, 2012). Vitamin B<sub>12</sub> deficiency is known to be prevalent when intake of these foods is low due to their high cost, lack of availability and cultural or religious beliefs. However, deficiency is certainly more prevalent in strict vegetarians (reviewed by de Benoist, 2008).

Absorption, Transport and Bioavailability

Absorption of vitamin B<sub>12</sub> is a complex process, involving a series of steps that can be affected adversely by intestinal disease, infections and medications (reviewed by Allen, 2008). Vitamin B<sub>12</sub> in food is bound to protein and is released in the stomach by the acid environment and by proteolysis of binders by pepsin (reviewed by Shane, 2008). It then subsequently binds to haptocorrin (HC) to form the complex HC-cobalamin (reviewed by Randaccio et al., 2010). In humans, the stomach contains specialized parietal cells that secrete a 50 kDa glycoprotein called intrinsic factor (IF) that can bind cobalamin (reviewed by Shane, 2008). In the small intestine, pancreatic proteases cleave HC and released cobalamin then binds to the IF
to form an IF-cobalamin complex (reviewed by Allen, 2008). The IF-receptor (cubulin) located at the distal ileum at the end of the small intestine recognizes this IF-cobalamin complex, not cobalamin or unligated IF and the complex is internalized by a receptor-mediated endocytotic process (Fedosov et al., 2005). Inside the enterocytes, the IF is degraded and the free cobalamin binds to a 38 kDa protein called transcobalamin II (TC-II) forming a TC-II-cobalamin complex that is released into plasma, where it is endocytosed by membrane receptors, R-TC-cobalamin (Quadros et al., 2009). Once endocytosed, the TC-II-cobalamin complex is degraded in the lysosome and the free cobalamin is transported out of the lysosome to the cytosol. Plasma also contains two additional vitamin B₁₂-binding glycoproteins or R-binders called haptocorrin (transcobalamin I, TC-I) and transcobalamin III (TC-III) which are less specific than TC-II and also bind vitamin B₁₂ analogs (reviewed by Shane, 2008). Once in the cytosol, cobalamin is processed by many proteins to produce the cofactors 5′-deoxyadenosylcobalamin in mitochondrion and methylcobalamin in cytosol (reviewed by Froese and Gravel, 2010). The vitamin is excreted via the urine and the bile (Fig. 25).

The bioavailability of vitamin B₁₂ from the diet is approximately 50% (reviewed by Simpson et al., 2010) whereas synthetic (crystalline) vitamin B₁₂ is known to be more efficiently absorbed; approximately 60% (reviewed by Allen, 2008). Normal body stores are about 1 to 3 mg; the turnover of the vitamin B₁₂ in healthy persons is about 0.1% per day; and signs of deficiency appear when the pool drops below 300 μg (reviewed by Allen, 2008).
Figure 25: Absorption and Cellular Transport Cycle of Vitamin B\textsubscript{12}

**THF**: Tetrahydrofolate; **TC-II-R**: Transcobalamine II Receptor; **TC-II-Cobalamine**: Transcobalamin II-Cobalamine


**Function**

Vitamin B\textsubscript{12} is known to play a vital role in one carbon metabolism (Katre et al., 2010). There are two major metabolic roles for vitamin B\textsubscript{12} where it acts as a cofactor: (a) synthesis of methionine from homocysteine; and (b) conversion of methylmalonyl coenzyme A to succinyl coenzyme A (reviewed by Klee, 2000). It is also critical for nucleotide synthesis and amino acid metabolism (Ronenberg et al.,...
It is known to have a close metabolic inter-relationship with folate as it is required for the conversion of 5-MTHF into tetrahydrofolate (THF), which is the active form of folate involved in the synthesis of DNA and the metabolism of homocysteine (reviewed by Wu et al., 2012) (Fig. 26). It also maintains normal folate metabolism, which is necessary for cell multiplication during pregnancy (Koebnick et al., 2002). It is known to have fundamental roles in the CNS function at all ages and also in the prevention of disorders of CNS development, mood disorders and dementias including Alzheimer's disease and vascular dementia in elderly people (reviewed by Reynolds, 2006).

Figure 26: Role of Vitamin B\textsubscript{12} as a Cofactor

![Diagram showing the role of Vitamin B\textsubscript{12} as a cofactor in the metabolism of homocysteine and folate metabolism.]

**Role of Vitamin B\textsubscript{12} in Pregnancy**

Maternal vitamin B\textsubscript{12} plays a role in intrauterine development which may impact birth weight, risk of diabetes and cognitive functioning (reviewed by Pepper and Black, 2011). It also has a role in DNA synthesis and cell multiplication during pregnancy (reviewed by Carmel et al., 2003). Their concentrations are known to
decline during pregnancy due to haemodilution (Metz et al., 1995). Vitamin $B_{12}$ is concentrated in the placenta and transferred to the fetus, with newborn vitamin $B_{12}$ concentrations approximately double than those of the mother (reviewed by Dror and Allen, 2012). A strong association is known to exist between maternal and infant plasma vitamin $B_{12}$ concentrations at delivery, indicating that maternal vitamin $B_{12}$ status affects the fetal vitamin status at birth (reviewed by Hovdenak and Haram, 2012). In India, the RDA of vitamin $B_{12}$ for pregnancy and lactation is 1.2 and 1.5 $\mu$g/day respectively (ICMR, 2009).

**Vitamin $B_{12}$ Deficiency**

Vitamin $B_{12}$ deficiency is mainly seen in pregnant women consuming a predominantly vegetarian diet (reviewed by Hovdenak and Haram, 2012). Studies in India have shown a low dietary consumption of vitamin $B_{12}$ due to dietary pattern of vegetarianism and poor consumption of milk and milk products resulting in deficiency of vitamin $B_{12}$ although concentrations of folate are adequate. Thus, this leads to an imbalance of plasma folate and vitamin $B_{12}$, which might have physiological consequences during pregnancy (Gadgil et al., 2014).

Elevated methylmalonic acid and total homocysteine concentrations are considered as sensitive metabolic markers for vitamin $B_{12}$ deficiency (Herrmann et al., 2000). Vitamin $B_{12}$ deficiency primarily in the elderly occurs due to malabsorption (reviewed by Allen, 2008). Deficiency of vitamin $B_{12}$ is also known to cause pernicious anemia (reviewed by Pepper and Black, 2011). Myelopathy and neuropathy are known to be main clinical manifestations of vitamin $B_{12}$ deficiency (reviewed by Cetin et al., 2010). Symptoms of vitamin $B_{12}$ deficiency include megaloblastic anaemia, tingling and numbness of the extremities, gait abnormalities, visual disturbances, memory loss and dementia. Furthermore, folic acid fortification
or supplementation with >1 mg/day is known to ‘mask’ clinical symptoms of vitamin B$_{12}$ deficiency (reviewed by Dror and Allen, 2012). Thus, vitamin B$_{12}$ supplementation may be especially needed in women on vegetarian diets, in malabsorption disorders and in communities or countries where undernutrition is common (reviewed by Hovdenak and Haram, 2012).

**Vitamin B$_{12}$ in Pregnancy Complications**

Maternal vitamin B$_{12}$ deficiency is known to be associated with increased risk for several adverse pregnancy outcomes for both mother and fetus (Vanderjagt et al., 2011) such as NTD (Molloy et al., 2009), IUGR (Muthayya et al., 2006), preeclampsia (reviewed by Allen, 2005) and early miscarriage (Hübner et al., 2008). Poor maternal B-vitamin status has been a major global cause of hyperhomocysteinemia and poor pregnancy outcomes (reviewed by Allen, 2005). Therefore, the next section describes homocysteine and its role in several pregnancy complications.

### 3.1.4 Homocysteine

**Structure**

Homocysteine is a non-protein forming, thiol-containing, four-carbon amino acid derived from the demethylation of the essential amino acid methionine (reviewed by Holmes, 2003) (Fig. 27). It is at the intersection of two metabolic pathways; (1) the remethylation cycle that includes remethylation to methionine, which requires folate, vitamin B$_{12}$ or betaine; and (2) transsulfuration to cystathionine, which requires pyridoxal-5'-phosphate (Miller et al., 2003). Thus, homocysteine may be either remethylated to methionine or eliminated from the cycle by formation of cysteine in the transsulfuration pathway (Mislanova et al., 2011). The methionine cycle takes
place in all cell types, whereas transsulfuration occurs in a limited number of tissues including liver, kidney, small intestine and pancreas (reviewed by Finkelstein, 2006).

**Figure 27: Structure of Homocysteine and Homocystine**

![Structure of Homocysteine and Homocystine](image)


Structurally, homocysteine closely resembles methionine and cysteine and all three amino acids contain sulfur. Homocystine is a dimer composed of two oxidized molecules of homocysteine linked by a disulfide bond (reviewed by Klee, 2000) (Fig. 27). Only 1 to 2% of total homocysteine circulate in blood freely in its reduced form, while 70 to 90% are protein-bound and the remaining are disulfides, homocystine and the mixed disulfide homocysteine-cysteine (reviewed by Herrmann, 2001). The sum of all the forms of homocysteine present in plasma is usually referred as total homocysteine (reviewed by Medina et al., 2001). It is an essential amino acid required for the growth of cells and tissues in the human body. The only source of
homocysteine in the human organism comes from the methionine in dietary proteins. Methionine is the only essential, sulfur containing amino acid in mammalian diets which are mainly of animal origin (reviewed by de la Calle et al., 2003). Homocysteine metabolism is strongly linked to its function as a methyl group donor in transmethylation reactions (Poirier, 2002). There are four key enzymes involved in homocysteine metabolism: cystathionine β-synthase, 5,10-methylentetrahydrofolate reductase, methionine synthase and methionine synthase reductase (Also-Rallo et al., 2005).

It is important to note that plasma homocysteine can also serve as a functional indicator of vitamin status and therefore is useful as a monitoring tool to measure the efficacy of a food-fortification program with B-vitamins (reviewed by Selhub, 2008). Circulating total homocysteine concentrations are a useful integral marker of one carbon metabolism and are known to be influenced by genetic and dietary factors (Katre et al., 2010). Homocysteine is an oxidant that can generate reactive oxygen species thereby damaging macromolecules, including DNA, proteins and lipids (reviewed by Wu et al., 2012). Homocysteine is highly cytotoxic and the intracellular homocysteine concentration is kept low by catabolism and by a cellular homocysteine export mechanism into plasma (reviewed by Herrmann, 2001). Plasma homocysteine is mainly metabolized in the kidney (about 70%) but very little is excreted into the urine (reviewed by Yeun, 1998).

**Hyperhomocysteinemia**

Hyperhomocysteinemia either results from a genetic defect in the enzymes that participate in homocysteine synthesis and metabolism or a deficiency of folic acid, vitamins B₆ and B₁₂ (Makedos et al., 2007). Elevated homocysteine levels are a
sign of disturbed remethylation of homocysteine (Geisel et al., 2005). Higher plasma total homocysteine concentrations are also associated with deficits in cognition, arterial and/or venous thrombosis, vascular dementia, neuropathies, risk of stroke and myocardial infarction (reviewed by Cetin et al., 2010). Studies have shown that hyperhomocysteinemia usually can be corrected by supplementation with folic acid (Yamamoto et al., 2012; Scorsatto et al., 2011).

**Homocysteine and Pregnancy**

In normal pregnancy, homocysteine concentrations fall with advancing gestational age (Guven et al., 2009) and are linked to many factors such as: physiological response to pregnancy, increase in oestrogen, hemodilution from increased plasma volume or increased demand for methionine by both the mother and the fetus (Davari-Tanha et al., 2008).

Elevated levels of homocysteine are associated with a greater risk of adverse pregnancy outcomes. The proposed mechanisms are: increase in oxygen free radical concentrations and reduction in nitrous oxide concentrations, leading to endothelial dysfunction. It also increases oxidative stress and results in placental ischemia, increases inflammatory response that is cytotoxic to endothelial cells and also leads to apoptosis of endothelial cells (reviewed by Allen, 2005). Further, placental development in early pregnancy is also known to be negatively influenced by increased maternal homocysteine concentrations (Steegers-Theunissen and Steegers, 2003).

**Homocysteine and Preeclampsia**

A number of studies have reported elevated levels of homocysteine in women with preeclampsia (Mujawar et al., 2011; Napolitano et al., 2008; Braekke et al., 2007). Hyperhomocysteinemia is suggested to play a key role in the pathogenesis of
preeclampsia through oxidative stress and endothelial cell dysfunction which is the central theme ultimately leading to hypertension and proteinuria during gestation (reviewed by Steegers et al., 2010; Falcao et al., 2009). Homocysteine is known to injure the vascular endothelium by generating hydrogen peroxide and by impairing basal nitric oxide production (Stamler et al., 1993). Further, hyperhomocysteinemia increases oxidative stress which is caused by an increase in the concentration of fibronectin, lipid peroxides and plasma triglycerides (reviewed by de la Calle et al., 2003). Elevated homocysteine levels which are associated with preeclampsia are also reported to be due to genetic abnormalities (reviewed by Selhub, 1999). A recent study demonstrates correlation between folate, vitamin B$_{12}$ and homocysteine that are required for the remethylation of homocysteine to methionine in the one carbon cycle (Mujawar et al., 2011).

### 3.1.5 One Carbon Cycle

The growing field of epigenetic research has highlighted the role of one carbon metabolites on the developmental programming of chronic disease (reviewed by Waterland and Michels, 2007). One carbon metabolism is a network of interrelated biochemical reactions that involve the transfer of one carbon groups from one site to another (reviewed by Choi and Mason, 2000). It is compartmentalized in the cell, occurring primarily within the cytoplasm and the mitochondria (reviewed by Beaudin and Stover, 2007). These reactions are significant for the production of DNA bases, the conversion of homocysteine into methionine, neurological and immunological function, growth and development and the formation of red blood cells (reviewed by Wu et al., 2012).

The vitamins, folate and B$_{12}$ serve as coenzymes in the one carbon metabolism (reviewed by Selhub et al., 2008). One carbon metabolism in mitochondria primarily
functions to generate one carbon units in the form of formate for cytoplasmic one carbon metabolism. In the cytoplasm, folate activated one carbon units act in an interdependent anabolic network comprised of 3 biosynthetic pathways: \textit{de novo} purine biosynthesis; \textit{de novo} thymidylate biosynthesis and the remethylation of homocysteine to methionine (reviewed by Stover, 2009). In this metabolism, a carbon unit from serine or glycine reacts with THF to form methylene-THF (reviewed by Selhub et al., 2008). This form of folate can be used for the synthesis of thymidine; oxidized to formyltetrahydrofolate for the synthesis of purines or reduced to 5-MTHF and used to methylate homocysteine to form methionine (reviewed by Ross, 2003).

Methionine is formed via the vitamin B$_{12}$-dependent transfer of a methyl group from 5-MTHF to homocysteine in the methionine synthase reaction (James et al., 2004). Methionine is then activated by methionine adenosyltransferase to form SAM, the primary methyl donor (Geisel et al., 2005). It is known to donate its labile methyl groups to more than 80 biological methylation reactions, including the methylation of DNA, RNA, proteins, phospholipids and neurotransmitters (reviewed by Choi and Mason, 2002). The transfer of the methyl group from SAM to the various methyl acceptors via numerous methyltransferases results in the formation of S-adenosylhomocysteine (SAH) (James et al., 2004). SAH is subsequently cleaved to homocysteine, which lies at an important metabolic branch point. Alternatively, homocysteine can be remethylated to reform methionine via either betaine-homocysteine methyltransferase or methionine synthase or can be exported to the extracellular space (Stead et al., 2006).

In some cell types and tissues, homocysteine can also be degraded by the transsulfuration pathway, through which homocysteine irreversibly condenses with serine to form cystathionine via a vitamin B$_{6}$-dependent enzyme cystathionine $\beta$-
synthase (reviewed by Beaudin and Stover, 2007). Pregnancy involves a marked acceleration in one carbon transfer reactions, particularly those required for nucleotide synthesis and thus cell division, which is the basis for the substantial increase in folate requirements during pregnancy (reviewed by Bailey, 2000). Impairments at various steps that affect remethylation of homocysteine to methionine or degradation of homocysteine to cysteine, including inadequate concentrations of folate or vitamin B_{12}, will result in elevated homocysteine concentrations (reviewed by Selhub et al., 2008).

One of the major methyl acceptors in the one carbon cycle are the phospholipids. Phosphatidylethanolamine-N-methyltransferase (PEMT) catalyzes the sequential methylation of phosphatidylethanolamine to phosphatidylcholine (reviewed by Vance et al., 1997). PEMT preferentially utilizes phosphatidylethanolamine that contains DHA molecule generating a phosphatidylcholine molecule containing DHA (DeLong et al., 1999). It has thus been suggested that the methylation of phosphatidylethanolamine to phosphatidylcholine by PEMT plays an important role in the transport of PUFA like DHA from the liver to the plasma and other tissues (Pynn et al., 2011; Selley, 2007). Further, it has been hypothesized that B-vitamin (folate, vitamin B_{12}) availability can directly modify liver PEMT activity and PEMT-dependent PUFA secretion. Thus, adequate dietary intake of these vitamins would be necessary to maintain normal plasma DHA concentrations and thus tissue availability (van Wijk et al., 2012). Further, animal studies in our department have also extensively demonstrated that alterations in maternal levels of folate and vitamin B_{12} affect the levels of plasma, brain, milk and placental DHA (Roy et al., 2012; Dangat et al., 2011; Kulkarni et al., 2011a). Besides, our studies on pregnant women have also discussed that micronutrients such as folic acid, vitamin B_{12} and DHA are
interlinked in the one carbon cycle (reviewed by Dhobale and Joshi, 2012; reviewed by Sundrani et al., 2011; Kulkarni et al., 2011b) (Fig. 28).

**Figure 28: One Carbon Cycle**

![One Carbon Cycle Diagram](image)

**5,10-MTHF**: 5,10-Methylenetetrahydrofolate; **5-MTHF**: 5-Methyltetrahydrofolate; **COMT**: Catecholamine-O-Methyltransferase; **DA**: Dopamine; **DHA**: Docosahexaenoic Acid; **DNA**: Deoxyribonucleic Acid; **EP**: Epinephrine; **MS**: Methionine Synthase; **MT**: Methyltransferase; **MTHFR**: Methyleneetahydrofolate Reductase; **NE**: Nor-Epinephrine; **PC-DHA**: Phosphatidylcholine-DHA; **PE-DHA**: Phosphatidylethanolamine-DHA; **PEMT**: Phosphatidylethanolamine Methyltransferase; **RNA**: Ribonucleic Acid; **SAH**: S-Adenosyl Homocysteine; **SAM**: S-Adenosyl Methionine; **THF**: Tetrahydrofolate.

Earlier cross-sectional studies carried out in our department in women with pregnancy complications such as preeclampsia indicate altered levels of LCPUFA and higher levels of homocysteine at delivery, which are associated with poor birth outcome (Dhobale et al., 2012; Kulkarni et al., 2011b, 2011c; Dangat et al., 2010; Mehendale et al., 2008). Furthermore, the department also reported a negative association between erythrocyte DHA and plasma homocysteine concentrations in preeclampsia (Kulkarni et al., 2011b). However, all these observations were made at
the end of pregnancy when the pathology had progressed. Thus, it would be very useful to analyze these levels in early pregnancy to examine changes over time, to understand their role in various pregnancy complications. There is also a need to examine longitudinally these associations during pregnancy as they are important determinants of the one carbon cycle, which play an important role in fetal programming and increase the risk of developing NCD such as type 2 diabetes (reviewed by Yajnik and Deshmukh, 2008) and CVD (reviewed by Martinelli et al., 2009; reviewed by Erkkilä et al., 2008) in later life.

This chapter therefore examines maternal plasma folate, vitamin B₁₂ and homocysteine levels at three different time points during gestation and their association with maternal fatty acid levels (plasma and erythrocyte, discussed earlier in Chapter 2) in women with preeclampsia and compare them with normotensive control women.

3.2 Methods and Materials

3.2.1 Study Subjects

The criteria for recruitment of study population and the inclusion and exclusion criteria are as mentioned in Chapter 2 (Section 2.2.1).

3.2.2 Sample Collection and Processing

Blood samples were collected and processed as described in Chapter 2 (Section 2.2.2). Folate in plasma is known to be unstable with long storage times, therefore analysis for folate, vitamin B₁₂ and homocysteine was carried out immediately. The storage cut-off was 3 months. Care was taken not to perform analysis on samples that were stored for a longer duration. Furthermore, hemolyzed samples were not used for analysis. Figure 29 shows the number of maternal plasma
samples analyzed for folate, vitamin B\textsubscript{12} and homocysteine levels at various time points.

**Figure 29: Flow Chart Showing Number of Maternal Plasma Samples Analyzed for Folate, Vitamin B\textsubscript{12} and Homocysteine Levels at Various Time Points**

NC: Normotensive Control; PE: Preeclampsia

### 3.2.3 Neonatal Measurements

Neonatal measurements were recorded as described in Chapter 2 (Section 2.2.3).

### 3.2.4 Folate, Vitamin B\textsubscript{12} and Homocysteine Estimations

Folate, vitamin B\textsubscript{12} and homocysteine levels were estimated by the chemiluminescent microparticle immunoassay (CMIA) technology (Abbott Diagnostics, Abbott Park, IL, USA) (reviewed by Lee and Griffiths, 1985) and described by our department earlier (Dhobale et al., 2012). Briefly, 100 μl of plasma...
was used for analysis of each folate, vitamin B$_{12}$ and homocysteine. The folate, vitamin B$_{12}$ and homocysteine assay was a two-step assay with an automated sample pre-treatment for determining the presence of folate, vitamin B$_{12}$ and homocysteine in human plasma. The reference range for plasma folate assay was 2.34-17.56 ng ml$^{-1}$, for plasma vitamin B$_{12}$ assay was 187-883 pg ml$^{-1}$ and for homocysteine assay it was 5.08-15.39 µmol L$^{-1}$.

Low plasma folate and vitamin B$_{12}$ concentrations were defined as <10 ng ml$^{-1}$ and <150 pg ml$^{-1}$ respectively and elevated plasma total homocysteine concentrations as a concentration >10 µmol L$^{-1}$ (Kulkarni et al., 2011b).

3.2.5 Statistical Analysis

The data was analyzed using the SPSS/PC+ package (Version 20, Chicago, IL, USA). Values are reported as mean ± SD. Skewed variables were transformed to normality using the log to the base 10 transformation (plasma folate, vitamin B$_{12}$, homocysteine). Independent t-test was used to compare mean values of the various parameters between normotensive control and preeclampsia (p<0.05) after adjusting for gestation and socioeconomic status. Correlation between variables was studied using Pearson’s correlation analysis after adjusting for gestation and socioeconomic status. The variable sample number (n) in different measures was due to insufficient sample volume available.

3.3 Results

3.3.1 Frequency of Consumption of Folate and Vitamin B$_{12}$ Rich Foods

In our cohort, the major source of folate was green leafy vegetables (e.g., spinach, ambat chukka) and legumes (e.g., cowpea, bengalgram, red gram). The rich sources of vitamin B$_{12}$ included dairy products and non-vegetarian food items. Dairy
products included whole milk and milk products (milk in tea and other beverages, yoghurt, buttermilk, ghee, ice cream and other milk based preparations). The non-vegetarian foods included meat, fish and eggs. The frequency of consumption of folate rich foods was similar in both the normotensive control and preeclampsia groups at T1 (p=0.134), T2 (p=0.995) and T3 (p=0.616). Similarly, the frequency of consumption of vitamin B$_{12}$ rich foods was similar in both the groups at T1 (p=0.990), T2 (p=0.484) and T3 (p=0.364). The percent women consuming folate and vitamin B$_{12}$ rich foods in both normotensive control and preeclampsia groups are in Table 12.

Table 12: Frequency of Consumption of Foods Rich in Folate and Vitamin B$_{12}$ at Three Time Points during Pregnancy

<table>
<thead>
<tr>
<th></th>
<th>Food Group</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>p</th>
<th>NC</th>
<th>PE</th>
<th>p</th>
<th>NC</th>
<th>PE</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>(n=143)</td>
<td>(n=52)</td>
<td>(n=114)</td>
<td>(n=37)</td>
<td>(n=131)</td>
<td>(n=42)</td>
<td>(n=2)</td>
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<tr>
<td>Folate Rich Foods</td>
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<td></td>
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<tr>
<td>Never</td>
<td>7(4.9)</td>
<td>7(13.5)</td>
<td>0.040</td>
<td>6(5.3)</td>
<td>2(5.4)</td>
<td>0.973</td>
<td>8(6.1)</td>
<td>2(4.8)</td>
<td>0.745</td>
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<tr>
<td>Weekly twice</td>
<td>82(57.3)</td>
<td>29(55.8)</td>
<td>0.844</td>
<td>61(53.5)</td>
<td>19(51.4)</td>
<td>0.819</td>
<td>66(50.4)</td>
<td>17(40.5)</td>
<td>0.264</td>
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<tr>
<td>Weekly 2-4 times</td>
<td>40(28)</td>
<td>14(26.9)</td>
<td>0.884</td>
<td>36(31.6)</td>
<td>12(32.4)</td>
<td>0.923</td>
<td>37(28.2)</td>
<td>16(38.1)</td>
<td>0.228</td>
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<tr>
<td>More than 4 times</td>
<td>14(9.8)</td>
<td>2(3.8)</td>
<td>0.181</td>
<td>11(9.6)</td>
<td>4(10.8)</td>
<td>0.837</td>
<td>20(15.3)</td>
<td>7(16.7)</td>
<td>0.828</td>
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<td>in a week</td>
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<tr>
<td>Vitamin B$_{12}$ Rich Foods</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Never</td>
<td>3(2.1)</td>
<td>1(1.9)</td>
<td>0.938</td>
<td>1(0.9)</td>
<td>0(0)</td>
<td>0.568</td>
<td>1(0.8)</td>
<td>2(2.4)</td>
<td>0.394</td>
<td></td>
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<tr>
<td>Weekly once</td>
<td>37(25.9)</td>
<td>14(26.9)</td>
<td>0.883</td>
<td>23(20.2)</td>
<td>5(13.5)</td>
<td>0.365</td>
<td>22(16.8)</td>
<td>26(9.5)</td>
<td>0.257</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weekly twice</td>
<td>53(37.1)</td>
<td>18(34.6)</td>
<td>0.753</td>
<td>41(36)</td>
<td>11(29.7)</td>
<td>0.488</td>
<td>46(35.1)</td>
<td>58(28.6)</td>
<td>0.434</td>
<td></td>
<td></td>
</tr>
<tr>
<td>More than 2 times</td>
<td>50(35)</td>
<td>19(36.5)</td>
<td>0.839</td>
<td>49(43)</td>
<td>21(56.8)</td>
<td>0.144</td>
<td>62(47.3)</td>
<td>87(59.5)</td>
<td>0.169</td>
<td></td>
<td></td>
</tr>
<tr>
<td>in a week</td>
<td></td>
<td></td>
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</table>

NC - Normotensive control; PE - Preeclampsia; n - Number of subjects; p - Significance; T1=16$^{th}$-20$^{th}$ week; T2= 26$^{th}$-30$^{th}$ week; T3= at delivery

3.3.2 Levels of Maternal Plasma Folate, Vitamin B$_{12}$ and Homocysteine across Gestation

Maternal plasma folate levels were similar between preeclampsia and normotensive control groups at all time points across gestation. Maternal plasma vitamin B$_{12}$ levels were significantly higher in preeclampsia (p<0.05) as compared with normotensive control at T2. On the other hand, maternal plasma homocysteine...
levels were higher in preeclampsia as compared with normotensive control at T1, T2 (p<0.05 for both) and T3 (p<0.01) (Fig. 30).

**Figure 30: Plasma Folate, Vitamin B<sub>12</sub> and Homocysteine Levels across Gestation**

![Bar charts showing plasma folate, vitamin B<sub>12</sub> and homocysteine levels across gestation.](image)

NC - Normotensive control; PE - Preeclampsia; T1=16<sup>th</sup>-20<sup>th</sup> week; T2= 26<sup>th</sup>-30<sup>th</sup> week; T3= at delivery; **p<0.01, *p<0.5 as compared with NC

The plasma folate levels were <10 ng ml<sup>-1</sup> in 38.88%, 42.24% and 54.76% of normotensive control women and 24.19%, 35.55% and 50% of women with preeclampsia at T1, T2 and T3, respectively. The plasma vitamin B<sub>12</sub> levels were <150 pg ml<sup>-1</sup> in 25.6%, 39.13% and 46.03% of normotensive control women and 17.74%, 29.54% and 39.13% of women with preeclampsia at T1, T2 and T3,
respectively, whereas the plasma homocysteine levels were >10 µmol L⁻¹ in 4.03%, 3.47% and 16% of normotensive control women and 6.55%, 4.54% and 23.91% of women with preeclampsia at T1, T2 and T3, respectively.

3.3.3 Associations between Folate, Vitamin B₁₂ and Homocysteine in Maternal Plasma

There was a negative association between maternal plasma folate and maternal plasma homocysteine at T2 (r = -0.189, p = 0.045, n = 115) in normotensive control. Similar negative association was observed between maternal plasma vitamin B₁₂ and maternal plasma homocysteine at T1 and T2 (r = -0.258, p = 0.004, n = 123; r = -0.264, p = 0.005, n = 114) in normotensive control. However, no associations were observed in the preeclampsia group.

3.3.4 Associations of Maternal Plasma Fatty Acids with Maternal Plasma Folate, Vitamin B₁₂ and Homocysteine

There was a positive association between maternal plasma folate and maternal plasma AA at T3 (r = 0.271, p = 0.003, n = 125) in normotensive control. While in preeclampsia there was a positive association between maternal plasma folate and maternal plasma DHA at T1 (r = 0.402, p = 0.003, n = 54).

A positive association was observed between maternal plasma vitamin B₁₂ and maternal plasma AA at T1 in normotensive control and preeclampsia (r = 0.203, p = 0.031, n = 122; r = 0.295, p = 0.035, n = 54 respectively). In preeclampsia, there was a positive association of maternal plasma vitamin B₁₂ with maternal plasma DHA and total omega-3 fatty acids at T1 (r = 0.327, p = 0.019, n = 54; r = 0.388, p = 0.005, n = 54 respectively).

There was a negative association of maternal plasma homocysteine with maternal plasma AA at T1 and T3 (r = -0.235, p = 0.013, n = 121; r = -0.180, p =
0.050, n = 124 respectively) and maternal plasma DHA at T3 (r = -0.185, p = 0.044, n = 124) in normotensive control. In preeclampsia, a negative association was observed between maternal plasma homocysteine and maternal plasma DHA at T2 (r = -0.447, p = 0.006, n = 44). Similar negative association was seen between maternal plasma homocysteine and maternal plasma total omega-3 fatty acids at T1 (r = -0.274, p = 0.054, n = 53) in preeclampsia.

3.3.5 Associations of Maternal Erythrocyte Fatty Acids with Maternal Plasma Folate, Vitamin B\textsubscript{12} and Homocysteine

There was a positive association between maternal plasma folate and maternal erythrocyte DHA at T1 and T3 (r = 0.280, p = 0.038, n = 58; r = 0.340, p = 0.032, n = 46 respectively) in preeclampsia. Similar positive association was seen between maternal plasma folate and maternal erythrocyte total omega-3 fatty acids at T1 (r = 0.287, p = 0.034, n = 58) in preeclampsia. However, there was no association observed in the normotensive control group.

There was a positive association of maternal plasma vitamin B\textsubscript{12} with maternal erythrocyte DHA and total omega-3 fatty acids at T3 (r = 0.454, p = 0.003, n = 46; r = 0.393, p = 0.012, n = 46 respectively) in preeclampsia. However, there was no association observed in the normotensive control group.

3.3.6 Associations of Maternal Plasma Folate, Vitamin B\textsubscript{12} and Homocysteine with Birth Outcome

There was a positive association of maternal plasma folate and maternal plasma vitamin B\textsubscript{12} with baby weight (r = 0.190, p = 0.040, n = 126; r = 0.224, p = 0.015, n = 126) at T3 in normotensive control. However, no associations were observed in preeclampsia.
3.4 Discussion

The present prospective study examined the levels of maternal plasma folate, vitamin B₁₂ and homocysteine across gestation in normotensive control women and women with preeclampsia. Further, their associations with maternal fatty acids (plasma and erythrocyte) were also examined. Our results for the first time indicate several interesting observations in preeclampsia which are as follows:

1) No change in the maternal plasma levels of folate at all time points
2) A significant increase in the maternal plasma levels of vitamin B₁₂ only at T2
3) A significant increase in the maternal plasma levels of homocysteine as gestation advances
4) Positive association of maternal plasma folate with maternal plasma and erythrocyte DHA at T1
5) Positive association of maternal plasma vitamin B₁₂ with maternal plasma DHA and total omega-3 fatty acids at T1
6) Negative association of maternal plasma homocysteine with maternal plasma DHA at T2 and total omega-3 fatty acids at T1
7) In addition, a positive association of maternal plasma folate and vitamin B₁₂ with baby weight at T3 was observed only in normotensive control

3.4.1 Maternal Plasma Folate Levels

In the present study, maternal plasma folate levels were not significantly different at all time points between the preeclampsia and normotensive control groups. These results are similar to earlier studies which did not find any significant difference in the folate levels between control and preeclampsia (Li et al., 2013; Thériault et al., 2013; Furness et al., 2012; Guven et al., 2009; Makedos et al., 2007;
Braekke et al., 2007; Rajkovic et al., 1997). It has been suggested that there may be some other factors in addition to this vitamin deficiency which play a role in the etiopathogenesis of preeclampsia (Acilmis et al., 2011).

In contrast, few other studies have reported either lower levels of folate (Salehi-PourMehr et al., 2012; Bergen et al., 2012; Mujawar et al., 2011; Patrick et al., 2004; Sanchez et al., 2001) or higher levels (Also-Rallo et al., 2005; López-Quesada et al., 2003; Powers et al., 2003) in preeclampsia as compared to controls. The discrepancy of these reports may be a result of various factors, including the timing of the intervention, the dose of folic acid, the other components in the supplements and the population (Li et al., 2013). As per the National Anemia Prophylaxis Programme all women in our cohort were routinely given iron (60 mg) and folic acid (500 µg) tablets during the first trimester of pregnancy. One possible explanation for similar levels could also be due to this Prophylaxis Programme, as in our study the first sample was collected at the end of first trimester. Similar observation has been reported by others in a cohort of pregnant women benefiting from a national policy of folic acid food fortification along with a high adherence to folic acid supplementation (Thériault et al., 2013).

3.4.2 Maternal Plasma Vitamin B_{12} Levels

In the current study, maternal plasma vitamin B_{12} levels were comparable between preeclampsia and normotensive control group at T1 and at T3. However, at T2, levels of maternal plasma vitamin B_{12} were higher in preeclampsia as compared to normotensive control. Studies carried out in preeclampsia are contradictory, with few reporting no change (Bergen et al., 2012; Acilmis et al., 2011; Guven et al., 2009; Makedos et al., 2007; López-Quesada et al., 2003; Sanchez et al., 2001; Rajkovic et al., 1997) and others reporting lower levels (Mujawar et al., 2011; Laivuori et al.,
There is one study which reports significantly higher vitamin B_{12} levels found in patients with preeclampsia bearing the wild type genotype of MTHFR gene involved in the folate-homocysteine metabolism. However, they suggest that these results are difficult to explain and should be confirmed in a larger group of patients (Also-Rallo et al., 2005).

In addition to the above studies, there is one report suggesting that vitamin B_{12} concentrations are specific to different races and do not differ by pregnancy outcome (Patrick et al., 2004). It has also been suggested that transcobalamin I is ubiquitous in most body fluids and its major importance is the problem it may cause with false positive increased vitamin B_{12} measurements (reviewed by Klee, 2000). Further, it has also been reported that because serum vitamin B_{12} levels are often influenced by factors unrelated to vitamin B_{12} intake, stores or deficiency, it is unclear whether the differences in concentrations actually reflect vitamin B_{12} status (Patrick et al., 2004).

Elevated levels of plasma cobalamin are known to be associated with functional cobalamin deficiency (Ermens et al., 2003). Increased levels of vitamin B_{12} are reported to be associated with inflammatory diseases (Geissbühler et al., 2000) and inflammation is known to contribute to the development of preeclampsia (López-Jaramillo et al., 2008). Thus, this may possibly have contributed to the increased levels of vitamin B_{12} observed in the present study and our earlier departmental cross-sectional study (Dhobale et al., 2012).

Despite similar frequency of consumption of folate and vitamin B_{12} rich foods between both the groups in our study, maternal vitamin B_{12} levels were higher in preeclampsia at T2. However, maternal vitamin B_{12} levels in Indians are known to be lower than that reported in western subjects (reviewed by Muthayya, 2009) and findings from the study support the same. This is because of low dietary consumption
of vitamin B$_{12}$ as vegetarianism has been widely practiced in India (reviewed by Antony, 2003).

3.4.3 Maternal Plasma Homocysteine Levels

The current prospective study reports higher maternal plasma homocysteine levels across pregnancy starting from 16$^{th}$ week of gestation till delivery in women with preeclampsia as compared with normotensive control women. Our results are in accordance with studies reporting higher levels of homocysteine at 11$^{th}$-16$^{th}$ week (Bergen et al., 2012), at the 11$^{th}$-14$^{th}$ week (Kaymaz et al., 2011), at the 24$^{th}$ and 34$^{th}$ week of gestation (López-Quesada et al., 2003). In addition, others too report that elevated homocysteine levels in early pregnancy are known to be associated with the later development of mild preeclampsia (Cotter et al., 2003). A study suggests that elevations in homocysteine levels precede the clinical manifestation of preeclampsia by approximately 8-16 weeks (Sorenson et al., 1999). In contrast, others indicate that homocysteine measured in second trimester cannot be used as a screening test for preeclampsia (Hietala et al., 2001). A number of other studies have also found higher levels of homocysteine in preeclampsia as compared with normotensive control (Kim et al., 2012; Acilmis et al., 2011; Mujawar et al., 2011; Guven et al., 2009; Makedos et al., 2007; Patrick et al., 2004; López-Quesada et al., 2003; Sanchez et al., 2001). However, the above mentioned studies were either carried out during early pregnancy, third trimester or at delivery.

Elevated plasma total homocysteine is known to arise from inadequate folate or vitamin B$_{12}$ status (reviewed by Molloy et al., 2008). In the current study there was a negative association of maternal plasma folate and vitamin B$_{12}$ with homocysteine in normotensive control group although these observations were not observed in the preeclampsia group. Reports indicate that elevated serum homocysteine levels were
not associated with deficiency of folic acid and vitamin B\textsubscript{12} in preeclampsia (Acilmis et al., 2011). Our findings support studies which report that vitamin B\textsubscript{12} is a predictor for maternal total homocysteine for the control group, but not in the preeclampsia group (Brække et al., 2007).

Studies suggest that increased homocysteine levels may cause oxidative stress and endothelial dysfunction that would ultimately lead to hypertension and proteinuria during gestation (Falcao et al., 2009). Few studies however believe that, in most cases, hyperhomocysteinemia may be a consequence rather than a cause of hypertensive disorders of pregnancy (Bergen et al., 2012; Steegers-Theunissen et al., 2004). Hyperhomocysteinemia observed in preeclampsia could also be explained partially by the pathologic process of hemoconcentration observed in preeclampsia (Acilmis et al., 2011). Thus, these elevated levels of homocysteine in preeclampsia observed in the current study support the dysregulation of the one carbon cycle in preeclampsia.

### 3.4.4 Associations of Maternal Fatty Acids (Plasma and Erythrocyte) with Maternal Plasma Folate, Vitamin B\textsubscript{12} and Homocysteine

The current study for the first time reports a positive association of maternal plasma folate and vitamin B\textsubscript{12} with maternal plasma and erythrocyte DHA during pregnancy in preeclampsia. Furthermore, there was a negative association between maternal plasma DHA and homocysteine in preeclampsia, suggesting the associations of folate, vitamin B\textsubscript{12} and homocysteine with DHA in the one carbon cycle as has been reported by us earlier in our departmental human and animal studies (Roy et al., 2012; Sable et al., 2012; Kulkarni et al., 2011a, 2011b; Dangat et al., 2011; Kale et al., 2010). Our findings indicate that homocysteine levels in preeclampsia are possibly influenced by DHA. It is well known that homocysteine itself is known to generate
reactive oxygen species and induce lipid peroxidation (Loscalzo, 1996). A number of animal and human studies have also suggested a link between hyperhomocysteinemia, lipid peroxidation and a decrease in omega-3 fatty acids (Assies et al., 2004). It has been suggested that by reducing homocysteine concentrations, folate may reduce the generation of reactive oxygen species and thus spare DHA, which is a target for lipid peroxidation (Durand et al., 1996). Furthermore, intervention studies and recent meta-analysis document that the high consumption of omega-3 fatty acids decreases plasma homocysteine levels (Huang et al., 2011).

3.4.5 Associations of Maternal Plasma Folate and Vitamin B$_{12}$ with Birth Outcome

A positive association was observed between maternal plasma folate and baby weight at T3 in the normotensive control group in the current study. It is well known that due to the role of folate in DNA synthesis and cell replication it can influence fetal growth (reviewed by Scholl and Johnson, 2000). Further, a number of observational studies suggest a possible beneficial effect of good maternal folate status on birth weight (Relton et al., 2005; Neggers et al., 1997; Frelut et al., 1995). The current study also reports a positive association of maternal plasma vitamin B$_{12}$ and baby weight at T3. Thus, our findings suggest that supplementation of both folic acid and vitamin B$_{12}$ may be useful to improve baby weight.

Summary

The present prospective data indicates altered levels of vitamin B$_{12}$, homocysteine and DHA, key components of one carbon cycle in preeclampsia. This study for the first time demonstrates that a disturbed one carbon cycle from early pregnancy may be the primary mechanism underlying pregnancy outcome.
Therefore, this study suggests that a balanced dietary supplementation of folate, vitamin B<sub>12</sub> and DHA during pregnancy may be beneficial.

As discussed in Chapter 2, we found altered levels of LCPUFA in preeclampsia. The dietary intake of normotensive control women and women with preeclampsia were similar suggesting that these alterations could be due to disturbed fatty acid metabolism. Therefore, the next chapter examines fatty acid desaturases and fatty acid transport proteins which are known to influence fatty acid metabolism.
A prospective study of maternal fatty acids, micronutrients and homocysteine and their association with birth outcome

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ABSTRACT

Our earlier studies both in animals and in humans have indicated that micronutrients (folic acid, vitamin B12) and long-chain polyunsaturated fatty acids, especially docosahexaenoic acid (DHA), are interlinked in the one-carbon cycle, which plays an important role in fetal 'programming' of adult diseases. The present study examines the levels of maternal and cord plasma fatty acids, maternal folate, vitamin B12 and homocysteine in healthy mothers at various time points during pregnancy and also examine an association between them. A longitudinal study of 106 normal pregnant women was carried out, and maternal blood was collected at three time points, viz., T1 = 16-20th week, T2 = 26-30th week and T3 = at delivery. Cord blood was collected at delivery. Fatty acids were estimated using a gas chromatograph. Levels of folate, vitamin B12 and homocysteine were estimated by the chemiluminescent microparticle immunoassay (CMIA) technology. Maternal plasma folate (P < 0.05), vitamin B12 (P < 0.01) and DHA (P < 0.05) levels were lowest, while maternal homocysteine levels were highest (P < 0.01) at T3. There was a negative association between maternal DHA and homocysteine at T2 (P < 0.05) and T3 (P < 0.01). There was a positive association between plasma DHA in maternal blood at T3 and cord blood. Furthermore, there was a positive association between maternal folate and vitamin B12 at T3 and baby weight, whereas maternal homocysteine at T1 were inversely associated with baby weight at delivery. Our study provides evidence for the associations of folic acid, vitamin B12, homocysteine with DHA and baby weight, suggesting that a balanced dietary supplementation of folate-vitamin B12-DHA during pregnancy may be beneficial.