GENETIC AND METABOLIC ALTERATIONS IN MATERNAL AND PATERNAL ONE CARBON METABOLISM AND DEVELOPMENT OF PREGNANCY COMPLICATIONS OF PLACENTAL ORIGIN

Júlia Haro Barceló

UNIVERSITAT ROVIRA i VIRGILI

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Doctoral thesis

Thesis supervised by Dr. Michelle Murphy

Department of Basic Medical Sciences

UNIVERSITAT ROVIRA i VIRGILI

Reus

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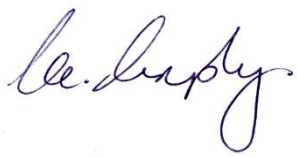
FAIG CONSTAR que aquest treball, titulat “Genetic and metabolic alterations in maternal and paternal one carbon metabolism and development of pregnancy complications of placental origin”, que presenta Júlia Haro Barceló per a l’obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament Ciències Mèdiques Bàsiques d’aquesta universitat.

HAGO CONSTAR que el presente trabajo, titulado Genetic and metabolic alterations in maternal and paternal one carbon metabolism and development of pregnancy complications of placental origin”, que presenta Júlia Haro Barceló para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento Ciencias Médicas Básicas de esta universidad.

I STATE that the present study, entitled “Genetic and metabolic alterations in maternal and paternal one carbon metabolism and development of pregnancy complications of placental origin”, presented by Júlia Haro Barceló for the award of the degree of Doctor, has been carried out under my supervision at the Department Ciències Mèdiques Bàsiques of this university.

Reus, 31 de juliol del 2020



Dra. Michelle Murphy

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There is no magic to achievement.

It's really about hard work, choices and persistence.

Michelle Obama

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Abstract

Pregnancy complications of placental origin such as gestational hypertension, intrauterine growth retardation (IUGR) and preeclampsia are major causes of perinatal morbidity and mortality affecting maternal and foetal health. Maternal characteristics including previous pregnancy complications, underlying illness, maternal age, genetics and lifestyle are the principal risk factors considered in prenatal care and research. It is well known that maternal status in folate and other one carbon (1C) metabolites can contribute to adverse pregnancy outcomes. The genetic polymorphisms methylene tetrahydrofolate reductase (*MTHFR*) 677 C>T and *SLC19A1* 80 G>A, (affecting the reduced folate carrier) affect folate metabolism and transport into the cell, respectively, and intervene in folate and 1C metabolic status. In many cases pregnancy complications cannot be explained by maternal factors. Those that stem from poor trophoblast invasion and impaired placentation, could also be caused by paternal factors. Nevertheless, paternal risk factors have been scarcely studied and in most cases (both in clinical practice and in research) are not considered.

In this thesis, we investigated whether maternal and paternal 1C metabolic status and the *MTHFR* 677 C>T and *SLC19A1* 80 G>A genotypes are associated with impaired placentation, intrauterine growth retardation and gestational hypertension.

Eight hundred and fifty six mothers receiving prenatal care in the University Hospitals Sant Joan, Reus and Joan XXIII, Tarragona were recruited before 12 GW. Their partners were also invited to participate and 66.1% agreed. A total of 748 mother-neonate dyads and 414 mother-father-neonate triads have been followed up to date from the Reus- Tarragona Birth Cohort study. Extensive lifestyle and obstetrical data was recorded from the first trimester throughout pregnancy and from separate interviews programmed for the

fathers. Fasting blood samples were collected during each trimester and from the father, and non-fasting samples at labour and from the cord. Plasma and red blood cell folate, plasma total homocysteine (tHcy) and plasma cobalamin were determined from maternal and paternal blood samples. *MTHFR* 677C>T and *SLC19A1* 80G>A genotypes were determined in the mother, the father and the cord. Associations between genetic and metabolic alterations in maternal and paternal 1C metabolism and development of impaired placentation, intrauterine growth retardation and gestational hypertension were investigated using multiple lineal and logistic regression analysis. Paternal *MTHFR* variant 677 C>T genotypes were associated with greater probability of impaired placentation compared to the CC genotype [OR (95% CI)], assessed by Doppler analysis of uterine artery resistance and wave forms: CT vs CC [4.0 (1.3, 12.6)] and TT vs CC [7.1 (1.6, 32.8)]. However, no association between maternal *MTHFR* 677 C>T genotypes and placentation was observed. Risk of impaired placentation [OR (95% CI)] was also increased when paternal tHcy was ≥P90 (14.1 μmol/L) compared to <P90, [2.7 (1.2, 6.0)]. Paternal smoking was significantly associated with uterine artery pulsatility index (β coefficient: 0.167, $p<0.05$). There was an interaction between mother's *MTHFR* genotype and maternal smoking in their relationship with birth weight ($P=0.024$). Therefore, the analysis was stratified by smoking habit during pregnancy. Maternal *MTHFR* 677 TT genotype was positively associated with birthweight only in smokers (β coefficient: 0.186, $p<0.05$), and late pregnancy tHcy was positively associated with birthweight in non smokers (β coefficient: 0.193, $p<0.001$). Neither paternal or maternal *MTHFR* 677 C>T genotypes were associated with IUGR risk. Paternal tHcy ≥P90 vs <P90 was associated with nearly five times greater probability of an IUGR-affected pregnancy [4.5 (1.7, 12.8)]. Mothers with the *MTHFR* 677 TT genotype were three times more likely to develop gestational hypertension compared with those with the CC genotype [2.9 (1.0, 8.4)]. Neither maternal or paternal tHcy concentrations, or

paternal *MTHFR* 677C>T genotype, were associated with risk of gestational hypertension. Neither maternal or paternal *SLC19A1* 80 G>A genotypes were associated with risk of any of the three outcomes studied.

In conclusion, the probability of impaired placentation was higher in pregnancies from fathers with the *MTHFR* 677 C>T variant (versus homozygote normal) genotypes and with elevated tHcy (versus <P90). This was also true for probability of IUGR, in the case of elevated paternal tHcy. Mothers with the TT genotype were at increased risk of developing gestational hypertension compared with the CC genotype. Paternal factors were not associated with gestational hypertension.

Keywords: homocysteine – MTHFR C677T polymorphism – father – pregnancy complications – intrauterine growth retardation – uterine artery doppler – uterine artery pulsatility index – gestational hypertension

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Abbreviations

Abbreviation	Definition
1C	One Carbon
BMI	Body mass index
CVD	Cardiovascular disease
DHF	Dihydrofolate
DNA	Deoxyribonucleic Acid
dTMP	Deoxythymidylate monophosphate
dUMP	Deoxyuridylate monophosphate
DVT	Deep vein thrombosis
FAD	Flavin adenine dinucleotide
GW	Gestational weeks
HLA	Human leukocyte antigen
HNF4A	Hepatocyte nuclear factor 4a
IGF	Insulin-like growth factor
IUGR	Intrauterine growth retardation
LBW	Low birth weight
MMA	Methyl malonic acid
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NHANES	National Health and Nutritional Examination Survey
NK	Natural Killer
NO	Nitric oxide
NTDs	Neural tube defects
PCFT	Proton coupled folate transporter
PTB	Preterm birth
RBC	Red blood cell

Abbreviation	Definition
RBCF	Red blood cell folate
RDA	Recommended Dietary Allowance
RFC	Reduced folate carrier
RNA	Ribonucleic acid
RTBC	Reus-Tarragona Birth Cohort
SAM	S-adenosylmethionine
SGA	Small for gestational age
tHcy	Plasma total homocysteine
THF	Tetrahydrofolate
WHO	World Health Organization

Enzyme and genetic polymorphism nomenclature

Abbreviation	International nomenclature	Definition
DHFR	EC 1.5.1.3	Dihydrofolate reductase
DNMT1	EC 2.1.1.37	DNA methyltransferase
MTHFD1	EC 1.5.1.5	Methylenetetrahydrofolate dehydrogenase
MTHFR	EC 1.5.1.20	Methylenetetrahydrofolate reductase
MTR	EC 2.1.1.13	Methionine synthase
SHMT	EC 2.1.2.1	Serine hydroxymethyltransferase
TET2	EC 1.14.11	Ten-eleven translocation
TYMS	EC 2.1.1.45	Thymidylate synthetase

Abbreviation	Reference SNP	Definition
MTHFR 677 C>T	rs1801133	Methylenetetrahydrofolate reductase 677C>T
SLC19A1 80 G>A	rs1051266	Reduced folate carrier 80G>A

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INTRODUCTION

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1. Introduction

Scientific evidence supports numerous roles for folate in maintaining health from early life to old age. Dietary folate is metabolised and participates in one-carbon (1C) metabolism. This metabolic network acts as a potential donor of methyl groups that are essential for Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA) biosynthesis and repair, basic in all stages of life, and involves amino acid metabolic pathways. Other vitamins, mainly B vitamins, also participate in this network and interact together. A complex regulation is required to prevent an imbalance in these vitamins, that can cause chronic disease in middle and old ages (1). Optimum maternal status in folate is especially required around the time of conception, to prevent neural tube defects (NTDs). Mandatory fortification of cereal grains with folic acid was first introduced in the USA and Canada in 1998, in order to decrease the recurrence of NTD-affected pregnancies. The measure was based on evidence from two clinical trials in which participants taking preconception folic acid supplementation had a reduced risk of NTDs up to 70% (2,3). Since the introduction of mandatory folic acid fortification, pregnancies affected by NTDs complications in the USA and Canada, have been reduced by approximately 50% (4,5). In Europe, many countries have not implemented this policy of mandatory fortification, but voluntary fortification provides folic acid from foods such as breakfast cereals and dairy foods, depending on cultural differences in dietary habits in each country. The recommendations for folic acid supplement use vary, but generally are aimed at the periconception period (6). Successful adherence to these recommendations requires that women are aware of these and planning a pregnancy. There are no recommendations for potential fathers. Our research group recently reported that 18.8% of adult, nonusers of B vitamin supplements,

representative of the population of our geographical region of Spain, had folate deficiency (≤ 7 nmol/L (7)) and this was also true in 24.2% of women of fertile age (8).

Folate participates in the remethylation of homocysteine to methionine (9). Homocysteine is a good indicator of 1C nutrient imbalance or deficiencies because it is sensitive to those changes and increases in these situations (10). One of the factors causing folate imbalance is the reduced activity of methylentetrahydrofolate reductase (MTHFR) observed in people with the *MTHFR* 677 C>T polymorphism (rs1801133). This enzyme catalyses the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the main form of circulatory folate and carbon donor for the remethylation of homocysteine into methionine (11). This polymorphism is associated with a thermolabile enzyme in the presence of the variant T allele, reducing enzyme activity up to 50-60% approximately in the homozygote (TT) genotype (12). The *MTHFR* 677 C>T polymorphism has been commonly studied in pregnancy complications and identified as a definite risk factor for the development of NTDs, as reported by van der Put N et al (1995) in the Netherlands in a case control study where participants with the homozygote variant have a threefold increased risk of spina bifida (13). Meta-analyses indicate that this genotype is associated with increased risk of other adverse health outcomes such as cardiovascular disease (CVD), elevated DNA damage and higher probability of a high resistance to flow in the uterine arteries, when folate status is low (1). This polymorphism may be a risk factor for developing pregnancy complications of placental origin. In a previous study, our research group observed that 18% of the adult population from our geographical region had the homozygote variant form of the polymorphism (8), similar to the general population in Spain and in other areas in Europe as Italy, France, and Hungary (14). However, the prevalence appeared to be lower (4%-6%) in the north of Europe, for example in a Helsinki study (14).

Previous studies have demonstrated that paternal factors may contribute to some pregnancy complications such as gestational hypertension, preeclampsia and intrauterine growth retardation (IUGR). Advanced paternal age (15), being born from a pregnancy complicated by preeclampsia (16), ethnicity (17) and genetic factors as a possible precipitating cause of an inadequate trophoblast invasion (18), are some of the paternal non-modifiable risk factors proposed so far. In addition, modifiable factors related with lifestyle and habits such as smoking (19), alcohol consumption (20) or poor nutritional status (21) have been related with impaired placentation leading to low birth weight or preterm babies. In a review, Dekker et al (2011) have described the "dangerous father" concept referring to fathers with some of the potential risk factors for preeclampsia including age over 45, unhealthy habits (tobacco, alcohol, drugs, etc.) and metabolic diseases (obesity, CVD, etc.) (22). However, the mechanism by which the father affects placentation is still not clear and there are discrepancies between authors in the results.

For all those reasons, this thesis sets out to study genetic and metabolic alterations in paternal one carbon metabolism affecting the development of pregnancy complications of placental origin. In order to develop primary prevention strategies, more information is needed regarding the effects of early life exposures on subsequent prenatal health and pregnancy outcome.

1.1. One Carbon metabolism: general insights

One-carbon metabolism encompasses a complex network of enzymatic reactions containing the folate and methionine cycles, among other metabolic cycles and pathways. Numerous amino acids and other essential nutrients, such as B-vitamins, are used as co-factors and co-substrates in the cycle, working in the connected metabolic pathways with both inter-dependent and disparate functions. For this reason, precise regulation of these is needed to maintain optimal functioning of the system to meet its roles as methyl group donor and in transmethylation and trans-sulphuration reactions among others necessary for purine synthesis, cell division, DNA repair, epigenetic processes, etc. Deficient status in the vitamins involved in the network can lead to impairment in its functioning and subsequently other complications in related biological processes (9).

The folate cycle, an integral part of the 1C metabolic network, is involved in the formation and transfer of carbon units to metabolic reactions and methyl groups to methylation reactions. Dietary folate is converted into intracellular metabolically active forms by physiological and metabolic processes including interconversion of polyglutamate and monoglutamate forms of folate, intestinal uptake, transport across cell membranes and metabolic reactions catalysed by specific enzymes (23). In parallel, 1C pathways exist in the cytosol and in the mitochondria and are connected by small metabolites that can readily cross the mitochondria. In the mitochondria, the catabolism of the amino acid serine, glycine, sarcosine, and dimethylglycine generate formate, the primary source of one-carbons for nuclear and cytoplasmic pathways (9). In the cytoplasm, 1C metabolism uses mitochondrial derived formate for the remethylation of homocysteine to methionine and also for the synthesis of purine nucleotides and thymidylate (dTMP) (9). Methionine is an essential

amino acid involved in the methionine cycle, an important methyl group donor in the body, involved in various biological processes such as DNA methylation, protein biosynthesis and is a precursor for the synthesis of S-adenosylmethionine (SAM) and homocysteine. SAM is an important cofactor and methyl group donor in numerous methylation reactions, including the methylation of DNA, RNA phospholipids, and proteins including histones (24). Homocysteine is a sulfur-containing amino acid, a demethylated derivative of methionine. Elevated fasting plasma homocysteine concentrations are influenced by genetic and environmental factors such as nutritional status, smoking habit, age, etc and are linked with some important health problems including vascular disease (25).

Dietary folate enters the cells via folate receptors, such as the reduced folate carrier (RFC), where is converted into dihydrofolate (DHF). DHF is subsequently converted to tetrahydrofolate (THF) the biologically active form of folate by dihydrofolate reductase (DHFR). Once in the cycle, THF is metabolized to produce methionine, purine and dTMP principally. THF is converted to 5,10-methyleneTHF in an NADPH-dependent reaction, and MTHFR catalyses the reaction where 5,10-methyleneTHF passes to 5-methylTHF (26). The one-carbon donor 5-methylTHF can be recycled by methionine synthase (MTR) to THF and methionine, in the remethylation of homocysteine to methionine in cobalamin dependent reaction (27). Alternatively, 5-methylTHF can be used to synthesize purine, which donates carbon units to the purine reactions (28).

Folate can also be metabolised to synthetize de *novo* dTMP by the participation of the methylation of deoxyuridylate (dUMP), DHFR, two serine hydoxymethyltransferase isozymes (SHMT1 and SHMT2α), and the trifunctional enzyme methyleneTHF dehydrogenase (MTHFD1). 5,10-MethyleneTHF is the required one-carbon donor for the TYMS-catalyzed

conversion of dUMP to dTMP and DHF; in this reaction 5,10-methyleneTHF serves as both a one-carbon donor and source of reducing equivalents (29). Moreover, as 1C metabolism is an important donor of methyl groups for post-translation modifications that can affect physiological functions and contributes to DNA stability and cellular biosynthesis, this metabolic network is crucial to other metabolic systems. Taking into account also that both the folate and methionine cycles are present in every human cell, derangements in 1C metabolism can have profound effects on cell function, metabolism, growth and proliferation (30).

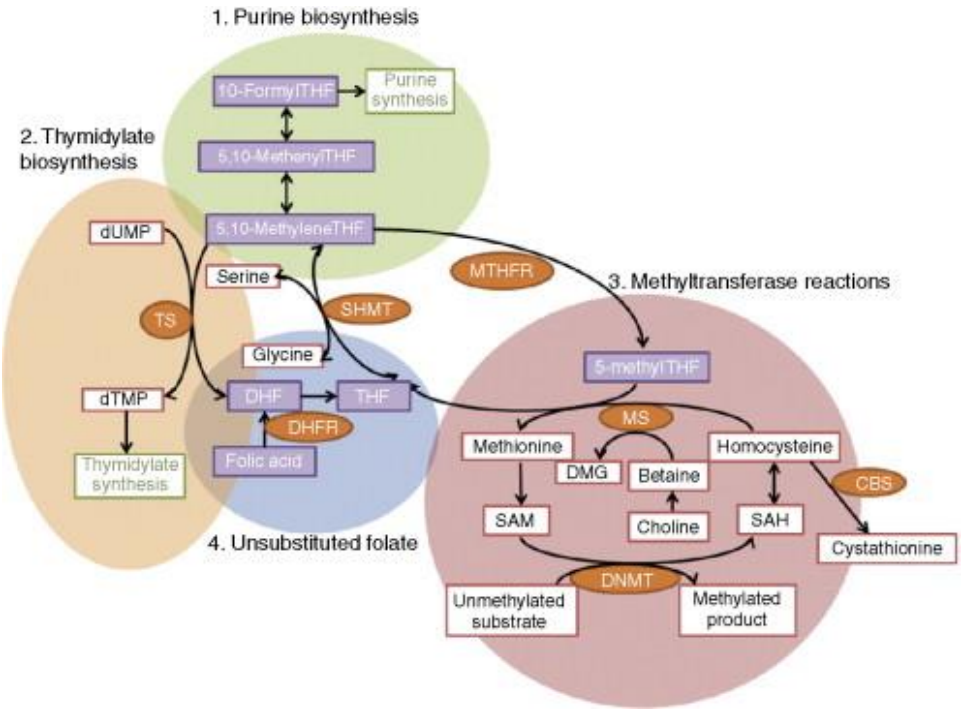

Figure 1. *Folate One-Carbon Metabolism scheme. From article "Folate and One-Carbon Metabolism and Its Impact on Aberrant DNA Methylation in Cancer"* Liu J et Ward RL (31).

In the literature it has been demonstrated that irregularities in one-carbon metabolism are associated with numerous diseases including anaemia (32), cancer (31), hypertension and cardiovascular disease (25), as well as pregnancy complications such as preeclampsia (33) or low birth weight (35, 36). Additionally, it is well established that folate deficiency during the periconception period is associated with increased risk of neural tube defects (36).

It has been proven that genetic polymorphisms, mainly affecting enzyme functions, in turn affect the 1C metabolic pathways affecting the complex regulation of the cycle. The genetic polymorphism with the most important effect on folate status (from the point of view of prevalence and effect) in the general population is the common 677 C>T variant in the gene encoding the methylenetetrahydrofolate reductase enzyme, MTHFR (1). This enzyme plays an important role in folate metabolism by catalyzing the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which acts as a methyl group donor for the remethylation of homocysteine to methionine (37). This enzyme is also important in the reactions maintaining the balance between thymidylate (for DNA synthesis) and methionine synthesis (methylation reactions) (38). Homozygous individuals (TT genotype) have reduced enzyme activity and a predisposition to mild hyperhomocysteinemia particularly when folate status is low (39). Several studies have reported that this genetic variant increases the risk for vascular disease and for neural tube defects (40).

Furthermore, the network is sensitive to status and availability of various amino acids (cysteine, serine and methionine) and B vitamins (folate, cobalamin, vitamin B_2, vitamin B_6) and lifestyle factors such as smoking or alcohol consumption. Arve Ulvik et al (2010) observed in a clinical trial

conducted in Norway that smoking significantly lowered circulating concentrations of folate and riboflavin (41). Modifiable factors such as nutrition, physical activity and toxic habits are the most important to take into account to prevent complications arising from impaired 1C metabolism.

1.2. Folates

Food folates and folic acid: sources, bioavailability and requirements

Folate is a water-soluble B vitamin, found naturally in foods. Different forms of folate occur due to the mixture of reduced forms of the vitamin (9). Folate cannot be stored in our body and we should eat folate-rich foods every day to maintain the required levels of this vitamin, if we are not supplementing our diet. The main food sources of folates are dark green vegetables including broccoli, spinach or lettuce, dried legumes such as chickpeas, beans or lentils, and some fruits like avocado or oranges. In the Spanish population, the most important sources of dietary folate are vegetables and cereals, legumes and fruits, contributing 20, 12.6 and 12% of total daily intake respectively (42). Folic acid refers to the synthetic form, a monoglutamate, found in the human diet only in fortified foods and supplements, but readily converted to the natural co-factor forms after ingestion (1). This folate form is commonly used in vitamin supplements due to its stability and actively reaching the intestine after its reduction. Folic acid is fully oxidized, but natural food folates are inherently less stable and not completely bioavailable (1). Low intake of folate-rich foods as well as low bioavailability of the naturally occurring folates in such foods (43), led some countries like the USA and Canada to implement the mandatory fortification of cereal grain products (bread, rice and pasta) with folic acid to improve intake of this vitamin in the population (45, 46). The proposal for mandatory fortification was made by the Food and Drug Administration and other public health services and was based on the established risk of NTDs associated with impaired folate status and NTDs (46). Multiple studies have assessed food folate bioavailability in humans, including studies measuring plasma, red blood cell (RBC) and urinary folate concentrations. The focus on folate bioavailability was needed to inform

dietary folate recommendations to provide optimal status as a priority for public health (47). The bioavailability of food folates is estimated to be ≤ 50% (48) and other evidence shows that folic acid ingested with food has about 85% of the bioavailability of free folic acid , 1.7 times more than natural folates (49). Many factors affect food folate bioavailability, for example, intestinal deconjugation of polyglutamyl folates, an obligatory step in folate absorption because only monoglutamyl forms can cross cell membranes. Small intestine absorption is also a limiting step because it is affected by optimal acidic pH in the jejunum, intestinal diseases, age, mutations in the transporter protein genes, etc. The food matrix can also influence folate bioavailability by trapping folate in the matrix, the instability of certain labile folates during digestion, and the presence of certain dietary constituents that may enhance folate stability during digestion (10, 51).

Estimates of folate requirements have been based on intake, associated with the maintenance of normal folate concentrations in plasma and erythrocytes (51). As said before, folic acid has different bioavailability to natural folate in food which is important to consider in dietary intake assessment. Dietary folate equivalents are used to express folate recommendations and are calculated as the µg natural food folate + 1.7 times the quantity of folic acid in the diet. This information is used by the National Academy of Sciences Institute of Medicine and other public health organizations for the Recommended Dietary Allowance (RDA) determination in some countries (52). In most European countries, however, this conversion factor is not applied and dietary folate intake is expressed simply as total folate in µg/d (1). The Spanish recommendations for daily folate intake are shown in *Table* (53). Pregnant women have different recommendations, with higher doses of folate intake, than other healthy adults in the population. Folate requirements are increased during pregnancy to ensure correct development of the foetus. As

it might be difficult to achieve the necessary increase in folate intake through food intake, they must resort to supplementation.

Some studies have demonstrated that women supplemented with folic acid in the preconception period reduce by 72% the risk of recurrent NTDs (2). Nevertheless, the recommendations can differ between countries. The majority recommend a healthy diet plus a folic acid supplement of 400 µg/d from preconception until the end of the first trimester of pregnancy, in line with the World Health Organization (WHO) recommendations (54).

***Table 1**. Spanish folate (µg/ day) recommendations in adult population.*

	Women	**Men**
10-13 years	250	250
14-69 years	300	300
>70 years	300	300
Preconception	400[1]	-
Pregnancy	500	-
Lactation	400	-

[1] From folic acid supplements.

Absorption, transport and metabolism

Folates are involved in a complex metabolic process that involves numerous reactions occurring in separate compartments. First, they must be transformed in order to be absorbed because food folates, mainly polyglutamates must be hydrolyzed to monoglutamate forms. Gamma-glutamylhydrolase located in the brush border membrane of the jejunal mucosa is responsible for this hydrolysis (55). When they have changed the form, folate monoglutamates can be absorbed across the intestinal mucosa by a saturable and energy dependent active transport mechanism. Several transmembrane carriers are involved but two of them, the reduced folate carrier and the proton coupled folate transporter (PCFT), are the major folate transporters into cells. Both transmembrane transporters are expressed on the apical membranes of the intestine, and their mRNAs are up-regulated in situations of folate depletion (56). The RFC is stimulated by high levels of intracellular organic anions and has a reduced affinity for folic acid. On the other hand, the PCFT functions at acidic pH and has similar affinity for reduced folates and folic acid (9). In situations of high folate concentrations, monoglutamates can also cross the membrane by passive diffusion (57).

Folate can also be taken up by the cell via three receptors in the apical membrane, α, β and ɣ. These folate receptors are expressed mostly in placenta and foetal tissues. They are found also in adult tissues and in some tumours (58). Transport via these receptors is a relatively slow process compared with the transmembrane carriers (59). Their well established role is in folate reabsorption by the kidney, and FR- α plays a role in embryo development and preventing neural tube defects (60).

The degree of metabolism in the intestinal mucosa depends on the dose of folate or folic acid consumed. If it is high, such as during supplementation, most of the transported vitamin appears unchanged in the portal circulation

(61). Folates are methylated in the mucous membrane cells before entering the portal circulation as 5-methyl-tetrahydrofolate. Under normal conditions, this metabolite is the primary circulating form of folate in plasma, binding to proteins, mainly albumin, which accounts for about 50% of bound folate. After folate absorption into the portal circulation, between 13-28 mg is retained in the liver and the rest is distributed to other tissues (9). Total body stores of folate in humans have been estimated to range from 10 to 100 mg (62). The half life of folates after entering into the enterohepatic circulation is approximately 100 days (47). This cycle of folate involves release of hepatic 5-methyl-thf into bile via methionine synthase reductase 2 (MTRR2) and then is reabsorbed in the small intestine for distribution to other tissues and liver, completing the enterohepatic cycle (63). Some studies clarify that the major role of this cycle is the maintenance of folate homeostasis (64). Little or no folate is lost in the urine at normal folate intakes.

When folate reaches the tissues, it is distributed among the different folate pools in the cells. Up to 50% enters the mitochondria, normally the glutamates with longer chain length. The 5-methyl-THF folate form is found in the cytosol pool (65). The nucleus also contains a folate pool that may be in equilibrium with this latter pool. In cells, folate is metabolized in the one carbon metabolic network. 5-methyl-THF, the predominant form, is used by MTR to remethylate homocysteine to methionine. This reaction also produces THF, which can be methylated by glycine and can also form 5,10-methyleneTHF or be catalyzed to generate formate and enter the purine synthesis pathway.

Concentrations of plasma folate are much lower than in red blood cells and almost all red blood cell folates (RBCF) are 5-methylTHF polyglutamates. The measurement of total folate provides information on the folate status of the individual. Folate plasma levels may be influenced by recent dietary intake (9).

For this reason, plasma folate has been used to predict short-term plasma status, while RBC folate is an indicator of folate long-term status or reservations. In addition, fasting plasma total homocysteine concentration is used as a non-specific functional biomarker for folate status (66).

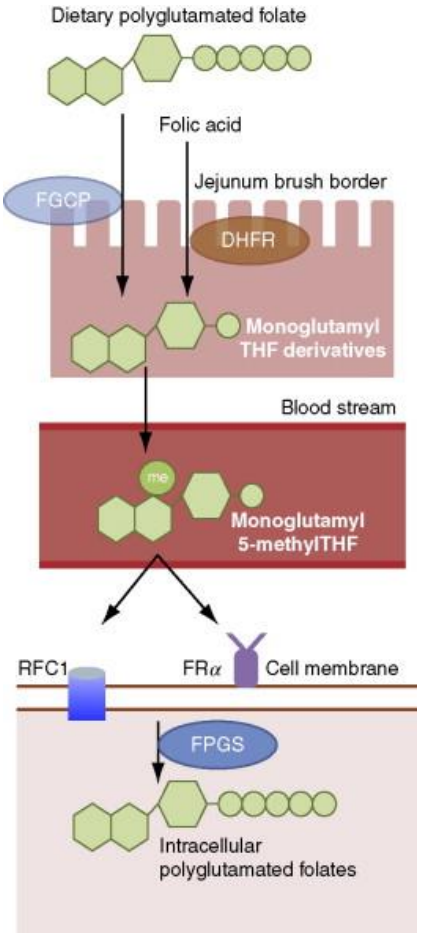

***Figure 2**. Folate absorption and distribution scheme. From article "Folate and One-Carbon Metabolism and Its Impact on Aberrant DNA Methylation in Cancer" Liu J et Ward RL* (31).

Maternal folate status and its determinants to preconception and pregnancy outcomes

As mentioned before, optimal folate status is required to prevent adverse health outcomes associated with folate deficiency, a potential public health problem that could affect many millions of people throughout the world. The prevalence of folate deficiency is not general around the world and is not associated with the level of development or the geographical location (67). However, in some countries like France (68) and Spain (8), where there is no mandatory fortification of food with folic acid, it is quite prevalent. People of any age can develop folate deficiency. Especially, in situations of increased requirements such as pregnancy, neoplastic diseases or in situations of malabsorption, the deficiency can appear (69). In addition, behavioural factors such as smoking, alcohol consumption, or oral contraceptive use are common causes of low folate status (70), because they are negatively associated with folate intake or absorption (69). Inadequate folate intake leads to decreased plasma and RBC folate concentrations and is associated with increased tHcy, a sensitive biomarker of impaired folate status. Cobalamin (B12) deficiency also leads to elevated tHcy so, elevated urinary or serum methylmalonic acid (MMA) concentration, affected by a pathway not shared by folate and cobalamin, is a specific indicator of impaired cobalamin status (69). Cobalamin is another vitamin involved in 1C metabolism, acting as a cofactor for key enzyme reactions in this cycle including the generation of methionine and tetrahydrofolate. Importantly, cobalamin and folate play inter-dependent roles in this specific pathway. The main and exclusive source of cobalamin is from foods of animal origin. Pregnant women are more likely to be B12 deficient than non-pregnant women (71).

The study of folates as a key nutrient for human health, and their importance before and during pregnancy (52), started in 1931 when Lucy Wills tested

marmite, which is a yeast extract rich in folate, as a cure for macrocytic anaemia in pregnant women (72). The increase in knowledge of the importance in avoiding folate deficiency over decades, motivated some countries to opt for folic acid fortification of cereal products (54). The impact of mandatory fortification is now reflecting a significant decline in the prevalence of NTDs in some countries. In the USA and Canada, the cases of some NTDs have decreased by 28% (73) and 46% (74), respectively. Nowadays, fortification with folic acid is mandatory in eighty one countries from global regions (75).

The National Health and Nutrition Examination Survey (US NHANES III) in line with the Institute of Medicine and in accordance with the WHO (76), has established the blood cut off concentrations for defining folate and B12 deficiencies. Values of serum folate <10 nmol/L and <340 nmol/L for RBC folate, as well as <150 pmol/L for plasma vitamin B12 are considered deficient (77). These data are based on the plasma vitamin concentrations below which plasma metabolites become elevated (total homocysteine concentration for folate and MMA for vitamin B12). However, until recently <7 nmol/L was widely used as the cut off for plasma folate deficiency (7). This cut off was based on the concentrations at which macrocytic anaemia was more likely to appear and was later revised due to its low precision.

As human's intake of folate and B12 depends on dietary sources, in some cases the ingestion of dietary supplements is needed to supply the required levels. Vegetarians, who don't eat animal products, are one of the examples of people who need to take supplementary vitamin B12. The periconceptional period is another of the recommended moments to supplement with folic acid and vitamin B12, to reduce associated pregnancy complications (71, 72). Nevertheless, according to NHANES I data, approximately 90% of the women of fertile age in the USA consume <400mg folate/day and only <10% meet the

RDA for pregnancy (78). Periconceptional maternal nutritional status is considered to be very important, due to its involvement in the development of the embryo and the fetus (79). Women with low dietary folate and cobalamin intake before and during pregnancy, resulting in vitamin deficiencies and an imbalance of the 1C metabolism, can develop pregnancy complications such as impaired placentation, intrauterine growth retardation and gestational hypertension. These are the main outcomes studied in this thesis and described in detail in subsequent sections of the introduction.

The focus of research into the relationship between 1C metabolism and pregnancy complications has been largely centred on the importance of folate in nucleic acid synthesis, required for the cell division stage of embryonic and fetal development. Marginal folate status during pregnancy can impair cellular growth and replication in the fetus or placenta (70). In 1975, Bryan Hibbard was one of the first authors to describe the importance of optimum folate status during pregnancy, especially in the early weeks when placentation and organogenesis occur. The author explained that fetal complications such as low birthweight, abortion and fetal malformation, occurred in women with disturbances in folate metabolism (80). Later, several authors continued researching the relationship between folate status and pregnancy complications. As is well established, Scott JM explained in a review that deficient folate status increased the risk of delivering a child with NTDs and argues that periconceptionally folic acid supplement use normalizes this risk (81). Poor folate status has also been associated with preterm delivery. In 1996, T O Scholl et al observed that women with a low mean daily folate intake (≤ 240 μ/d) had an approximately twofold greater risk of preterm delivery (82). Low folate status has also been associated with gestational hypertension and preeclampsia (83). De Ocampo M et al reported an attenuated risk with early and late folic acid supplement use and observed

also a decreased risk to develop those complications when the duration of this supplementation increased (83). In a review from 1987, Kramer MS described folic acid as one of the nutritional factors predicting indirectly intrauterine growth retardation, among other determinants such as genetic, demographic and obstetric factors (84).

Folate deficiency leads to elevated tHcy, which has also been reported to be a risk factor for pregnancy complications (70) in several studies. Cotter A et al (2003) observed in matched case-control studies, that elevated tHcy plasma levels (>10 µmol/L) in early pregnancy increase the risk of non-severe (85) and severe (86) preeclampsia compared with the control population (mean homocysteine of 7.07 ± 1.5 µmol/L). Rajkovic A et al found similar results in an observational study, showing significantly elevated homocysteine plasma concentrations in the preeclamptic group compared to control group (8.66 ± 3.05 versus 4.99 ± 1.11 µmol/L) (87). Increased maternal plasma homocysteine concentrations are also associated with increased risk of habitual spontaneous abortion. W L Nelen et al observed that women in the highest homocysteine concentration percentile (over the 95th, concentrations greater than 18.3µmol/L) tended toward 3.6 times increased risk (88). In a recent report from the Reus Tarragona Birth Cohort we also confirmed that elevated early pregnancy tHcy (≥ P90 (7.1 µmol/L)) is associated with increased risk of miscarriage (89). Increased plasma homocysteine levels are also related with low birth weight as well as intrauterine growth restriction. Murphy MM et al (2004) reported that mothers in the highest tHcy tertile at 8 weeks of pregnancy were three times, and at labour were four times, more likely to give birth to a neonate in the lowest birth weight tertile (34). In addition, Denise Furness et al in a prospective cohort study shown that the combination of both low folate concentrations and high homocysteine are associated with the subsequent development of IUGR (90). However, in a

multivariate regression analysis the authors revealed that RBC folate was a strong predictor of this pregnancy complication.

On the other hand, it has been shown that cobalamin status is closely related with folate levels (91). Even when folate levels are high, if the status of cobalamin is poor, metabolic signs of cobalamin deficiency can appear involving high MMA and tHcy plasma levels, combined with low holotranscobalamin (holoTC) levels, one of the plasma B12 bound proteins (92). This suggests that an imbalance in the folate-cobalamin pool adversely affects cobalamin metabolism, potentially leading to adverse perinatal health when cobalamin status is low in pregnancy (93). Low cobalamin status has been related with adverse pregnancy outcomes such as small for gestational age (SGA) (94), preterm birth and low birth weight (95). In a prospective observational cohort study in south Indian women, low vitamin B12 intake (<1.2 µg/day) was independently associated with a higher risk of SGA. However, in a subgroup of folic acid supplement users, those with the lowest B12: folate ratio (low intake of vitamin B12 in the presence of a high intake of folate) had a higher risk of SGA compared to those with the highest ratio. This suggests that in addition to vitamin B12 and folate deficiencies alone, there may be adverse birth outcomes associated with imbalanced vitamin B12 and folate intakes or status during pregnancy (94). On the other hand, Yurdanur G et al described no association between maternal B12 status and risk of preeclampsia. They only found an association with other B12 related biomarkers such as plasma homocysteine or folate (96).

Compromised cobalamin status during pregnancy may also affect the lactation period, leading to deficient status, and impaired development of the baby (97). Finally, the combination of both elevated folate and low cobalamin status have been linked with increased risk of gestational diabetes (98), increased insulin resistance (99), and small-for-gestational-age status (94) in the offspring.

All these evidences suggest a relationship between impaired 1C metabolism and adverse pregnancy outcomes. Even though the biological mechanisms are still unclear, a possible mechanism by which folate status and other B vitamins acting in the cycle can influence disease predisposition is by epigenetic processes, such as methylation, that can modify gene expression. These biological reactions will be discussed later. Another possible mechanism proposed is by DNA damage associated with elevated maternal homocysteine concentrations (90). Or endothelial dysfunction due to elevated homocysteine levels (negatively correlated with nitric oxide (NO)) that contributes to vasodilatation that occurs during normal pregnancy (100).

During pregnancy, as folate requirements are higher, vitamin supplement use is useful to achieve optimal folate status, as well as other minerals and vitamins. Our research group have recently shown that folic acid supplementation is associated positively with plasma and RBC folate concentrations and lower tHcy throughout pregnancy. In addition, when supplementation continues throughout pregnancy plasma folate status decrease between 15 and 24–27 GW and remain stable thereafter, whereas in the absence of continued supplementation plasma folate decreased throughout the remainder of pregnancy. Contrarily, when the supplementation stops plasma folate concentration decline throughout pregnancy (93). If women have poor folate status at the beginning of pregnancy, plasma and RBC folate levels will decrease further throughout.
In 2002 our research group, Murphy MM et al reported that tHcy levels decreased as a physiologic response to pregnancy, even further in mothers who use folic acid supplements (101). These results were complemented with further information in 2004, regarding the increase in tHcy during the third trimester, and further by labour. In women that were taking folic acid

supplements, tHcy concentrations by labour were similar to those at preconception point (34).

On the other hand, Murphy MM et al as well as other authors, have demonstrated that during pregnancy cobalamin blood levels drop (100, 101). In a longitudinal study, our research group also observed that the decrease in plasma cobalamin by 32GW is not merely due to a physiological effect of pregnancy but also due to possible mobilisation of maternal cobalamin stores. This is reflected by the rise in methylmalonic acid during the second half of pregnancy in the face of continuing haemodilution and increased glomerular filtration rate that occur during pregnancy. The levels of plasma Methylmalonic Acid (MMA) are higher by 32GW and labour than at preconception *(Figure 3).* This rise is higher in women with suboptimal early pregnancy cobalamin status than in those who are replete (102). Cobalamin is also negatively associated with tHcy, and as it happens with MMA the association became stronger as pregnancy progressed (93). This suggests that maternal cobalamin stores, even in non-vegetarian women, can be strained during pregnancy.

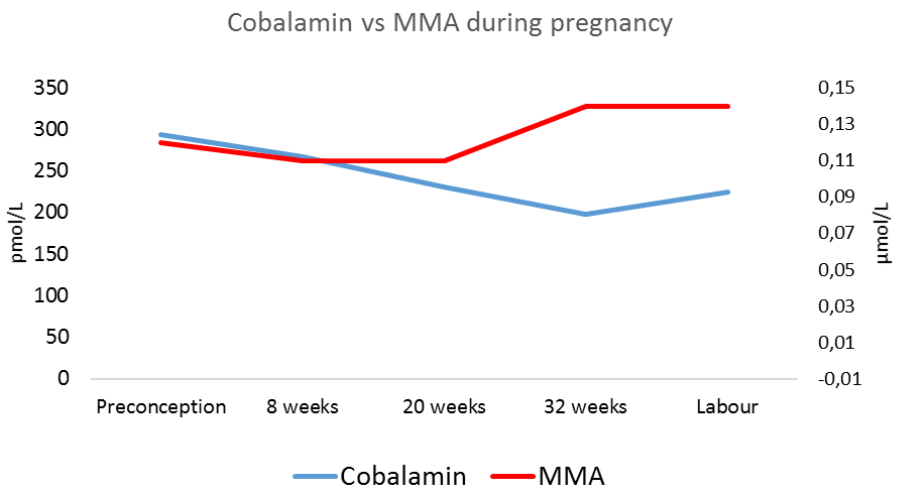

Figure 3. *Cobalamin (blue line) and MMA (red line) concentrations during preconception, three points of pregnancy (8, 20 and 32 GW) and at labour are shown*(102).

In a recent publication, our research group found that folate could modify the relation between cobalamin and MMA status during pregnancy. We observed that women with elevated plasma folate in the 1st trimester have higher MMA concentrations than the group of women with low folate status, if they have suboptimal B12 status. In women with normal cobalamin status, the plasma MMA concentrations are not affected by folate status *(Figure 4)* (93).

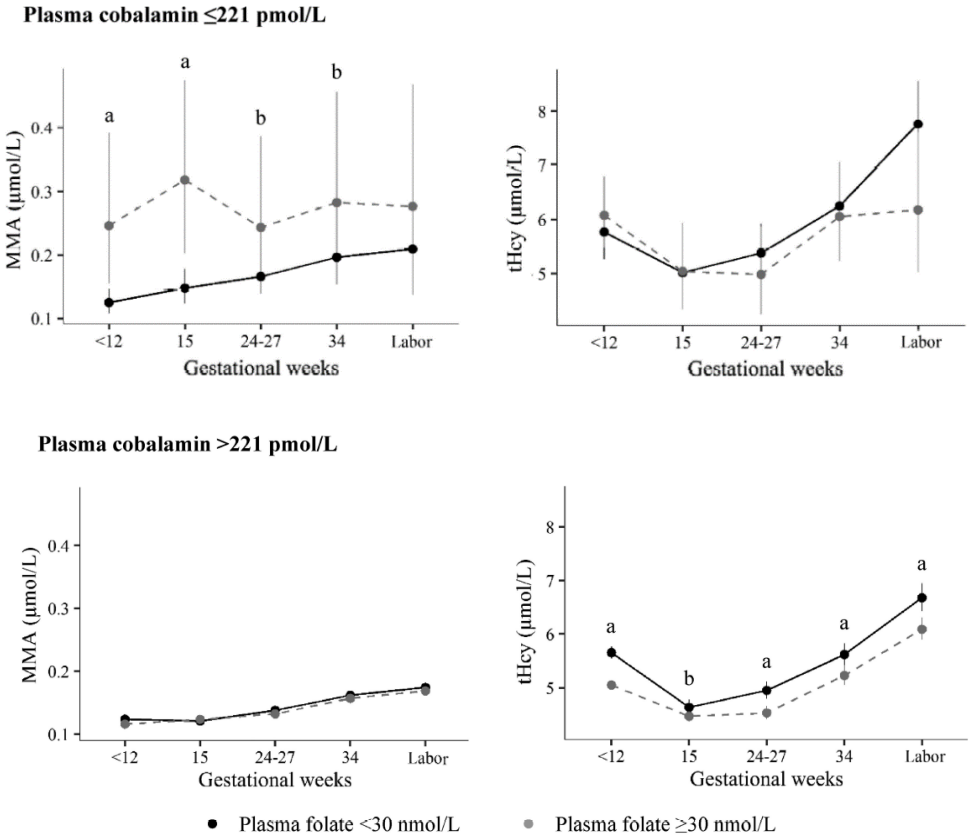

Figure 4. *Estimated marginal means (95% CIs) for plasma MMA and tHcy throughout pregnancy according to first-trimester plasma cobalamin and folate status. Linear mixed models were used. MMA, methylmalonic acid; MTHFR, methylenetetrahydrofolate reductase; tHcy, plasma total homocysteine. Figure from article "Early pregnancy folate-cobalamin interactions and their effects on cobalamin status and hematologic variables throughout" Pol Solé-Navais et al. 2018* (93).

There are other modifiable factors related with maternal lifestyle that can negatively affect pregnancy outcomes. Some of them are inadequate gestational gain, inadequate prenatal care, smoking, alcohol and drug use (104). However, other non-modifiable factors may also influence pregnancy complications. This is the case of history of spontaneous abortion, preterm delivery, low birth weight or intrauterine growth restriction in previous pregnancies that can affect the current pregnancy (105). Genetics is also a non-modifiable factor that may influence pregnancy complications and folate status. There is potential evidence that polymorphisms, affecting genes coding for proteins or enzymes involved in folate uptake or metabolism, can modify plasma or body folate levels leading to health and pregnancy problems commented before (69). *MTHFR* C677T is a common polymorphism that leads to instability of methylenetetrahydrofolate reductase (106). As explained before, this enzyme is known to catalyse the reactions involved in transferring the methyl groups to the principal methyl donor (methionine), that are necessary for DNA synthesis, methylation, cell division and tissue growth (107), and is essential for placenta and foetal development. The homozygote variant genotype (TT) is associated with elevated tHcy plasma levels, as well as decreased levels of DNA methylation compared with those with the *CC* genotype (homozygote normal) (66). The effect of the polymorphism on tHcy is intensified under low folate and cobalamin conditions, and it has been related with increased risk of NTDs and other adverse pregnancy outcomes (108). The effect of this polymorphism on pregnancy and other complications is discussed further below.

Apart from the effect of maternal factors on pregnancy outcome, some literature supports the implication of paternal factors as one of the causes of pregnancy complications of placental origin. Examples are impaired placentation, IUGR and gestational hypertension. Paternal lifestyle as well as

nutrition and poor folate status, are some of the modifiable factors that can lead to potential paternal precursors of pregnancy complications (109). Likewise, non-modifiable genetic factors affecting 1C metabolism and folate status in the father, may lead to pregnancy complications (110). The paternal involvement in the development of adverse pregnancy outcomes, as a main objective of this thesis, will be commented in the next chapters.

1.3. *MTHFR* C677T polymorphism

The *MTHFR* C677T polymorphism and pregnancy complications

Methylenetetrahydrofolate reductase (MTHFR) polymorphisms and adverse pregnancy outcomes such as IUGR, preterm delivery, preeclampsia, pregnancy loss and others has been the focus of interest by various authors for many years (111). The *MTHFR* gene catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the main form of circulatory folate, which is the co-enzyme of methionine synthase together with vitamin B12 and plays a key role in the remethylation of homocysteine into methionine (11).

A cytosine to thymine substitution at position 677 (*MTHFR* C677T) is a quite common polymorphism in this gene, leading to the substitution of valine for alanine in the corresponding protein resulting in a reduction of the enzyme activity (112). Consequently, the conversion of homocysteine to methionine is impaired, leading to elevated tHcy (113), which is a risk factor for cardiovascular disease (114), and reduced DNA methylation due to less available free methyl groups (66). The polymorphism is relatively common in the Spanish adult population (18.1 %) (8) and has been suggested to be involved in thrombotic events or impaired methylation during pregnancy (115).

Elevated maternal plasma tHcy levels, due to the reduced enzyme activity may contribute to placenta-mediated pregnancy complications (114). Increased tHcy may lead to endothelial dysfunction (116), inflammatory processes (117), oxidative stress (11), arteriolar constriction (11) and placental thrombosis (113). These conditions might be associated with impaired blood flow and prothrombotic changes in the uterine artery wall, inadequate trophoblast invasion into the uterine vasculature, and placental hypo perfusion that

subsequently leads to pregnancy complications (11). Furthermore, these complications may also result from the increased requirements for DNA methylation and protein synthesis involved in the growth of the baby and the placenta, taking into account that methyl groups are decreased in the TT genotype of the *MTHFR* polymorphism (118).

Chaudhry et al (2019) reported that a 5 µmol/L increase in tHcy (4 standard deviations of tHcy approximately) was associated with elevated probability of IUGR and preeclampsia but not with placental abruption or pregnancy loss, in a Canadian cohort after adjusting for gestational age at the time of blood draw and explanatory maternal characteristics (114). Mishra et al (2018) studied the *MTHFR* gene methylation patterns of placental tissue in preeclamptic Indian women, and observed lower *MTHFR* gene methylation on the foetal side of preeclamptic placenta compared to gestation-matched controls (115). Hypomethylation of genes involved in placentation is reported to be associated with impaired trophoblastic invasion (119), leading to unsuccessful placentation. In the same study, preeclampsia cases with the CT genotype had higher levels of global DNA methylation compared to controls with the CT genotype (115). This suggests that heterozygote women with hyper global DNA methylation are more prone to preeclampsia (115). In addition, a meta-analysis performed by Han Wu et al (2017) aimed at clarifying the association between maternal *MTHFR* C677T genotype and susceptibility to preterm birth and low birth weight. The maternal *MTHFR* C677T polymorphism increased the risk of preterm birth and low birthweight in homozygote TT versus CT or CC genotypes. Therefore, they concluded that identification of the maternal *MTHFR* C677T mutation might be useful in identifying women needing primary prevention of these pregnancy complications (11).

Other studies have investigated the relationship between the *MTHFR* C677T polymorphism and pregnancy loss. Mehandjiev et al (2019) studied the

association between the *MTHFR* C677T polymorphism and intervillous and decidua pathology in patients with pregnancy loss, and they reported that the T allele and *MTHFR* TT genotype are associated with severe intervillous and decidua pathologies such as fibrin deposits and thrombosis in those patients (118). Turgal et al (2018) also studied the effect of the *MTHFR* C677T polymorphism on pregnancy loss, and reported that the *MTHFR* C677T polymorphism increases early pregnancy loss, with the highest rates occurring in the *MTHFR* homozygote variant genotype (120). They proposed an impaired homocysteine/methionine metabolism disorder due to the polymorphism as a potential risk factor for this complication. Yajuan Xu et al (2018) observed in a case-control study significantly higher frequencies of the T allele of the *MTHFR* C677T polymorphism in the unexplained recurrent pregnancy loss group compared to the control group (121). On the other hand, the presence of CC and CT genotypes was significantly reduced in the unexplained recurrent pregnancy loss compared to the control group. Finally, our research group recently reported from the Reus-Tarragona Birth Cohort study that mothers with first trimester tHcy ≥ P90 (7.1 µmol/L) have increased risk of miscarriage (OR 95% CI, 2.5 [1.1, 5.7]). The prevalence of the *MTHFR* 677 TT genotype was higher in this group compared with mothers with tHcy < P90 (89).

The *MTHFR* C677T polymorphism and cardiovascular diseases

Apart from its reported involvement in some pregnancy complications, numerous studies have also reported an association between the *MTHFR* C677T polymorphism and cardiovascular diseases such as coronary artery disease, stroke or hypertension (122). The homozygous mutant genotype is associated with a 40% greater risk of cardiovascular disease compared to the homozygous normal genotype (123). Most of studies have focused on homocysteine metabolism disorders as the putative risk factor associated with this polymorphism (124). In homozygotes, with the *MTHFR* 677 TT genotype, impaired folate metabolism and higher homocysteine concentrations has been reported (125). Homocysteine produces reactive oxygen species during the thiol oxidation process. This species are toxic to the endothelium and may cause endothelial injury in various organs (126). Many studies have been performed in the general population showing that mildly elevated tHcy is associated with increased risks of atherosclerosis and venous thrombosis, such that hyperhomocysteinemia is considered an independent risk factor for cardiovascular diseases (127). Endothelial cell dysfunction has been identified as a key mechanism by which vascular risk factors may mediate their effect on vascular disease risk (128).

Shiran et al (2015) observed higher tHcy in the presence of endothelial dysfunction detected by flow-mediated dilatation, in individuals with B12 deficiency (B12 <150 pmol/L) with the *MTHFR* 677 TT compared to the CC genotype. They also confirmed the interaction between B12 deficiency and the *MTHFR* C677T mutation and observed that among individuals with B12 deficiency, TT homozygosity was associated with higher tHcy compared to the CC or CT genotypes. In addition, endothelial function was more affected in B12 deficiency patients with the TT genotype, compared to those with normal levels of B12 due to the role of hyperhomocysteinemia in oxidative stress and

reduced endothelial cell proliferation (129). Tang et al (2014) studied a cohort of coronary slow flow phenomenon patients and found higher frequencies of the *MTHFR* 677 TT and CT genotypes and *MTHFR* 677 T allele in the patients compared to controls (130). They also verified that tHcy plasma levels were higher in the patients with the homozygote genotype compared to CT and CC genotypes. Other studies have investigated the relationship between the *MTHFR* C677T polymorphism and deep vein thrombosis (DVT), caused by an imbalance between vascular relaxing factor and vascular contraction. It was shown in two populations, Iranian and Chinese, that the frequencies of the TT genotype and T alleles of the polymorphism were higher in the DVT group compared with those in the control group (130, 131). These results suggest that the *MTHFR* C677T polymorphism may be a risk factor for DVT. In another study with 160 ischemic stroke patients divided into two groups with or without hypertension, Ariana et al (2019) observed that the TT genotype was more prevalent in ischaemic stroke patients with hypertension (16 patients; 69.5%) compared with the group without hypertension. All patients with ischaemic stroke in the group with hypertension have the *MTHFR* 677 TT genotype (5 patients; 100%)(133). As this paper shows, many other studies have proposed this genetic variant as an independent risk factor for hypertension, with an increased risk of between 24 and 87% in homozygotes for the *MTHFR* C677T polymorphism compared to the *MTHFR* 677 CC and CT genotypes (123). Lynch et al (2019) reported in 75 cardiovascular disease patients that those with the TT genotype had higher systolic and diastolic blood pressure at baseline compared to those with the CC genotype (134). In a study conducted in Southwest Cameroon, Ghogomu et al (2016) also reported that the frequency of the T allele of the *MTHFR* C677T polymorphism was higher in the hypertensive group than in the normotensive group. On the other hand, the C allele was higher in the normotensive than in the

hypertensive subjects (135). These results are comparable to those obtained in populations from Spain (136), Australia (137) and Turkey (138).

Our research group also investigated the relationship between the *MTHFR* C677T polymorphism and moderately elevated tHcy and hypertension in an adult population study (139). There was no association between either of the variant *MTHFR* C677T genotypes and diagnosed hypertension in the overall representative sample (randomly selected from population records) of the adult population, that was unexposed to mandatory fortification with folic acid. However, the TT genotype was associated with greater probability of having hypertension than the CC genotype in participants ≤50 years. On the other hand, moderately elevated tHcy was associated with hypertension in the overall population and specifically in people over 50 years of age. Therefore, both moderately elevated tHcy and the *MTHFR* C677T polymorphism are associated with risk of hypertension, and these associations differ in subgroups of the population (139). However, our data do not directly support that the mechanism linking the *MTHFR* genotype to hypertension is via elevated tHcy as other studies do. Several studies show that homocysteine is a predictor of secondary, but not primary events (140), suggesting that is a product rather than a cause of vascular disease. The inconsistent results may be due to the methodology used for each study as the matching of correct controls with the cases, inadequate measurements or adjustments for the risk factors, sample storage and determinations at baseline, etc (128). Moreover, intervention studies performed to lower tHcy have shown little or no corresponding blood pressure response. This could be due to the fact that there is no mechanistic association between homocysteine and blood pressure (123), or could be because intervention is ineffective once the disease is established. Few or no studies have investigated the effects of lowering tHcy as a primary preventive strategy. Thus, *MTHFR* polymorphism

may be related to blood pressure via a mechanism independent of homocysteine.

The effect of nutritional status on the *MTHFR* C677T polymorphism

It is well established that TT homozygotes for the *MTHFR* C677T polymorphism have elevated homocysteine concentrations and lower folate status compared to the common genotype, indicating a gene-nutrient interaction (128). The prejudicial effect of the homozygous variant genotype is significant only when folate status is low, such as in countries with no mandatory food fortification, like in Spain. In regions with folic acid fortification policies the apparent effect of the *MTHFR* genotype has been proposed to be null (141). Therefore, dietary folate can influence the phenotype and thus disease risk (pregnancy complications, cardiovascular diseases, etc.) (123). It is proven that folic acid supplementation is an effective method to lower plasma homocysteine levels (123).

Recently, riboflavin (vitamin B2) is taking more attention due to its important role in one-carbon metabolism because it acts in the form of flavin adenine dinucleotide (FAD) as a cofactor for the MTHFR enzyme (123). Low riboflavin status, more frequent in individuals with the homozygous mutant TT genotype (125), is associated with elevated tHcy and low folate concentrations (123). Molecular studies have shown that MTHFR activity is reduced in the presence of the *MTHFR* 677 TT genotype due to its reduced affinity for the FAD cofactor (142). In addition, in human studies, riboflavin supplementation of individuals with the TT genotype can reduce homocysteine levels (143). This suggests that optimal riboflavin status can stabilize the variant enzyme and restore MTHFR activity (123).

Recent evidence suggests that riboflavin may be an important modulating factor via a novel effect on blood pressure (123). Lynch et al (2019) confirmed the involvement of riboflavin in ensuring optimum MTHFR activity, suggesting that this vitamin may interact with the *MTHFR* gene to influence blood pressure. They observed that riboflavin supplementation, at dietary level (1.6 mg/day), can decrease blood pressure in TT hypertensive adults compared with controls (134). They did not find a significant reduction in the CC genotype group. In addition, people with the TT genotype and poor riboflavin status, can reduce plasma tHcy levels with riboflavin supplementation (143). In a randomized trial, Wilson et al (2013) demonstrated in hypertensive individuals with the *MTHFR* 677 TT genotype treated with antihypertensive drugs, that those supplemented with riboflavin decrease blood pressure more effectively than those in the placebo group (treatment with current antihypertensive drugs alone)(144).

Increasing the activity of the MTHFR enzyme by riboflavin supplementation is only observed in those individuals with the homozygous *MTHFR* 677 TT genotype, with no response evident in CC or CT genotype groups (143). This effect might lead to improved endothelial function through increased bioavailability of nitric oxide via increases in 5-methyltetrahydrofolate (123).

This emerging evidence of riboflavin supplementation in lowering tHcy and blood pressure, suggests that nutritional and pharmacological interventions based on folic acid and other B vitamin supplementation can provide useful targeted therapy to reduce pregnancy complications and cardiovascular diseases in genetically at risk people. However, large-scale clinical intervention studies are needed to confirm the effects and correct doses to prevent negative effects.

1.4. Placentation and pregnancy complications of placental origin

Trophoblast invasion and placentation

The origin of a successful pregnancy and fetal development stems from fertilisation. The resulting zygote cell goes through several stages of division and proliferation resulting in different cellular stages. During the first steps after conception, cellular division leads to the 32 cells considered the morula (145) as it passes through the fallopian tube. Two days after fertilization, the cells reach the uterine cavity in the next cell stage, the blastocyst. The blastocyst is a vesicle composed of between 107-256 cells that make up the early embryo, composed by two types of cells; the inner layer cell mass called the embryoblast that will develop into fetal and embryonic tissues (146) and the outer layer of cells called the trophoblast that is the forerunner of the placenta and the cord (147). From the cluster of the embryonic disc (inner cell mass) grows another cell layer to line the cavity of the yolk sac, an extraembryonic membrane that plays an important role in the early nutrition of the embryo, and is the source of early hematopoiesis (148). On the other hand, the placenta is the principal organ required for embryonic development, because is an autonomous and transitory organ that allows nutritional and gas exchanges between the fetus and the mother, provides an immunological barrier between both, and produces and secretes a number of hormones, cytokines and sing molecules (149).

During the blastocyst stage, by day 6-7 after fertilization, this group of cells adheres to the maternal endometrium and the embryo develops a fluid-filled central cavity. On the 10th day, the single layer of the trophoblast proliferates and it differentiates into two types of cells; the inner layer called cytotrophoblast and the outer layer syncytiotrophoblast (145). The cytotrophoblast is composed of individual, well defined and rapidly

proliferating cells (150). Contrarily, the syncytiotrophoblast comprises multinucleated cells with indistinct cell borders (145). The cytotrophoblast cells proliferate and create cell columns with invasive properties into the maternal tissues. The blood vessels surrounding the blastocyst are broken, and trophoblast invasion occurs so that the vessels required to initiate circulation between the uterus and the placenta are formed (151). Trophoblast invasion is the main process of placentation, starting in early pregnancy and lasting until the 20th GW, so that contact with the maternal blood is established (152) and allowing the physiological transformation of the structure of the intrauterine arteries, required for a successful pregnancy (149). The trophoblast will also contribute to placental hormonal production (149). After the first phase of implantation into the maternal uterus, the trophoblast differentiates along two main pathways; the villous or the extra villous (153). The first one covers the chorionic villi, the site of maternal-fetal gas and nutrient exchange, and the extra villous trophoblasts invade the uterus wall to anchor the placenta in the uterus (145). To guarantee an adequate blood flow to the foetus, the blood vessels in the outer endometrium (the outer epithelial part of the uterus) have to change their form into large flaccid tubes (154), and transform the normal musculoelastic structure of the arterial wall to amorphous fibrinoid material in which trophoblast cells are embedded (155). This physiological transformation of the spiral arteries requires trophoblast cell invasion and the activation of macrophages and maternal Natural Killer (NK) cells that secrete growth factors and produce apoptosis in the endothelial cells. They strip off the muscular wall of the spiral arteries and substitute the endothelial cells that line these vessels (154). The arteries are converted into dilated conduits to maximize the delivery of oxygen and nutrients from maternal blood to the intervillous space, to ensure optimal foetal development (156). During this process, maternal–fetal interaction creates a state of mild systemic inflammation (157), in part

due to the HLA antigens such as HLA-G expressed by the trophoblast extravillous cells, that may influence maternal rejection of the fetus (158). Apart from the NK cells and the trophoblast cells, many molecules interact together to regulate trophoblast growth, migration and invasion to maintain a healthy utero-placenta equilibrium. These molecules include growth factors, inflammatory cytokines, growth factor binding proteins and leukocytes (159). For this reason, a complex network of soluble autocrine and paracrine factors, signalling pathways and regulatory transcription factors are needed to regulate it (160).

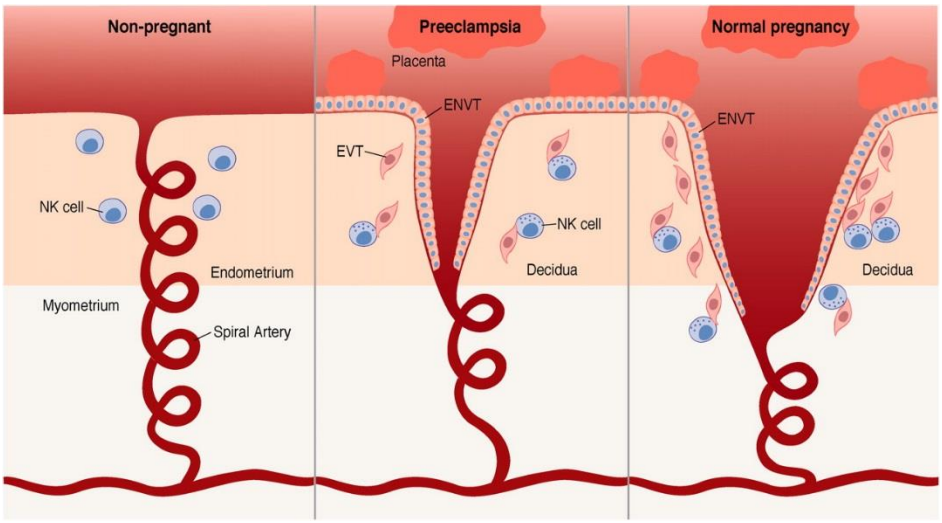

***Figure 5**. Uterine arteries in three different times are represented. On the left, uterine arteries with spiral form in non-pregnant women. In the middle, unsuccesful remodelation during pregnancy leading to preeclampsia. On the right, remodelled uterine arteries in a normal pregnancy. Figure from article "NK Cells and Trophoblasts" Parham P* (154).

Briefly, placentation is a complex process and one of the most important determinants of pregnancy outcome. Trophoblast invasion, as the main process of placentation, is the key to avoiding pregnancy complications related with inadequate transformation of the spiral arteries that are important to ensure adequate fetal oxygen and nutrient supply. Extended

literature shows that successful placentation will prevent adverse pregnancy outcomes such as severe forms of intrauterine growth restriction and gestational hypertension (156). Both complications are explained below, as they are the main outcomes studied in this thesis.

Pregnancy complications of placental origin: impaired placentation, intrauterine growth retardation and gestational hypertension

Impaired placentation is a common complication in severe pregnancy outcomes, and in part is caused by impaired physiological transformation of the spiral arteries in the placenta, leading to high resistance to blood flow in the uterine arteries (161). The Doppler ultrasound measurement of the intrauterine arteries is a physiological test to measure the blood flow in these arteries. This technique is used to assess the resistance of the blood flow between the mother and the fetus, determined by placental volume, the number of spiral arteries and their transformation (162). It was introduced in the 80's by Campbell et al (163) and still used due to its efficiency as a non-invasive measurement of fetal pathologies as well as feto-placental and utero-placental circulation (164). Currently, Doppler sonography of the uterine arteries is an established screening method during the second trimester and has been advanced to the first trimester, in many clinical settings, as an early detection strategy of impaired uterine artery flow.

In the first trimester of pregnancy, the waveforms of the uterine arteries present high resistance and a pronounced decrease of the blood flow velocity at the beginning of the diastole, known as a notch (165). In normal pregnancy, uterine artery pulsatility index decreases gradually as pregnancy progresses, the notch tends to disappear (166) and blood flow to the placenta increases. Pathological conditions are diagnosed as bilateral uterine artery notches

(confirmed by image) and/ or mean pulsatility index of the left and right uterine arteries above the 95th percentile (P95th >= 1.5250) (167).

These conditions are the result of high resistance to blood flowing into the placenta such as occurs in the non-pregnant uterus, because blood vessels in the placenta are not enlarging or dilating as they should. Patients with bilateral notches in the Doppler wave from of the uterine arteries and high resistance index at 12 and 20 GW, have higher risk of developing adverse pregnancy outcomes but the condition can still normalise and the risk can decrease (168). However, when the abnormal Doppler waveforms persists into the third trimester of pregnancy, it shows a high risk of high impedance to flow and the resulting placental complications (161). Preeclampsia and intrauterine growth retardation are two of the main adverse outcomes reported in the literature to be associated with failed trophoblast invasion and spiral artery transformation (155, 159).

The definitions of IUGR and small for gestational age (SGA) are similar in most of the literature, when the estimated fetal weight is <10th percentile of population curves for the given gestational week and sex (169). IUGR refers to a growth restriction *in utero* due to maternal, fetal or placental pathology and stems from placental insufficiency that causes failure to achieve growth in line with the genetically programmed trajectory (170). IUGR-affected pregnancies will result in a low birth weight baby with adverse perinatal outcomes (171). Using an ultrasound scan, biometric measurements can be calculated to estimate the fetal weight in the third trimester. Therefore, IUGR can be diagnosed during pregnancy, with antenatal measurements of head circumference, femur length, abdominal circumference, and bi-parietal diameter, taking into account the real gestational age (172). In case of low fetal weight (below the 10th percentile) the clinician will do the growth follow-

up (173). In Spain, it is estimated to affect nearly 10% of pregnancies, and it is associated with 30% of all perinatal mortality and severe morbidity (170).

IUGR occurs in up to 30% of pregnancies that are affected by preeclampsia, and being small at birth has also been associated with an increased risk for developing cardiovascular disease and hypertension in adulthood (174).

Babies with IUGR can be classified into two categories: asymmetric IUGR when the babies tend to have normal heads sizes and small bodies, or symmetric IUGR when the whole baby is small (175). The first one is caused by maternal or placental risk factors, whereas the second one is due to fetal factors. Maternal risk factors are those affecting the fetus development like maternal history of previous IUGR, maternal age (<16 or >35 years old), maternal insufficient weight gain, maternal hypoxia, malnutrition or toxic substance abuse (176). Some of these factors are modifiable; consequently, maternal habits during pregnancy have an important impact. In addition, placental risk factors affect the placental capacity to provide oxygen and nutrients to the fetus required for its development. Abnormal placental vasculature, placental infection, placental abruption and dysfunction or molar pregnancy are some of the known factors (18). Normally, these factors cannot be changed during pregnancy. Finally, there are risk factors of foetal origin leading to symmetric IUGR, for example, multiple gestation, chromosomal abnormalities, genetic syndromes, congenital abnormalities or congenital heart defects, infections and metabolic disorders (177).

Urgent induction of delivery may be the principal treatment for IUGR babies. Although the maintenance of the fetus as long as possible in the uterus is the current preference, due to the risk associated with premature births, in IUGR cases the risk posed for the fetus may be higher *in utero*. Due to their small size and potential prematurity, these babies have four times more risk of having severe fetal complications (178) including perinatal asphyxia during

delivery, hypothermia, metabolic and hematologic alterations, cerebral palsy and higher risk of cardio pathologies (179).

Gestational hypertension described as the first appearance of systolic blood pressure >= 140 mmHg and/or diastolic blood pressure >= 90 mmHg after 20 gestational weeks in women that had normal blood pressure up until 20 GW or a difference in first trimester blood pressure and that after 17GW of >=30mmHg for systolic and/or >= 15mmHg for diastolic (180), is the first clinical sign of preeclampsia. Severe hypertension is considered for systolic blood pressure >=160 mmHg and/or diastolic blood pressure >=110 mmHg (96). Preeclampsia is a serious maternal disorder causing new onset hypertension and proteinuria (>300mg/24 hours) only during pregnancy, after 20 GW, and in some cases lasts up to 6 weeks after delivery (182). This syndrome of placental origin is associated with significant maternal and neonatal morbidity (159). Risk factors that may increase the risk of developing preeclampsia include first pregnancy, more than one foetus, poor trophoblast invasion, unsuccessful placentation and family history of preeclampsia (183). Other factors such as elevated maternal age (>35 years old), history of previous hypertension, diabetes and obesity, genetics, environmental factors or nutrition, especially low folate status and high homocysteine concentrations, may also predispose to development of the disease (96). The prevalence of this disease is relatively low in Spain, between 1-2%, compared with other developed countries where it is double (182).

The exact causes are unclear, but the origin might come from abnormal placenta development. As explained before, in a normal pregnancy the spiral arteries dilate to 10 times their normal size. However, in preeclampsia the uterine arteries become fibrous and thus narrower, causing less blood flow to the placenta (184). In such conditions, pro-inflammatory proteins are released into the maternal circulation and predispose the endothelial cells to become

dysfunctional, causing vasoconstriction and more salt retention by the kidney (185), which can result in hypertension. Vasoconstriction may also affect other local areas leading to decreased blood flow in certain parts of the body. In the kidney, less blood flow causes oliguria and proteinuria. In the retina, it causes blurred vision, flashing lights and the development of scotoma. In the liver, poor blood flow may cause injury, swelling and elevation of liver enzymes (186). Endothelial injury increases vascular permeability, allowing the water to leak from the vessels between endothelial cells and to enter the tissues. Because there is also less protein in the blood, due to the proteinuria, even more fluid from the blood vessels enters the tissue and causes generalized edema in the legs, face and hands (186). If there is a pulmonary edema, other complications including cough and shortness of breath can appear. If the edema is in the brain, the symptoms may be headaches, confusion and seizures. We talk about eclampsia when the seizures appear (182). In addition, the poorly perfused placenta can also lead to intrauterine growth retardation and even placental abruption, haemorrhagic stroke and fetal death in severe cases (186). However, in some cases the symptomatology of preeclampsia can be mild. The delivery of the fetus and the placenta is the main treatment for preeclampsia. It will depend on gestational age of the fetus as well as the severity of disease (how it affects both, maternal and fetal health) (187). If the preeclampsia is mild, it is recommendable to finish the gestation on time and control regularly the mother to prevent severe complications. If it is severe, blood pressure should be controlled using medication to prevent a worst scenario like eclampsia and other serious signs, and control the fetal health to decide the optimal moment for delivery (182).

1.5. Paternal factors affecting placentation and pregnancy complications

Pregnancy complications described earlier, such as impaired placentation, intrauterine growth retardation and gestational hypertension may influence short and long term maternal and fetal health. A variety of genetic and environmental factors interact to produce adverse pregnancy outcomes. Established maternal risk factors associated with pregnancy complications are age (>35 years), multiparity, gestational diabetes, obesity and others. These factors are usually studied in the context of the materno-fetal-placenta unit, without taking into account paternal factors (18). However, maternal risk factors alone may not explain many preeclampsia cases and other pregnancy complications. For example, poor trophoblast invasion and deficient placentation are important risk factors for these complications, and the causes in many cases are unknown or may not be explained by maternal factors (188). If we consider that the placenta comes from the trophoblast cells, of embryonic origin, we can assume that paternal and maternal factors interact in the placentation process. In fact, paternal genetic factors predict good trophoblast development, and has a potential bearing on the process of placentation failure (189), a key process in fetal development. For this reason, the physiopathological process that leads to pregnancy complications may involve the paternal-maternal-fetal unit (190). In addition, paternal transmission of diseases may result in the exposure of male germ cells to environmental factors such as under and over nutrition. In particular, environmental paternal exposures can affect the epigenome, including DNA methylation, and lead to heritable alterations in fetal gene expression (191). The epigenetics process, as a possible mechanism linking 1C metabolism to pregnancy complications is explained in detail in the next section.

Regarding the risk of developing preeclampsia during pregnancy, recent studies have proposed some paternal factors that may be involved. These include the length of the relationship with the same father, the compatibility of the mother to paternal antigens, primipaternity, etc. There is also a paternal phenomenon related with the development of preeclampsia, described as "the dangerous father", commented before (22). In addition, paternal family history of preeclampsia may affect pregnancy complications, as shown in a study where fathers born from preeclampsia- affected pregnancies have an increased probability of fathering a pregnancy affected by this complication (16). It was also observed that the risk of developing preeclampsia in subsequent pregnancies was reduced when mothers with a previous history of preeclampsia, became pregnant by a different partner (192).

There is also evidence for paternal involvement in other pregnancy complications such as IUGR. Some articles have shown a relationship between advanced paternal age (>45 years) and IUGR (178), suggesting paternal genetics as a possible mechanism inducing the complications because of the increasing number of mutations with age (191). However, there are controversial results due to the poor association between age and IUGR in other studies (193)(191). In the case of Hurley et al (2017), they found no association probably because of the small sample size compared to other studies that found a relationship between paternal age and IUGR, and due to the inaccuracy of paternal age or missing data recorded from the assisted reproductive technology pregnancies (194). Furthermore, paternal low birth weight, IUGR or SGA, have been reported to be potential factors enhancing the probability of fathering an IUGR baby (20, 193). Some studies have also investigated the effect of mixed paternal and maternal ethnicity on the risk of IUGR and report increased likelihood in these circumstances. Possible mechanisms proposed include maternal chronic stress associated with

everyday interpersonal and institutional racism (17) and differential prenatal and maternity care (196).

There is a clear paternal implication in successful placentation that can prevent pregnancy complications and guarantee optimal health in both the mother and the baby. The main paternal factors studied, so far, can be classified as modifiable or non-modifiable. Modifiable paternal factors are related with lifestyle such as smoking, alcohol consumption, poor nutrition status, stress, socioeconomic status, and the non-modifiable ones are age, ethnicity and genetics, etc.

Modifiable paternal factors affecting placentation and pregnancy complications

Many modifiable factors, stemming from paternal habits and lifestyle, may influence the development of pregnancy complications. It's well known that paternal, as well as maternal toxic habits including smoking (19), alcohol consumption or drug use (20), are related with impaired placentation leading to low birth weight or preterm babies. Other paternal behaviour factors such as sedentary lifestyle, stress or depression, have been reported to increase the risk of developing preterm birth (197). Family socioeconomic status (198) or paternal educational level (199) have been also studied as possible risk factors of pregnancy complications, due to their close relationship with nutritional status. Nutritional deficiencies are an important detonator of pregnancy complications because of the increased nutrition requirements for fetal development. Evidence from animal studies suggests that paternal folate and 1C metabolism status, before conception, are possible modifiable factors involved in adverse pregnancy outcome and long term offspring health (191). Maloney et al (2011) in a study with rats fed with folic acid and methionine

deficient diet (methyl-deficient diet) during the periods of oocyte and embryo development, observed life-long changes in proteins involved in the processes of energy metabolism in the adult offspring (200). Cherif et al (2019) investigated also rats fed with a methyl donor deficiency diet during gestation and lactation and reported an association between this diet and the development and function of energy balance circuits, affecting the expression of neuropeptides and related receptors in the hypothalamus, resulting in growth and weight deficits (201).

In human studies, paternal deficiencies or excess of macronutrient during pre and post conception periods have also been proposed as potential factors affecting pregnancy related complications (202). In a Chinese population, Chen et al (2017) reported that a high quality protein paternal diet, with eggs and meat, was associated with lower incidence of preterm SGA (21). In a case control study with men from infertile couples, Eslamian G et al (2012) showed that those fed with a western-type diet with processed meat, sweets, refined grains and snacks were associated with reduced sperm motility (203).

The importance of the paternal 1C metabolic network and nutrition on successful placentation and optimum fetal development have recently come to light. As men contribute to half of the embryo's genetic material, paternal 1C metabolism is also important for pregnancy opportunities and outcomes (204). This network has an important role in biological processes such as cellular division, synthesis and DNA repair and is an important source of methyl groups for epigenetic programming of the paternal seminal fluid and subsequently the embryonic genome. Some nutrients such as folate, cobalamin and methionine are involved in this network acting as substrates or cofactors for the enzymes that participate in the cycle. The deficiency of one or more of these nutrients can alter the general network working, therefore, paternal folate status is crucial for optimal paternal 1C metabolism (109).

Some studies have investigated the relationship between impaired paternal 1C metabolism and complications related with pregnancy. Studies in humans have largely focused on the influence of paternal folate status on semen parameters and they have confirmed that plasma and seminal optimal folate concentrations positively affect semen quality (205). Boonyarangkul et al (2015) showed that after 3-months of treatment with 5 mg of folic acid per day the percentage of motile semen increased from 11.4% to 20.4% (206). Other studies focused on homocysteine semen concentrations have reported higher homocysteine concentrations in infertile men's sperm compared with fertile men. They also reported poor embryo quality in the group of high homocysteine sperm concentrations (207). In a study of 280 couples, Govindaiah et al reported 7 times more risk of spontaneous abortion in those couples with paternal hyperhomocysteinemia (values higher than P.95th, ≥19.6 µmol/L, measured after the pregnancy) compared to those with normal tHcy (110). In a study of mice, epigenetic alterations in DNA methylation in two types of folic acid diets were reported. One was a folic acid deficient diet and the other one highly supplemented, and both affected sperm DNA methylation, sperm counts and the offspring's abnormal development and death (191). Similarly, Lambrot et al (2013) reported that low paternal folate status may alter the sperm epigenome and result in adverse pregnancy outcomes in mice (208). In addition, in a human study, it has been published recently that both low and high long term paternal folate status, assessed by RBC folate levels from a blood sample taken between 7 and 9 GW, are associated with reduced embryonic growth (109).

Adverse pregnancy outcomes originating from nutritional deficiencies, such as folate deficiency, are interrelated with both, maternal and paternal deficiency. Ratan et al (2008) reported lower folate levels in fathers of children born with NTDs, compared to controls (209). One possible explanation is that folate

status during spermatogenesis influences the paternal epigenome of the embryo, with further consequences for the embryonic development (109). Another one may be that the final consequences originate from deficient intracellular folate concentration in germ cells (71). Current strategies for primary prevention proposed to prevent pregnancy complications and malformations such as NTDs are aimed at the mother, following the prenatal care protocol in Catalonia (180) and European countries. Maternal nutritional interventions with folic acid supplementation is the main strategy used. No strategies focused on the father are implemented yet, but there is more interest in paternal involvement in pregnancy complications. This problem is overcome in countries with mandatory folic acid food fortification, but in countries where the fortification is absent and only some food is supplemented, such as Spain, people have to be aware of the benefits of taking folic acid.

Moreover, 1C network regulation also depends on nutrient-gene interactions. *MTHFR* 677C>T and *SLC19A1* are two important polymorphism determinants for folate and homocysteine status, because of their roles in folate metabolism and entrance into the cell (8). Therefore, we should consider non-modifiable paternal factors such as genetics, due to their important role in guaranteeing an optimal nutritional status and in preventing pregnancy complications.

Non-modifiable paternal factors affecting placentation and pregnancy complications

As previously stated, paternal genetics, age and ethnicity are some of the non-modifiable factors that have been related with adverse pregnancy outcomes such as SGA, preterm birth, IUGR and preeclampsia. In the context of this thesis, we are very interested in the genetic factors that may provide links between paternal and pregnancy health. Since foetal genes are derived from each of a maternal and a paternal copy, the non-imprinted expression in the placenta and in the fetus can be affected by the paternal genotype (18). In the literature, some articles have proposed genetic factors as a possible precipitating cause of inadequate trophoblast invasion, which in turn affects pregnancy health (160). Imprinted genes, for example, are important for embryo growth and development, with evidence generally showing that paternally expressed genes promote fetal growth, while maternally expressed genes suppress it (210).

Genetic factors are closely related with nutritional status due to gene-nutrient interactions. Some polymorphisms are affected by nutrients and may modify the activity of the enzyme, protein or biological molecule they are affecting. In our group, we are interested in studying 1C metabolism, the vitamins involved in the cycle and the genes involved in the regulation and optimum functioning of the pathway. We focus our investigation on two polymorphisms affecting folate metabolism (*MTHFR* 677C>T) and transport (*SLC19A1* 80G>A). Data from a case-control observational study, show that the homozygote mutant genotype (TT) of the paternal *MTHFR* 677C>T polymorphism was linked with 2.4 times increased risk of miscarriage, compared with the control group (110). However, another study with 151 cases and 157 controls studied two months after the last pregnancy did not obtain comparable data (211).

On the other hand, the reduced folate carrierencoded by the *SLC19A1* gene, is the main entrance of folate to the cell and it is strongly associated with the final folate status. Fetal folate availability is dependent on maternal folate levels and placental folate transport capacity. To date, several studies demonstrated that maternal *SLC19A1* 80AA polymorphism, affecting the RFC transport activity, was associated with pregnancy complications such as NTDs and fetal malformations, due to elevated folate requirements during pregnancy (211, 212). In addition, this risk was influenced by a gene–environment interaction between the *SLC19A1* 80AA genotype and maternal periconceptional use of folic acid reducing the occurrence of NTDs (214). In 2015, Pei L et al observed a significant interaction between maternal GG/GA genotype and the risk of developing an NTDs in the offspring (215). The *SLC19A1* 80AA polymorphism has also been related with an elevated risk of other adverse pregnancy outcomes including recurrent pregnancy loss (216), IUGR (217) and preeclampsia (218). Protein expression of the RFC was decreased in placentas from pregnancies with IUGR and preeclampsia compared with placentas of uncomplicated pregnancies (216, 217). However, we did not find any evidence in the literature focusing on paternal RFC expression or activity and pregnancy complications.

With the current evidences, the hypothesis that paternal factors may contribute to pregnancy complications of placental origin seems to be clear. However, there is limited and sometimes conflicting evidence in the literature. We still have a limited understanding of the influence of paternal factors on human offspring. The most frequently proposed biological mechanisms in the literature, to explain the role of the father are based on the immunological tolerance of the mother and the paternal genes. The invasion of trophoblasts into the decidua and myometrium appears to be primarily controlled by immune mechanisms, for this reason the mother has to tolerate the paternal

antigens to avoid unsuccessful placenta development due to poor vascular adaptation. Paternal genes inducing angiogenesis of the placenta, trophoblast apoptosis, oxidative stress, etc may increase the predisposition of the mother to suffer pregnancy complications. In addition, paternal epigenetic changes may also affect genetic expression involved in vascular dysfunction, inflammation and other causes of pregnancy diseases (22). Further research is needed to validate this hypothesis and establish the paternal biological mechanisms involved in the development of pregnancy complications. This way, it will be possible, if necessary, to establish primary prevention strategies against pregnancy complications focusing on the father. In the present thesis project, we want to study this topic focusing our hypothesis and investigation on the possible role of paternal one carbon metabolism, considering paternal genetics and folate status, in the development of some adverse pregnancy outcomes described before.

1.6. Epigenetics, a potential link between 1CM and outcomes

A potential mechanism proposed to explain the association between one carbon metabolism and the outcomes studied are epigenetic changes, which can regulate gene expression. Some nutrients can modulate gene expression induced by epigenetic modifications. Epigenetic factors are DNA modifications that can affect gene expression without altering the sequence (219). Some of them, including DNA methylation, histone acetylation, as well as micro RNA modifications can modify chromatin structure making it accessible or not for transcription mechanisms. Chromatin is the genetic material packed into eukaryotic cells, composed by the association of DNA with histones and other proteins. Depending on the chromatin structure, euchromatin as the active form or heterochromatin as inactive, the gene will be expressed or not (220). DNA can be only modified by methylation, but histones can suffer changes by acetylation, methylation, phosphorylation and others.

The 1C network is an important methyl group donor system that can contribute to epigenetic modifications of DNA and histones, especially by methylation (220). Methylation is the covalent addition of a methyl group at a nitrogenous base, mediated by the enzyme methyl transferase. In the CpG islands, in the zone of the eukaryotic genome with regulatory and promoter parts of the genes, the cytosine of the dinucleotide CpG is methylated. S-Adenosyl methionine, synthetized in our body by methionine coming from diet, is the methyl group donor. Methylation of DNA sequence at the CpG islands is linked to downstream gene silencing by recruiting histone deacetylases that contribute to chromatin condensation restricting the access of regulatory transcription factors (219). It is a heritable mechanism to inactivate genes in a stable way, therefore, an important process to control gene expression. On the other hand, histone modifications are post-translational affecting the N-terminal part of some histones in the

nucleosome. These modifications affect the condensed status of chromatin (221). Apart from methylation, histones can be acetylated by the acetyl transferase in lysine residues. When histones are acetylated, chromatin has a non-condensed status and transcription can be activated. They can also be deacetylated, catalyzed by histone deacetylase. The elimination of acetyl groups blocks the union between transcriptional factors and DNA (219). Both, histone deacetylation and DNA methylation decrease gene expression by increasing chromatin condensation and lowering the accessibility of transcriptional factors to DNA.

Vitamins and amino acids acting in one carbon metabolism can be responsible for the epigenetic changes explained. As commented before, methionine is the precursor of the methyl group donor, and is an essential amino acid obtained exclusively from the diet because it cannot be synthetized in our body (222). Meat, fish, eggs, dairy products and other foods rich in proteins are good sources of methionine (223). In addition, the transfer of the methyl group from the donor to cytosine also requires the participation of folates and cobalamin (222). Niacin (vitamin B3) is also needed for DNA methylation. This vitamin is involved in the synthesis of some factors that participate in the inhibition of enzymes that regulates DNA methylation. Niacin is also needed for NAD+ synthesis, required for DNA reparation (224). Vitamin B3 is found in eggs, fish, legumes, lactic products, enriched bread, and cereals (225). We can suppose that diets poor in this nutrient, or deficiencies, causing an imbalance in one carbon metabolism can reduce DNA methylation and modify gene expression. Such alterations can induce health problems, and if they occur during prenatal or pregnancy periods they can lead to pregnancy complications such as impaired implantation, intrauterine growth retardation or gestational hypertension (221).

There is evidence in the literature linking epigenetic changes with adverse pregnancy outcomes. Related with preeclampsia, epigenetic changes play a crucial role in the development and the progression of the disease, particularly by methylation process. In a review, Kamrani et al (2019) reported evidence of the methylation pattern changes in pregnancies affected by preeclampsia compared to healthy pregnancies. In preeclamptic placentas, changed global DNA methylation models are related to maternal blood pressure. They review DNA methylation of some genes in relation to preeclampsia. For example, the TET2 enzyme involved in the regulation of trophoblast cell invasion through regulating MMP-9 promoter demethylation is hypermethylated in preeclamptic patients. Furthermore, the hypermethylation of the IGF-1 promoter by DNMT-1 reduces IGF-1 in the hypoxic trophoblast and causes preeclampsia (221). Herzog et al (2017) performed a case-control study to compare the epigenetic patterns of placental and fetal tissues between preeclamptic pregnancies and three control groups (uncomplicated pregnancies, normotensive fetal growth restricted and normotensive preterm birth pregnancies). They have demonstrated significant differences in genome-wide white blood cells and placental DNA methylation between the group with preeclampsia and the control preterm birth group, but no differences were found with the other control groups. The differences in the hypermethylated genes between groups were those closely associated with cardiovascular and metabolic developmental pathways (226).

Regarding IUGR, placental epigenetic dysregulation has been proposed as a cause. In a study with monozygotic twins, with the same genotype at conception and a shared maternal environment, Roifman et al (2016) studied the differences in genome-wide placental DNA methylation patterns between severely growth-discordant twins and normal twins to identify novel candidate genes. They observed differentially methylated regions in the promoters of eight genes related with fatty acid beta-oxidation and

transcriptional regulation in the IUGR compared to the control group, suggesting that IUGR in monozygotic twins is associated with impaired lipid metabolism and transcriptional regulation (227). Other studies also indicate epigenetic dysregulation associated with IUGR. Einstein et al (2010) collected samples from women who delivered IUGR or infants with appropriate growth to detect global patterns of epigenetic changes. They found that changes in cytosine methylation occur in response to IUGR of moderate degree and involving a restricted number of loci. They also identify specific loci that are targeted for dysregulation of DNA methylation, in particular the hepatocyte nuclear factor 4a (HNF4A) gene, a well-known diabetes candidate gene and other loci encoding HNF4A-interacting proteins (228).

In addition, paternal nutritional imbalance in 1C metabolism can also alter genotype expression and induce abnormal phenotypes, following the fundamental epigenetic gene-nutrients link commented before (71). The father can transmit epigenetic modifications to the oocyte, modulating gene expression during fetal development and consequently, affecting postnatal and adult health. The epigenetic process can be considered as a reprogramming of the DNA methylation patterns in the zygote, and signifies an environmental regulation of the expression pattern of some genes that persist throughout life (229). Paternal folate status is also involved in the epigenetic programming of the seminal and subsequent embryonic genome. Steegers-Theunissen et al (2013) reviewed evidence indicating that paternal nutrition and other lifestyle factors during the periconception period can affect reproductive performance via 1C metabolism pathways (204). Restricted dietary provisions of 1C substrates and cofactors around the time of conception could lead to genome-wide epigenetic modifications to DNA methylation in offspring (204). In a human study with idiopathic infertility in men, Arabi et al (2015) revealed that 6 months treatment with high-dose

(5mg) folic acid supplements significantly changed the overall seminal DNA methylation pattern (230). Interestingly, the most marked loss of DNA methylation was found in sperm form patients with the homozygous variant genotype of the *MTHFR* C677T polymorphism. A study in mice also showed changes in the seminal epigenome when paternal dietary folate intake was low. Lambrot et al (2013) fed male mice either a folate deficient or folate sufficient diet throughout life. They found differential methylation in sperm of genes implicated in the development of chronic diseases such as cancer, diabetes, autism and schizophrenia (208). This study indicates that paternal dietary folate is essential for offspring health, because there are environment sensitive regions in the sperm epigenome that respond to diet and transfer an epigenomic map that influences development. Similarly, Lundi Ly et al (2017) designed a mouse study to expose male germ cells to low, high and very high folate levels throughout male germ cell development. Female mice were fed, before and throughout pregnancy, diets with four different folic acid doses (0.3 mg/kg, 2 mg/kg, 20 mg/kg and 40 mg/kg) and F1 males were weaned to their respective prenatal diets to allow for diet exposure during all windows of germline epigenetic reprogramming. DNA methylation at a global level and at the differentially methylated regions of imprinted genes in F1 sperm and placenta was assessed. The results indicate that both folic acid deficiency and high dose supplementation can be detrimental to germ cell development and reproductive fitness, in part by altering DNA methylation in sperm (191).

All this data provides further support for paternally transmitted environmental effects, by epigenetic changes and reprogramming in germ cells, associated with pregnancy complications and offspring abnormalities.

Emerging evidence indicates that derangements in 1C metabolism can lead to epigenetic modifications to DNA methylation implicated in long-term programming of offspring health. This provides a mechanistic explanation for at least some of the associations between reproductive failure, age-related

diseases and modifications induced by nutritional factors and lifestyle (204). This information opens new avenues of understanding the potential mechanisms underlying inter-generational disease transmission and preventing developmental defects via paternal routes.

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Júlia Haro Barceló

HYPOTHESIS AND AIMS

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2. Hypothesis and Aims

2.1 Hypothesis

Alterations in maternal or paternal 1C metabolism, caused by *MTHFR* 677C>T and *SLC19A1* 80G>A polymorphisms and/ or deficiencies or imbalances in folate or cobalamin status, may be associated with the development of pregnancy complications stemming from inadequate placental perfusion such as IUGR and gestational hypertension.

2.2 Aims

Main aim

To investigate the association between genetic, nutritional and metabolic components of the maternal and paternal 1C metabolic network and the probability of impaired placentation, intrauterine growth retardation and gestational hypertension.

Specific aims

- To compare the likelihood of impaired placentation, intrauterine growth retardation or gestational hypertension between the maternal or paternal *MTHFR* 677C>T *(*TT *versus* CC*,* CT *versus* CC*)* and *SLC19A1* 80 G>A *(*AA *versus* GG*,* AG *versus* GG*)* genotypes*.*

- To investigate the association between elevated early pregnancy maternal tHcy, elevated paternal tHcy and the probability of impaired

placentation diagnosed by pathological Doppler of uterine arteries at 20 gestational weeks, intrauterine growth retardation or gestational hypertension.

- To explore whether the associations between the genotypes and the three pathologies is modulated by the status in the 1C metabolism nutrients.

MATERIAL AND METHODS

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3. Material and methods

3.1. Design and study population

This thesis is based on The Reus-Tarragona Birth Cohort (RTBC) study. The RTBC is an ongoing population based longitudinal cohort study from early pregnancy until mid-childhood carried out by the Area of Preventive Medicine and Public Health from the "Universitat Rovira i Virgili" in collaboration with the Obstetrics and Gynaecology Units of the University Hospitals Sant Joan Reus and Joan XXIII Tarragona. The fathers of these pregnancies are also invited to participate in the study and investigating their contribution to pregnancy outcome is the main objective of this thesis. The study was designed to identify early pregnancy nutrition and parental genetic factors influencing fetal growth and pregnancy outcomes of placental origin, as well as growth, development and mental performance in their offspring at 7.5 years. The project for this thesis is based on the pregnancy phase and the influence of both maternal and paternal 1C metabolism in the establishment and functioning of the materno-feto-placental unit.

The study was carried out according to the Declaration of Helsinki and was approved by the Ethical Committees from each of the two participating University Hospitals (Sant Joan, Reus and Joan XXIII, Tarragona). Signed informed consent was obtained from all study participants.

3.2. Mother and baby

Pregnant women attending their first check-up, before 12 GW, with a confirmed viable fetus and singleton pregnancy were eligible to participate and were recruited by the Areas of Obstetrics and Gynecology from the two University Hospitals. Exclusion criteria were multiple pregnancy, chronic illness or surgical interventions affecting nutritional status or use of any medication that affects folate or vitamin B12 metabolism.

Following the Spanish Obstetrics and Gynaecology Society guidelines (181) pregnant women with low obstetrical risk were recommended to take daily supplements containing 400µg of folic acid until the end of the first trimester. The recommended prenatal supplement also contained 2µg of cyanocobalamin. Women with a history of a previous pregnancy affected by NTDs, other congenital defects or complications or with a first-degree relative with NTDs were recommended to take 4-5 mg of folic acid per day until the end of the first trimester. As a preventive measure against anaemia, low dose iron supplements (40 mg/d) were recommended from 12 GW throughout the second and third trimesters of pregnancy to all participants. Women that were already anaemic were treated with iron supplements containing 80 or 120 mg/d according to the treatment regime determined by the obstetricians.

Participant recruitment started in January 2005 and 2006 in the Sant Joan, Reus and Joan XXIII, Tarragona University Hospitals respectively. All pregnancies completed before March 2020 were included in the thesis. This represented 856 pregnancies. Of these, 672 fathers were eligible to invite.

Fasting blood samples were taken from mothers at <12, 24-27, and 34 GW, and non-fasting blood samples on admission to hospital with confirmed labour and from the umbilical cord vein. Blood samples were programmed to be

collected at the same time of those routinely collected during prenatal monitoring of pregnancy according to the regional health authorities protocol (181).

Medical and obstetrical history and lifestyle data collection

Maternal age and BMI were recorded at the first antenatal check-up. Detailed information regarding supplement use and lifestyle data before and during pregnancy were collected by questionnaire at 20 and 32 GW (coinciding with programmed ultrasound scans) and covers the six months preconception and each trimester of pregnancy.

The questionnaires specifically targeted folic acid, cobalamin and iron use and were designed to obtain detailed information regarding supplement brand, composition, timing of initiation, frequency and duration of use. Other multivitamin/ mineral supplement use was also recorded with the same detail. We used this information to estimate the daily intake of folic acid, cobalamin and iron from supplements during the first trimester and between the 4^{th} and 7^{th} months of pregnancy.

Smoking habit during the 5 years prior to pregnancy and throughout the pregnancy itself was assessed by interviews at 20 and 32 GW (number of cigarettes/d), medical history and plasma cotinine concentrations (>10 ng/mL confirmed active smokers) from blood samples at <12, 24-27 GW and from cord blood. Similarly, alcohol consumption (duration, drinks/w, timing of cessation before or during pregnancy) and illegal drug use in the previous 5 years (type and frequency of use in the 12 months prior to conception and at the time of the interview) was also recorded.

Information on participant's education, occupation and salary were recoded to classify them according to socioeconomic status in three different categories (low, mid and high) based on guidelines for the socioeconomic measurement (231).

Information on parity, previous adverse obstetrical outcomes, gestational age at labour and offspring's sex and birthweight were obtained from the obstetrical records. Gestational hypertension was also registered and was defined as systolic blood pressure >140 mmHg and/or a diastolic blood pressure >90 mmHg occurring for the first time after the 20^{th} GW in at least in two check-ups (6 hours apart) (181).

Preeclampsia was defined as high blood pressure and proteinuria (>300mg/24 hours) occurring after 20 GW (182).

Gestational diabetes was diagnosed according to a two-step approach stipulated in the pregnancy monitoring protocol established in both Hospitals (181). The first screening was performed with a 50 g oral glucose challenge test (GCT) at 24-27 GW in low risk pregnancies. If positive (glucose concentration >7.8 mmol/L), a 100 g Oral Glucose Tolerance Test was performed (232). Blood samples for glucose determination were taken at baseline (fasting) and at 1, 2 and 3 hours after glucose administration. Gestational diabetes was diagnosed if at least two glucose determinations were above 5.8, 10.6, 9.2 or 8.1 mmol/L at the respective sample collections. In pregnancies with previous gestational diabetes, a glucose challenge test was performed at the first obstetrical visit and, if negative, at 24-27 GW and again at 30 GW. In the case of a positive glucose challenge test, the same protocol described before used for the diagnosis in low risk pregnancies was then applied in those patients.

Preterm birth was defined according to WHO criteria (birth at less than 37 GW) (233). Intrauterine growth restriction was defined as birth weight below the 10th percentile according to sex and gestational age standardized birth weight curves from the Spanish Obstetrics and Gynaecology Society (234).

Placentation was assessed using Doppler evaluation of the intrauterine artery waveforms and pulsatility index. Pathological Doppler was defined as a mean of pulsatility index of right and left uterine arteries ≥P95 and/or the presence of a bilateral notch in the waveforms (167). The notch is the result of elevated resistance to blood flowing into the placenta (as occurs in the non-pregnant uterus).

Blood sample collection

Fasting blood samples were collected at <12, 24-27 and 34 GW and non-fasting at labour into ethylenediaminetetraacetic acid (EDTA)-K_2 treated vacutainers (10 ml), a dry vacutainer for serum (10 ml) and small (EDTA)-K_2 treated vacutainers (5 ml). In the case of cord samples, blood was collected from the umbilical vein into 2 X 10 ml EDTA vacutainers and 2 X 10 ml dry vacutainers before the placenta was expulsed.

All samples were processed at the “Institut d’Investigació Sanitària Pere Virgili Biobank”, in the laboratories of “Facultat de Medicina de l’Universitat Rovira i Virgili”, and in the Joan XXIII University Hospital research laboratory and the central hospital laboratories of both hospitals (cord and labour samples) according to a strict standardized protocol. This ensured that samples were kept at 4ºC until processed within less than an hour of collection to separate whole blood, plasma, serum, washed red cells and leukocyte fractions for storage in the biobank at -80º C. Times of sample collection and separation of

plasma were recorded as a quality control measure to ensure that this protocol was complied with.

Whole blood from EDTA tubes was prepared for posterior red blood cell folate analysis by diluting it 1:10 with 1% freshly prepared ascorbic acid solution. The mixture was kept at room temperature for 30 minutes in order to allow the ascorbic acid erythrocyte lysates to release the serum conjugase, which converts the folate polyglutamates to assayable monoglutamate forms. Two aliquots of 250 µl were prepared and stored at -80º C.

Plasma was separated from the EDTA vacutainer at 1800 g for 15 minutes at 4ºC and stored in aliquots of 1 ml at -80º C. Serum was separated after coagulation of blood at room temperature (approximately 30 minutes after its collection) and aliquoted and stored at -80º C.

In order to isolate leukocytes, phosphate buffered solution was added to the remaining buffy coat and erythrocytes in the EDTA tube and was thoroughly mixed by inversion. The mixture was transferred to a Falcon tube containing 30 ml of hemolysis solution. It was mixed by inversion and then kept at room temperature for 20 minutes to lyse the remaining erythrocytes. The Falcon tube was centrifuged for 5 minutes at 2200 g at room temperature and the supernatant discarded. The pellet was re-suspended in 20 ml of hemolysis solution. The tube was centrifuged as described above and as a result, the leukocytes were isolated from the other cell types. The supernatant was discarded again and 450 µl of PBS and 10 ml of Cell Lysis Solution (Qiagen GmbH, Hilden, Germany) were added to the leukocyte aliquot and preserved at -80º C. This tube was kept at room temperature and protected from light for at least 1 month and a maximum of 6 months.

Leukocyte DNA was extracted and quantified in preparation for maternal and cord genetic polymorphism determinations. Auto pure Protein Precipitation

Solution (3.33 mL) was added and mixed with vortex to obtain a homogenous mix before incubation in iced water for 15 minutes. The mixture was centrifuged at 2200 g at 4º C for 12 minutes. The supernatant containing the suspended DNA was removed and added to a 50 ml Falcon tube with 10 ml of cold 100% isopropanol. The tube was gently mixed by inversion until the precipitated DNA was visible. After centrifuging at 2200 g at 4º C for 5 minutes, the supernatant was discarded (pellet contained the DNA). The tube was dried for 30-40 minutes on absorbent paper, and 1200 µl of DNA Hydration Solution were added to the Falcon tube, mixed and further incubated at room temperature for 3-4 days in a mixer incubator. DNA quantification was performed using a spectrophotometer Nanodrop 1000 at 260 nm wavelength using 2µl of hydrated DNA.

Biochemical and genetic determinations

Plasma aliquots of 0.5 ml from each pregnancy sample and the cord and 120 ng of lyophilized DNA from mother and cord were shipped on dry ice to BeVital A/S (Bergen, Norway) and analyzed, ensuring that all samples from the same pregnancy were analyzed in the same batch, within <18 months after sample collection.

Red blood cell and plasma folate

Folate concentrations in plasma and whole blood were determined by an automated gold standard microbiological assay using a microtiter plate and a chloramphenicol-resistant strain of *Lactobacillus casei* (235). Performances for inter- and intra-assay coefficient of variation were 4 and 5% respectively and the lower detection limit was 2nmol/L.

RBC folate values were calculated using the following formula (236):

Whole blood folate – [Plasma folate*(1-hematocrit/100)] / (Haematocrit/100)

Whole blood folate was corrected for dilution prior to this.

Plasma cobalamin

Total cobalamin concentrations were determined by an automated microbiological assay using a microtiter plate and a colistin sulphate-resistant strain of *Lactobacillus leichmannii* (237). The lower detection limit was 30 pmol/L, and inter- and intra-assay coefficients of variation 4 and 5% respectively.

Plasma total homocysteine and cotinine

Plasma total homocysteine, methylmalonic acid and cotinine were determined by liquid chromatography–tandem mass spectrometry (238). Cotinine, an oxidized metabolite of nicotine, is a marker of recent tobacco exposure. Participants with plasma cotinine concentration >10 ng/mL were considered smokers, and ≤10 ng/mL non-smokers. The lower limits of detection were 0.1 and 0.03µmol/L and 1nmol/L, inter-assay coefficient of variation 2, 3-8 and 6% and intra-assay coefficient 1-2, 1-4 and 2-3% respectively for total homocysteine, methylmalonic acid and cotinine determinations.

Plasma creatinine

Creatinine concentrations were determined by Jaffé reaction (Química Clínica Aplicada, SA, Amposta, Spain) and were used as an estimation of renal function (239). Inter- and intra-assay coefficients of variation were 2.1 and 1.7% respectively.

Genetic polymorphisms

DNA isolated from both mother and cord blood leukocytes were used to determine genetic polymorphisms affecting folate metabolism and cobalamin metabolism and transport. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was used to analyze the *methylenetretrahydrofolate reductase* 677C>T, and the *solute carrier family 19 member 1* 80G>A genotypes, by Bevital (Norway).

3.3. Recruitment and follow-up of fathers

Eligible fathers were invited to participate in the study. In the first instance, we informed the mothers of our interest in investigating the association of paternal genetic and metabolic factors with pregnancy outcome. We asked them if they agreed to provide us the contact details for the father. When the mothers agreed, the fathers were contacted by phone, following a recruitment protocol. The aims of the study, their contribution and what participation would entail was explained to them and they were asked whether they were interested in participating. The time of contact with the father and the time of participation depended on when we spoke to the mother (preferably coinciding with a prenatal visit where possible), how long it took to secure contact with the father and when it suited the father to give a fasting blood sample.

Those that accepted, came to the “Hospital Universitari Sant Joan de Reus” to provide informed signed consent, a fasting blood sample and complete a lifestyle questionnaire.

Ideally, we invited them to participate as near as possible to conception. To evaluate the association of biochemical results at that moment with possible paternal origin complications during placentation, it would be better for the study to have the fasting blood sample just before the conception. To reduce the effect of this limitation, we asked all fathers if they had changed their nutritional habits or lifestyle from the six months before pregnancy until the moment of participation. On the other hand, the results of the genetic analysis will have been unaffected with lifestyle changes, if any, over a relatively short period of time.

Medical history and lifestyle data collection

On the day of their blood sample, a member of our team administered a questionnaire (exemplar in appendices) to fathers regarding basic elements of their medical history as well as supplement use, lifestyle and toxic habits. The questionnaire was specifically designed to cover the time around pre-conception and conception. Any change six months before pregnancy and during pregnancy in paternal diet, supplement use, or habits was recorded to take into account if considered to have a relevant effect on nutritional status. Concrete vitamin or mineral supplementation use, timing of initiation, duration, doses, and brand was recorded. We used this information to estimate the real intake of folic acid or other vitamins and minerals apart from diet. As with the mothers, smoking habit data covered the 5 years before pregnancy and during pregnancy, was assessed by questionnaire (number of cigarettes/d), and whether the habit was maintained, reduced or given up during pregnancy. Plasma cotinine concentrations (>10 ng/mL confirmed active smokers) from blood was checked to corroborate their answers. Similarly, alcohol consumption (duration, drinks /w, timing of cessation before or during pregnancy) and illegal drug use in the previous 5 years (type and frequency of use in the 12 months prior to conception and at the time of the interview) was recorded.

Paternal age and BMI were calculated from the information collected during the visit (birth data, weight and height). BMI calculated as Kg/m^2, was classified as normal weight, overweight or obesity.
Medical history and blood group was obtained by the self-reported information.

Before carrying out the analysis to study the objectives proposed for this thesis, parental verification was performed to prevent introducing errors into the analysis. A battery of 19 polymorphisms was used. If the cord genotype did not match with parental genotypes for more than three polymorphisms, the mismatched mother-cord or father-cord sets would have been excluded from the analysis.

Blood sample collection

The blood sample was programmed as near as possible to conception (determined by ability to contact the father and compatibility of his work schedule). Protocol for collection and processing of samples for biochemical and genetic determinations were the same as for the pregnancy phase and the same laboratories were used.

3.4. Statistical analysis

All statistical analysis were performed using SPSS (SPSS Inc, Chicago, IL, USA) for Windows, version 25.0.

The normality of continuous variables was checked with the Kolmogorov-Smirnov test. Variables not normally distributed were natural log transformed when necessary, to meet the necessary criteria for the application of parametric statistical tests. Descriptive characteristics comprising geometric means (95% CI) or median (P_{10}-P_{90}) and relative frequencies (95% CI), where applicable, are reported for all the participants. Genotype and allele frequencies (95% CI) of the studied genetic variants are also reported. Confidence intervals of categorical variables expressed in % are calculated using the "CIA" programme (Southampton, UK). The McNemar test was used to compare the prevalence of plasma and red blood cell folate deficiency between different time points of pregnancy (<12, 15, 24-27, 34 GW, labour). Post-hoc Bonferroni correction for multiple comparisons of the P values was applied. ANOVA for repeated measures was performed to compare means between different pregnancy time points (<12, 15, 24-27, 34 GW, labour) and post-hoc Bonferroni correction for multiple comparisons of the P values was applied. Maternal, paternal and cord 1C metabolism status (plasma folate, erythrocyte folate, total plasma homocysteine and plasma cobalamin) were compared between the different genotypes for *MTHFR* 677C>T and *SLC19A1* 80 G>A, as well as the combination of both. Statistical significance was accepted from p. values <0.05. Percentages (95% CI) of the outcomes included in the study and according to genotypes are also reported.

BMI was calculated as weight (kg) divided by height squared (m^2). Smoking pattern was categorized by women who smoke throughout pregnancy versus never or women who smoke in the first trimester versus never. Previous

pregnancy was categorized as confirmed previous live newborn or none. Socioeconomic status was categorized as low versus mid-high status considering maternal and paternal status together. Variables of the polymorphisms were used comparing the homozygote normal versus the heterozygote or the homozygote genotypes: CC vs CT or TT for *MTHFR* 677 C>T and GG vs GA or AA for *SLC19A1* 80G>A. The variable used as control for the three clinical outcomes was created with participants without any pregnancy complication such as gestational hypertension, gestational diabetes, IUGR, etc.

The 3 main outcomes studied were: pathological doppler of uterine arteries at 20GW, intrauterine growth restriction and pregnancy induced hypertension. Multiple linear and logistic regressions were used to examine the associations between maternal and paternal genetic, nutritional and metabolic components of 1C metabolism and continuous and categorical variables associated with the development of pregnancy outcomes and complications respectively.

Interaction terms were calculated to assess possible interactions between independent variables, in their relationship with the dependent variable of interest. The product of the independent variables being tested was calculated and included in the linear or logistic regression analysis. When the interaction term was significant, we proceeded to perform the regression analysis on stratified sets of data to account for the interaction.

Multiple linear regression analysis was performed to identify maternal and paternal predictors of pulsatility index of uterine arteries (dependent variable, natural log transformed). The models will be described in detail in the results section. Generally, for each analysis we designed 2 models. The first model explored the association of the 1CM variables of interest and pulsatility index.

For the genotype models, this included the *MTHFR* C677T and *SLC19A1* G80A genotypes as well as first trimester plasma cobalamin and red blood cell folate status (low tertile versus the others). Subsequently maternal clinical and lifestyle factors (smoking during pregnancy versus never, socioeconomic status, age, BMI, late pregnancy anaemia (3rd trimester haemoglobin <11 g/dL), previous pregnancies longer than 20 GW versus none, gestational age (weeks at birth and sex of the baby) were added to form the complete model. In the tHcy models, only tHcy (representing overall 1CM status) at the corresponding time of pregnancy was included in the first model. Subsequently, the next model was adjusted for the same maternal clinical and lifestyle factors described for the complete 1CM model above.

To study the involvement of paternal factors, we added the paternal variables to the complete maternal models described above. Therefore, paternal *MTHFR* 677C>T genotype, cobalamin and red blood cell folate status and paternal tHcy ≥P90 were included in the corresponding models.

Multiple logistic regression analysis was used to identify maternal and paternal factors associated with Pathological doppler of uterine arteries at 20 GW (dependent variable). The models to examine the association between genotypes and impaired placentation included *MTHFR* 677C>T and *SLC19A1* 80G>A genotype, red blood cell folate and plasma cobalamin concentrations at <12 and 15 GW. In the models focused on the association between tHcy and impaired placentation, the categorical tHcy variable (≥P90 tHcy in early pregnancy versus other percentiles) replaced the genotypes and red blood cell folate and plasma cobalamin variables in each model, at <12 and 15 GW. The models were adjusted for smoking habit, socioeconomic status, 1st trimester BMI, age, parity and gestational age at doppler measurement. Adjusting for study centre did not change the results in any of the models so it was not included.

We checked for outliers and influential cases (Cook's distance >4/n) and excluded them from the models when identified.

Paternal factors were also studied in the logistic regressions. Paternal *MTHFR* 677C>T genotype, smoking habit, age, red blood cell folate concentrations and elevated paternal tHcy levels (≥P90) were included in the same models used for the mothers.

Multiple linear regression analysis was performed to identify maternal and paternal predictors of birthweight (dependent variable) with a similar strategy to those described for uterine artery pulsatility index above. In all multiple linear regression analyses, participants with gestational diabetes were excluded due to its potential effect on birth weight.

Multiple logistic regression analysis was used to identify maternal and paternal factors associated with IUGR (dependent variable) applying a similar strategy as described for pathological doppler above. In this case we did not exclude participants with gestational diabetes to avoid loss of statistical power.

Multiple logistic regressions analysis was used to study the association between maternal and paternal genetic and 1CM related factors and gestational hypertension. We excluded pregnancies affected by gestational diabetes or intrauterine growth retardation from the analysis and used similar model designs as described above for pathological Doppler and IUGR.

RESULTS

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4. Results

4.1. Descriptive results

Eight hundred and fifty six women were screened at their first antenatal check-up. 16 women were not eligible to participate (twin pregnancies, drug abuse, donated oocytes and GA >20 GW). From the 840 mothers invited, 21 were not interested and 8 refused for language or cultural reasons. 811 mothers accepted and 748 were identified, as pregnancy progressed, to invite the father (the others had miscarried, moved hospital or left the study). From those participants, 3 were donor fathers, we could not establish contact with 60 mothers and 13 had adverse outcomes before establishing contact with them (12 pregnancy terminations due to foetal anomalies and 1 fetal death). Given the nature of these reasons, paternal contact details could not be requested from the mothers due to discontinuation of prenatal follow up and loss of contact with the study team. A total of 672 fathers were eligible to invite. A further 46 fathers were ineligible for recruitment because 39 mothers refused to provide their contact details and we could not establish the contact with 7 of them. A total of 626 fathers were invited to participate. Of these, 212 fathers refused. 414 fathers agreed to participate by December 2019 allowing us to complete 414 mother-father-neonate triads (66.1 of those eligible) (*Figure 6*).

Maternal and paternal demographic and lifestyle descriptive characteristics are shown in *Table 2*. A total of 25.7% of the mothers smoked at some time of pregnancy and 36.7% of the fathers were smokers. A total of 36.0% of the women reported taking folic acid before pregnancy and 94.2 % during the first trimester. 51.8% did not meet the recommended red blood cell folate status

of 906 nmol/L for NTDs prevention. Only 6.8% of men reported taking multivitamin or mineral supplements.

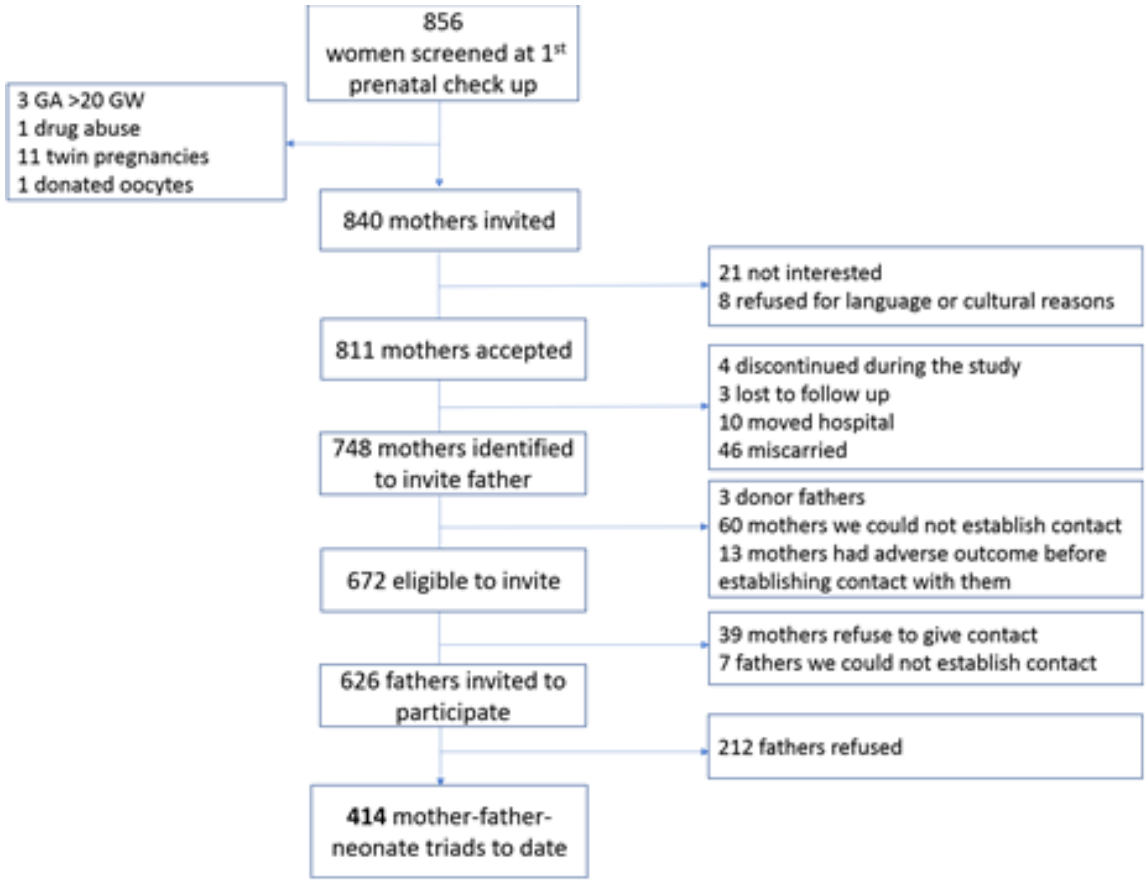

Figure 6. *Participant flow chart from recruitment until triads included.*

***Table 2**. Maternal and paternal characteristics during the first trimester.*

Basic characteristics	Mothers	Fathers
Age (years)[1]	32.0 (26.0, 39.0) [752]	34.0 (29.0, 41.0) [414]
BMI (kg/m^2)[1]	23.3 (19.6, 29.8) [752]	25.7 (22.1, 31.2) [413]
Socioeconomic status[2,3]		
Low	12.2 (10.0, 14.7) [93/764]	
Middle	45.8 (42.3, 49.4) [350/764]	
High	42.0 (38.6, 45.5) [321/764]	
Smoking status[2]		
Never	74.3 (71.1, 77.3) [569/ 766]	
1st trimester only	9.4 (7.5, 11.7) [72/ 766]	
Throughout pregnancy	16.3 (13.9, 19.1) [125/766]	36.7 (32.1, 41.4) [151/412]
Previous Pregnancies[2]	52.4 (48.9, 55.8) [411/785]	
Folic acid supplement use[2,4]		
Preconception	36.0 (32.5, 39.7) [249/691]	
1st trimester	94.2 (92.2, 95.7) [662/703]	6.8 (4.7, 9.6) [28/413]
RBC folate <906 nmol/L[2]	51.8 (48.2, 55.3) [391/755]	

Abbreviations: BMI, Body Mass Index. [1]Median (P_{10}, P_{90}). [2]Percentages (95% confidence interval). [3]Combined maternal and paternal. [4]Information only available from mothers that completed the relevant information in the questionnaires. [n].

Maternal plasma folate, red blood cell folate, total plasma homocysteine and cobalamin concentrations throughout pregnancy at <12, 15, 24-27, 34 GW and at labour are reported in *Table 3.* The same determinations were also performed in the cord blood. During the first trimester (<12 GW) plasma folate status was the highest, with a median of 28.9 nmol/L and similar at 15 GW. By

24-27 GW, it had fallen to 14.4 nmol/L and continued to decrease until the end of pregnancy. Plasma folate concentration in the cord was higher than in the mother during labour. Erythrocyte folate concentrations increased from <12 to 15 GW but had started to decline by 24-27 GW. Median tHcy decreased from 5.3 µmol/L at <12 GW to 4.5 µmol/L by 15 GW. During late pregnancy, it increased and reached 6.0 µmol/L by labour. Median cord blood tHcy was lower than maternal tHcy at labour. Median plasma cobalamin decreased gradually from 364 pmol/L in the first trimester to 233 pmol/L by labour and cord cobalamin was higher than the mothers at labour. Folic acid supplement use interacts significantly with plasma folate concentrations from 15 GW to the end of pregnancy. This interaction was also observed between folic acid supplementation and red blood cell folate and tHcy levels at 24-27 and at 34 GW. No interaction between folic acid supplementation and B12 concentrations was observed (data not reported in the table).

Table 3. *Maternal 1CM folate, cobalamin and tHcy status during pregnancy and in the cord.*

	<12GW	15GW	24-27GW	34GW	Labour	Cord
Plasma folate (nmol/L)	28.9 (10.0, 61.2)[1]	26.6 (9.9, 53.8)[1]	14.4 (5.8, 39.7)[1]	11.0 (5.0, 38.6)[1]	10.0 (4.9, 36.5)[1]	26.3 (12.2, 56.5)[1]
	27.0 (25.7, 28.4)[2] [773]	24.9 (23.4, 26.6)[2] †† [448]	15.3 (14.4, 16.2)[2] *** ††† [665]	12.8 (12.0, 13.7)[2] *** †† [633]	12.1 (11.3, 12.9)[2] **† [611]	26.0 (24.8, 27.3)[2] [577]
Erythrocyte folate (nmol/L)	936 (466, 1841)[1]	1266 (665, 2204)[1]	1138 (602, 2119)[1]	949 (464, 2065)[1]	-	-
	942 (906, 980)[2] [753]	1248 (1191, 1307)[2] *** [446]	1150 (1105, 1196)[2] *** ††† [651]	975 (932, 1021)[2] ***††† [616]	-	-
Plasma total homocysteine (µmol/L)	5.3 (4.0, 7.1)[1]	4.5 (3.5, 5.9)[1]	4.6 (3.5, 6.3)[1]	5.2 (3.8, 7.3)[1]	6.0 (4.3, 8.6)[1]	4.8 (3.4, 6.7)[1]
	5.3 (5.2, 5.4) [2] [774]	4.5 (4.4, 4.6)[2] *** [449]	4.6 (4.6, 4.7)[2] ** ††† [666]	5.2 (5.1, 5.3)[2] *** † [634]	6.1 (6.0, 6.3)[2] *** [613]	4.8 (4.7, 4.9)[2] [578]
Plasma B12 (pmol/L)	364 (240, 525) [1]	332 (213, 467)[1]	277 (178, 424)[1]	252 (160, 388)[1]	233 (143, 369)[1]	336 (115, 635)[1]
	360 (351, 369)[2] [773]	324 (314, 334)[2] *** [448]	274 (267, 281)[2] *** [665]	250 (244, 257)[2] *** [633]	231 (224, 238)[2] *** [606]	316 (299, 333)[2] [550]

Abbreviations: GW, gestational week. [1]Median (P_{10}, P_{90}). [2]Geometric mean (95% confidence interval). [n]. Geometric means between the different time points of pregnancy were compared using ANOVA for repeated measures analysis. Post-hoc Bonferroni correction for multiple comparisons was applied. Intrasubject factor: time of pregnancy; intersubject factor folic acid supplement use (>400 µg/ d in the first trimester and continued use in the second and third trimesters). Different to previous time point: *P<0.05, **p<0.01, ***p<0.001. Intersubject factor folic acid supplement use †P<0.05, ††p<0.01, †††p<0.001.

The following graphs illustrate the percentage of participants deficient in plasma and red blood cell folate during pregnancy, at <12, 15, 24-27, 34 GW and labour. Deficiency was defined as plasma folate concentrations ≤ 7 nmol/L (7), and red blood cell folate concentrations ≤ 340 nmol/L. The prevalence of plasma folate deficiency increased as pregnancy progressed, mainly after 15 GW. In the case of red blood cell folate deficiency, the prevalence was the lowest at 15 and 24-27 GW but it had increased by 34 GW.

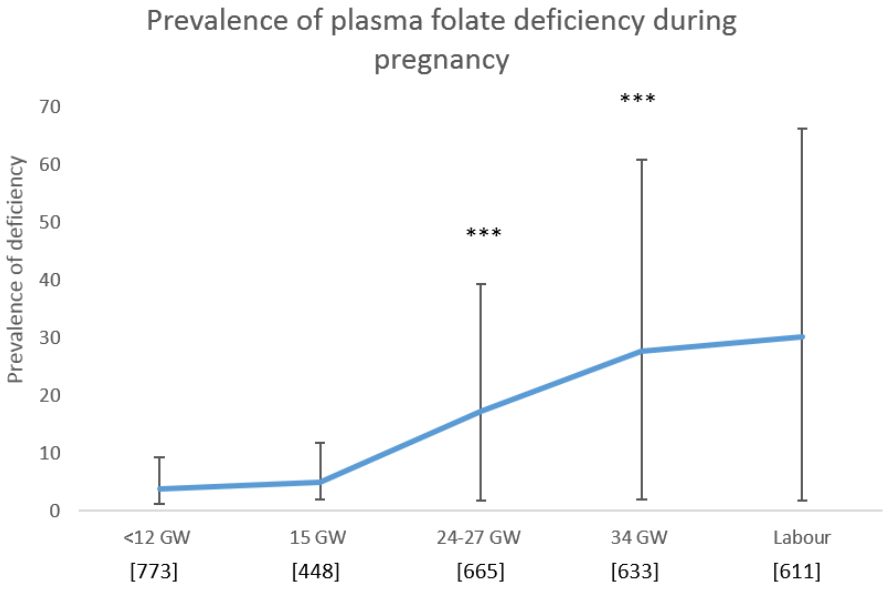

Figure 7. Prevalence of plasma folate deficiency (≤ 7nmol/L) in participants during pregnancy and at labour are represented. [n]. Means (dots) and 95% Confidence Interval (bars) are illustrated. Prevalences of deficiency with the preceding time point were compared using Chi-square (McNemar) and post-hoc Bonferoni correction of P values for multiple comparisons was applied. *P<0.05, ***P<0.001.

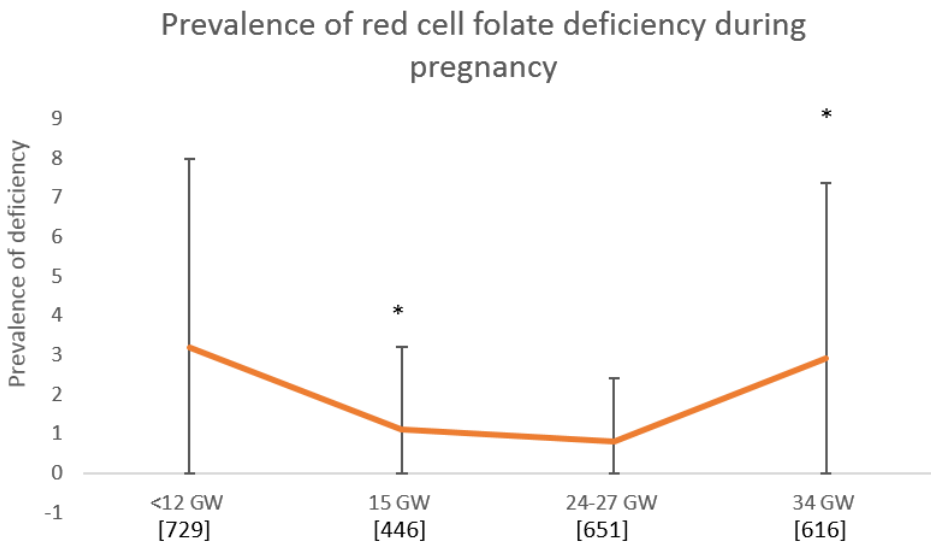

Figure 8. Prevalence of red blood cell folate deficiency (≤ 340 nmol/L) in participants during pregnancy are represented. [n]. Means (dots) and 95% Confidence Interval (bars) are illustrated. Prevalences of deficiency with the preceding time point were compared using Chi-square (McNemar) and post-hoc Bonferoni correction of P values for multiple comparisons was applied. *P<0.05, ***P<0.001.

Paternal 1CM nutrient and metabolite status is reported in *Table 4.* Median plasma folate was relatively low at 8.8 nmol/L. Median erythrocyte folate concentration was 456.8 nmol/L, tHcy was 9.6 µmol/L and plasma cobalamin 367.2 pmol/L.

***Table 4**. Paternal status in 1CM nutrients and metabolites.*

	Fathers	N
Plasma folate (nmol/L)	8.8 (4.5, 16.8)[1]	405
	8.7 (8.3, 9.2)[2]	
Erythrocyte folate (nmol/L)	456.8 (205.4, 802.9)[1]	384
	425.9 (404.6, 448.4)[2]	
Plasma total homocysteine (µmol/L)	9.6 (7.5, 14.0)[1]	404
	9.9 (9.7, 10.2)[2]	
Plasma B12 (pmol/L)	367.2 (282.5, 570.4)[1]	403
	380.3 (369.6, 391.4)[2]	

[1] Median (P_{10}, P_{90}). [2] Geometric mean (95% confidence interval).

Maternal, paternal and cord genotypes and allele frequencies for the *MTHFR* 677C>T and *SLC19A1* 80 G>A genetic variants and the combination of both are shown in *Table 5*. All the observed genotype frequencies were in Hardy-Weinberg equilibrium (p>0.05). The homozygote variant genotype for the *MTHFR* C677T polymorphism was 17.0, 14.1 and 18.6% for the mother, father and cord respectively. For the *SLC19A1* G80A polymorphism, the homozygote variant genotype frequency was 27.2, 28.9 and 26.9% for the mother, father and cord respectively. Regarding the combined genotypes, the CT-GA combination was the most frequent, found in >20% of mothers, fathers and cords.

***Table 5**. Frequencies of maternal, paternal and cord MTHFR 677* C>T *and SLC19A1* 80 G>A *genotypes.*[1]

	Mother	Father	Cord
***MTHFR 677 C>T* genotype**			
CC	34 (30.5, 37.3) [249]	36.3 (31.6, 41.2) [139]	35.4 (31.5, 39.6) [188]
CT	49.3 (45.7, 52.9) [363]	49.6 (44.6, 54.6) [192]	46.0 (41.8, 50.2) [244]
TT	17.0 (14.4, 19.8) [125]	14.1 (11.0, 17.9) [54]	18.6 (15.6, 22.2) [99]
T allele frequency	41.6 (39.1, 44.1) [613/1474]	38.9 (35.5, 42.4) [298/766]	41.6 (38.7, 44.6) [442/1062]
***SLC19A1 80 G>A* genotype**			
GG	27.2 (24.2, 30.6) [200]	27.9 (23.6, 32.6) [106]	25.6 (22.1, 29.5) [135]
GA	45.5 (41.9, 49.1) [334]	43.2 (38.3, 48.2) [164]	47.4 (43.2, 51.7) [250]
AA	27.2 (24.2, 30.6) [200]	28.9 (24.6, 33.7) [112]	26.9 (23.3, 30.9) [142]
A allele frequency	50.0 (47.4, 52.6) [734/1468]	50.5 (47.0, 54.1) [384/760]	50.7 (47.6, 53.7) [534/1054]
***MTHFR 677 C>T + SLC19A1 80 G>A* genotype**			
CC-GG	10.6 (8.6, 13.1) [78]	10.0 (7.4, 13.4) [38]	10.3 (8.0, 13.2) [54]
CC-GA	15.0 (12.6, 17.8) [110]	17.4 (13.9, 21.5) [67]	16.6 (13.6, 20.0) [87]
CC-AA	8.3 (6.5, 10.5) [61]	9.2 (6.7, 12.5) [35]	8.8 (6.6, 11.5) [46]
CT-GG	11.9 (9.7, 14.4) [87]	13.2 (10.1, 16.9) [50]	10.9 (8.5, 13.8) [57]
CT-GA	24.3 (21.3, 27.5) [178]	21.6 (17.7, 26.0) [83]	21.3 (18.0, 25.0) [112]
CT-AA	12.9 (10.7, 15.6) [96]	14.5 (11.3, 18.4) [57]	13.5 (10.9, 16.7) [72]
TT-GG	4.8 (3.4, 6.6) [35]	4.7 (3.0, 7.4) [18]	4.6 (3.1, 6.7) [24]
TT-GA	6.3 (4.7, 8.3) [47]	4.2 (2.6, 6.7) [16]	9.3 (7.1, 12.1) [49]
TT-AA	6.0 (4.5, 8.0) [44]	5.3 (3.4, 8.0) [20]	4.8 (3.2, 6.9) [25]

[1] All data shown are percentages (95% confidence interval).

Abbreviations: *MTHFR*, methylenetetrahydrofolate reductase; *SLC19A1,* solute carrier family 19 member 1. [n]

Maternal one carbon metabolism status (plasma folate, erythrocyte folate and total plasma homocysteine) according to *MTHFR* 677 C>T and *SLC19A1* 80 G>A genotypes and the combination of both at 5 different points of pregnancy (<12, 15, 24-27, 34 GW and labour) are reported in *Figure 9* and *tables 6* and *7*. A similar pattern to that observed for change in plasma folate (*Table 3)* was observed for all the *MTHFR* 677 C>T genotypes and plasma folate did not differ between them (*Figure 9*). In the women with available RBC folate measurements at 15 GW, we observed that it increased between the first trimester and 15 GW and then gradually declined for the rest of pregnancy. In contrast to plasma folate, we did observe lower RBC folate status in the *MTHFR* 677TT compared to CC genotype from 24-27 GW and also compared to the CT genotype at 34 GW (*Figure 9*). THcy decreased between the first trimester and 15 GW and then gradually increased for the rest of pregnancy, reaching the highest concentration at labour. THcy was higher in the *MTHFR* 677TT compared to CC and CT genotypes from 15GW throughout the rest of pregnancy (*Figure 9*). No significant differences were observed in plasma or tHcy status between *SLC19A1* 80 G>A genotypes (*Table 6*). Regarding the combination of both genotypes, only tHcy at <12, 24-27 and 34 GW and at labour differed among these (*Table 7*). People with the combination of *MTHFR* 677 + *SLC19A1* TT-GG, TT-GA genotypes had the highest tHcy out of all of the combinations. People with the combinations of *MTHFR* 677 + *SLC19A1* CC-GA and CC-AA genotypes had the lowest. People with the combinations of *MTHFR* 677 + *SLC19A1* CC-GG, CT-GA and CT-AA had lower tHcy compared to the TT-GG and TT-GA genotype combinations but higher than those with the CC-GA and CC-AA combinations. Plasma

cobalamin concentrations and changes during pregnancy did not differ between genotypes (data not shown).

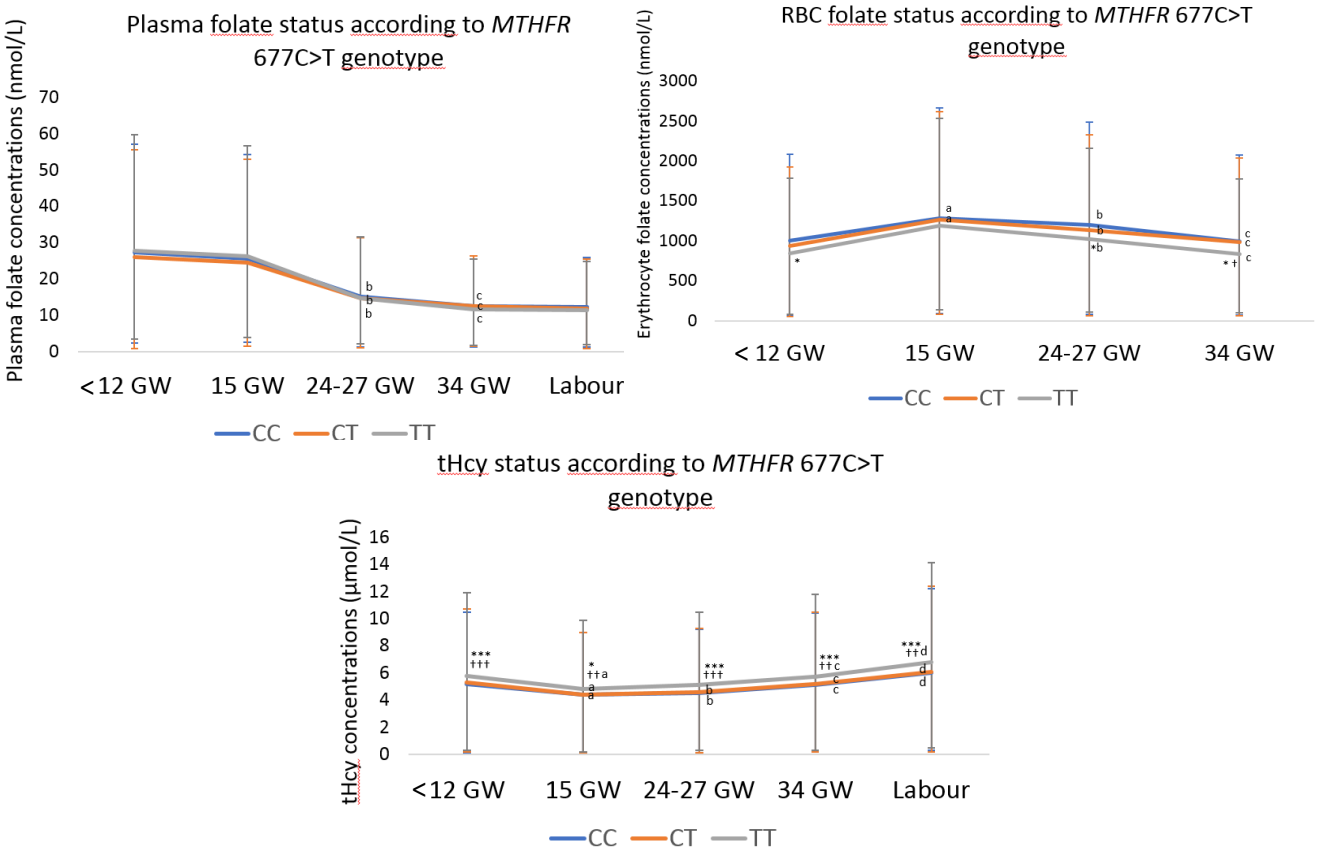

Figure 9. Maternal one carbon metabolism status according to *MTHFR* 677 C>T genotype. Geometric means (95% confidence interval) are shown. Blue line represents CC genotype; plasma folate and tHcy at <12GW n= 248, 15GW n=148, 24-27GW n=217, 34GW n=202 and at labour n=206. RBC folate at <12GW n= 241, 15GW n=146, 24-27GW n=212, 34GW n=194. Orange line represents CT genotype; plasma folate and tHcy at <12GW n=355, 15GW n=214, 24-27GW n=309, 34GW n=299 and at labour n=289. RBC folate at <12GW n= 345, 15GW n=214, 24-27GW n=301, 34GW n=291. Grey line represents TT genotype; plasma folate <12GW n= 122, 15GW n=83, 24-27GW n=108, 34GW n=102 and at labour n=94. RBC folate at <12GW n= 121, 15GW n=83, 24-27GW n=107, 34GW n=101. THcy at <12GW n= 123, 15GW n=84, 24-27GW n=109, 34GW n=103 and at labour n=95. ANOVA with post-hoc Bonferroni correction for multiple comparisons were used to compare the differences in geometric means between the genotypes and to compare plasma concentrations of 1C metabolites between different times of pregnancy and labour. $^{*}p<0.05$ $^{**}p<0.01$ *** $p<0.001$ TT compared to CC genotype. $^{\dagger\dagger}p<0.01$ $^{\dagger\dagger\dagger}p<0.001$ TT compared to CT genotype. ^{a}p <0.001 compared with <12 GW, ^{b}p <0.001 compared with 15GW, ^{c}p <0.001 compared with 24-27GW, ^{d}p <0.001 compared with 34GW. GW, gestational week; *MTHFR*,

***Table 6**. Maternal one carbon metabolism status according to SLC19A1 80 G>A genotype.*

	<12GW	15GW	24-27GW	34GW	Labour
Plasma folate (nmol/L)					
SLC19A1 GG genotype	30.4 (9.2, 61.5)[1]	28.2 (10.2, 45.6)[1]	13.5 (5.6, 36.6)[1]	9.7 (4.9, 35.6)[1]	9.0 (4.6, 37.7)[1]
	27.3 (24.6, 30.4)[2] [198]	25.9 (23.1, 29.1)[2] [123]	14.8 (13.3, 16.6)[2bbb] [175]	11.8 (10.4, 13.3)[2ccc] [169]	11.3 (9.9, 12.8)[2] [163]
SLC19A1 GA genotype	27.2 (10.1, 59.7)[1]	25.2 (10.1, 55.5)[1]	13.5 (5.7, 39.8)[1]	11.2 (5.0, 40.6)[1]	10.1 (4.9, 34.6)[1]
	26.7 (24.7, 28.8)[2] [330]	25.0 (22.7, 27.5)[2] [201]	14.8 (13.6, 16.2)[2bbb] [292]	12.8 (11.6, 14.2)[2ccc] [269]	12.0 (10.9, 13.2)[2d] [273]
SLC19A1 AA genotype	30.3 (10.5, 58.9)[1]	27.0 (9.6, 54.7)[1]	13.1 (5.9, 39.7)[1]	9.9 (5.1, 36.9)[1]	10.0 (4.9, 37.3)[1]
	26.4 (24.0, 29.1)[2] [194]	24.5 (21.6, 27.8)[2] [118]	14.7 (13.0, 16.5)[2bbb] [164]	12.2 (10.8, 13.7)[2] ccc [162]	12.2 (10.7, 14.0)[2] [150]
p value	0.885	0.802	0.986	0.525	0.631
Erythrocyte folate (nmol/L)					
SLC19A1 GG genotype	1003.5 (454.7, 1840.3)[1]	1380.3 (789.9, 2245.2)[1]	1170.6 (584.9, 2006.6)[1]	904.1 (462.9, 1691.1)[1]	-
	970.7 (897.8, 1049.5)[2] [193]	1330.5 (1221.9, 1448.7)[2aaa] [122]	1107.3 (1019.9, 1202.2)[2bbb] [172]	869.8 (801.3, 944.2)[2] ccc [166]	-
SLC19A1 GA genotype	941.6 (466.0, 1778.0)[1]	1237.5 (622.3, 2196.1)[1]	1137.1 (580.9, 2099.7)[1]	1028.2 (461.4, 2166.3)[1]	-
	937.1 (884.4, 992.9)[2] [323]	1228.0 (1145.5, 1316.6)[2aaa] [201]	1134.2 (1068.4, 1204.0)[2bbb] [284]	1006.3 (936.2, 1081.6)[2] ccc [264]	-

***Table 6**. Maternal one carbon metabolism status according to SLC19A1 80 G>A genotype.*

	<12GW	15GW	24-27GW	34GW	Labour
SLC19A1 AA genotype	886.0 (468.8, 1931.4)[1]	1247.4 (681.3, 2221.4)[1]	1092.5 (599.4, 2083.3)[1]	815.1 (432.9, 1958.1)[1]	-
	914.7 (842.0, 993.6)[2] [188]	1234.4 (1129.4, 1349.2)[2aaa] [117]	1113.8 (1025.7, 1209.6)[2bbb] [161]	925.9 (847.2, 1011.9)[2ccc] [153]	-
p value	0.564	0.343	0.865	0.199	-
Plasma total homocysteine (µmol/L)					
SLC19A1 GG genotype	5.3 (4.2, 7.4)[1]	4.4 (3.6, 6.0)[1]	4.7 (3.5, 6.5)[1]	5.3 (3.9, 7.9)[1]	6.2 (4.1, 9.2)[1]
	5.4 (5.2, 5.6)[2] [198]	4.5 (4.3, 4.7)[2aaa] [123]	4.8 (4.6, 5.0)[2bb] [175]	5.4 (5.2, 5.6)[2ccc] [169]	6.3 (6.0, 6.6)[2ddd] [163]
SLC19A1 GA genotype	5.3 (4.0, 7.2)[1]	4.5 (3.5, 5.7)[1]	4.6 (3.5, 6.3)[1]	5.1 (3.8, 7.0)[1]	6.0 (4.3, 8.5)[1]
	5.3 (5.2, 5.5)[2] [331]	4.5 (4.4, 4.6)[2aaa] [202]	4.6 (4.5, 4.7)[2 bb] [293]	5.2 (5.1, 5.4)[2ccc] [270]	6.1 (5.9, 6.3)[2ddd] [275]
SLC19A1 AA genotype	5.3 (3.8, 6.9)[1]	4.6 (3.3, 5.9)[1]	4.7 (3.4, 6.3)[1]	5.3 (3.6, 7.1)[1]	5.9 (4.4, 8.8)[1]
	5.3 (5.1, 5.4)[2] [194]	4.6 (4.4, 4.8)[2aaa] [116]	4.6 (4.5, 4.8)[2] [132]	5.1 (4.9, 5.3)[2ccc] [130]	6.1 (5.9, 6.4)[2ddd] [120]
p value	0.587	0.605	0.347	0.190	0.598

Abbreviations: GW, gestational week; *SLC19A1, solute carrier family 19 member 1.*[1] Median (P_{10}, P_{90}). [2]Geometric mean (95% confidence interval). Geometric means were compared between time points using ANOVA for repeated measures and between genotypes using ANOVA (post hoc Bonferroni p value are shown). [a]p <0.05, [aa]p <0.01, [aaa]p <0.001 compared with <12 GW, [b]p<0.05, [bb]p<0.01, [bbb]p <0.001 compared with 15GW, [c]p <0.05, [cc]p <0.01, [ccc]p <0.001 compared with 24-27GW, [d]p <0.05, [dd]p <0.01, [ddd]p <0.001 compared with 34GW.

***Table 7**. Maternal one carbon metabolism status according to maternal combination of both genotypes.*

	<12GW	15GW	24-27GW	34GW	Labour
Plasma folate (nmol/L)					
MTHFR 677 CC +	30.6 (10.2, 59.2)[1]	28.0 (12.8, 43.3)[1]	15.3 (6.2, 40.0)[1]	10.2 (5.0, 34.2)[1]	9.5 (5.1, 38.4)[1]
SLC19A1 GG	27.3 (23.4, 31.7)[2]	26.2 (22.3, 30.8)[2]	16.1 (13.5, 19.2)[2bbb]	11.9 (9.8, 14.4)[2c]	12.6 (10.2, 15.5)[2d]
genotype	[78]	[43]	[68]	[62]	[64]
MTHFR 677 CC +	29.3 (10.8, 53.0)[1]	24.6 (10.9, 70.3)[1]	12.9 (5.8, 42.4)[1]	12.3 (5.1, 40.8)[1]	10.9 (5.1, 33.9)[1]
SLC19A1 GA	27.3 (24.1, 30.9)[2]	26.1 (21.6, 31.4)[2]	15.2 (13.1, 17.6)[2]	13.3 (11.1, 15.9)[2ccc]	12.5 (10.5, 14.8)[2]
genotype	[110]	[67]	[96]	[88]	[90]
MTHFR 677 CC +	31.5 (11.0, 55.3)[1]	28.8 (9.6, 49.9)[1]	12.0 (6.5, 39.8)[1]	8.4 (5.2, 37.3)[1]	8.2 (4.9, 42.6)[1]
SLC19A1 AA	27.8 (23.5, 32.9)[2]	24.3 (19.7, 29.8)[2]	13.5 (11.3, 16.3)[2bbb]	11.8 (9.5, 14.6)[2cc]	11.4 (9.0, 14.3)[2dd]
genotype	[60]	[38]	[53]	[52]	[52]
MTHFR 677 CT +	28.1 (8.2, 79.4)[1]	27.4 (6.8, 46.8)[1]	11.1 (5.4, 36.8)[1]	8.6 (5.1, 39.1)[1]	8.2 (4.1, 39.8)[1]
SLC19A1 GG	26.1 (21.8, 31.3)[2]	24.5 (20.0, 29.9)[2]	13.9 (11.6, 16.5)[2bbb]	11.6 (9.7, 14.0)[2cc]	10.4 (8.5, 12.6)[2dd]
genotype	[85]	[55]	[76]	[75]	[70]
MTHFR 677 CT +	26.9 (9.8, 62.2)[1]	25.2 (9.3, 55.9)[1]	13.6 (5.5, 36.5)[1]	10.4 (4.9, 44.5)[1]	9.7 (4.7, 35.3)[1]
SLC19A1 GA	25.9 (23.3, 28.9)[2]	24.5 (21.5, 27.9)[2]	14.6 (12.9, 16.5)[2bbb]	12.8 (11.2, 14.7)[2cc]	11.7 (10.3, 13.3)[2dd]
genotype	[175]	[104]	[155]	[143]	[148]
MTHFR 677 CT +	28.8 (10.5, 60.0)[1]	26.5 (9.6, 46.2)[1]	16.5 (6.2, 39.7)[1]	11.2 (5.0, 35.4)[1]	11.6 (5.8, 37.6)[1]
SLC19A1 AA	26.1 (22.8, 29.9)[2]	23.9 (20.2, 28.2)[2]	15.7 (13.1, 18.8)[2bbb]	12.8 (10.9, 15.0)[2]	13.4 (11.2, 16.1)[2]
genotype	[92]	[52]	[75]	[78]	[68]
MTHFR 677 TT +	36.8 (11.8, 57.9)[1]	29.3 (10.4, 69.7)[1]	14.9 (5.7, 34.1)[1]	12.3 (3.6, 41.7)[1]	10.7 (4.5, 31.3)[1]
SLC19A1 GG	30.8 (24.7, 38.4)[2]	28.8 (22.2, 37.4)[2]	14.7 (11.3, 19.1)[2bbb]	11.9 (8.7, 16.2)[2ccc]	10.8 (8.2, 14.2)[2]
genotype	[35]	[25]	[31]	[32]	[29]
MTHFR 677 TT +	25.8 (9.5, 81.1)[1]	28.2 (7.8, 49.1)[1]	14.3 (5.2, 38.2)[1]	11.4 (4.6, 35.5)[1]	12.1 (4.9, 32.8)[1]
SLC19A1 GA	28.1 (22.2, 35.6)[2]	24.2 (19.2, 30.6)[2]	14.8 (11.7, 18.7)[2bbb]	11.9 (9.1, 15.5)[2]	11.9 (9.1, 15.7)[2]
genotype	[45]	[30]	[41]	[38]	[35]

***Table 7**. Maternal one carbon metabolism status according to maternal combination of both genotypes.*

	<12GW	**15GW**	**24-27GW**	**34GW**	**Labour**
MTHFR 677 TT +	30.2 (8.9, 60.4)[1]	25.2 (8.5, 89.0)[1]	12.3 (5.4, 47.5)[1]	9.5 (4.9, 49.6)[1]	8.3 (3.3, 46.3)[1]
SLC19A1 AA	25.4 (19.7, 32.6)[2]	26.2 (18.6, 36.8)[2]	14.4 (10.7, 19.3)[2bbb]	11.4 (8.5, 15.3)[2]	11.2 (7.5, 16.8)[2]
genotype	[42]	[28]	[36]	[32]	[30]
p value	0.955	0.974	0.946	0.965	0.766
Erythrocyte folate (nmol/L)					
	1038.7	1385.6	1218.7	953.3	-
MTHFR 677 CC +	(452.7, 1836.0)[1]	(799.9, 2245.2)[1]	(665.3, 2130.1)[1]	(466.4, 1703.2)[1]	
SLC19A1 GG	991.2	1366.4	1215.1	942.8	
genotype	(871.7, 1126.9)[2]	(1189.8, 1569.2)[2aaa]	(1089.3, 1355.5)[2bb]	(831.2, 1069.5)[2ccc]	
	[76]	[42]	[66]	[60]	
	997.0	1171.7	1215.1	1061.9 (472.2,	-
MTHFR 677 CC +	(520.2, 1872.2)[1]	(602.8, 2299.9)[1]	(651.2, 2402.2)[1]	2449.8)[1]	
SLC19A1 GA	1023.9	1246.7	1217.5	1094.8	
genotype	(927.9, 1129.8)[2]	(1104.2, 1407.5)[2aaa]	(1099.5, 1348.2)[2]	(960.8, 1247.5)[2ccc]	
	[107]	[67]	[93]	[85]	
	975.9	1186.6	1122.0	867.4	-
MTHFR 677 CC +	(519.7, 1960.3)[1]	(770.7, 2006.2)[1]	(662.1, 1846.1)[1]	(525.7, 1936.6)[1]	
SLC19A1 AA	987.1	1258.9	1160.2	902.3	
genotype	(856.7, 1137.4)[2]	(1101.4, 1439.0)[2aa]	(1011.4, 1330.8)[2]	(777.6, 1046.9)[2ccc]	
	[58]	[37]	[53]	[49]	
	1064.6	1391.7	1118.0	904.6	-
MTHFR 677 CT +	(441.6, 1868.5)[1]	(775.5, 2167.6)[1]	(527.6, 2019.8)[1]	(426.4, 1782.2)[1]	
SLC19A1 GG	982.7	1290.0	1121.8	952.3	
genotype	(869.5, 1110.6)[2]	(1136.4, 1464.3)[2aaa]	(996.7, 1262.6)[2]	(843.8, 1074.8)[2ccc]	
	[83]	[55]	[76]	[74]	

***Table 7**. Maternal one carbon metabolism status according to maternal combination of both genotypes.*

	<12GW	**15GW**	**24-27GW**	**34GW**	**Labour**
MTHFR 677 CT + *SLC19A1 GA* genotype	939.9 (457.8, 1711.1)[1] 916.8 (847.4, 991.9)[2] [171]	1342.4 (756.3, 2155.8)[1] 1278.8 (1161.6, 1407.9)[2aaa] [104]	1136.4 (573.1, 1991.1)[1] 1126.6 (1039.1, 1221.3)[2bb] [150]	1026.2 (521.9, 2067.7)[1] 1014.2 (923.5, 1113.8)[2ccc] [141]	-
MTHFR 677 CT + *SLC19A1 AA* genotype	910.3 (443.9, 1895.0)[1] 915.2 (814.7, 1028.2)[2] [88]	1248.1 (658.6, 2195.3)[1] 1215.9 (1062.8, 1391.1)[2aaa] [52]	1103.9 (576.1, 2151.2)[1] 1135.3 (1007.3, 1279.6)[2bb] [72]	912.6 (500.0, 2018.2)[1] 966.8 (856.0, 1091.8)[2cc] [73]	-
MTHFR 677 TT + *SLC19A1 GG* genotype	843.0 (478.3, 1929.3)[1] 899.2 (754.3, 1072.0)[2] [34]	1339.6 (648.3, 3080.7)[1] 1347.4 (1080.8, 1679.9)[2aaa] [25]	1116.7 (452.4, 1987.5)[1] 1072.7 (885.9, 1298.8)[2b] [30]	761.4 (382.4, 1562.8)[1] 829.5 (681.4, 1009.9)[2ccc] [32]	-
MTHFR 677 TT + *SLC19A1 GA* genotype	854.7 (386.0, 1511.1)[1] 824.8 (696.1, 977.4)[2] [45]	992.3 (510.9, 2026.4)[1] 1031.8 (853.5, 1247.4)[2aaa] [30]	997.6 (446.4, 1829.7)[1] 989.7 (827.3, 1183.9)[2] [41]	838.5 (357.4, 1690.4)[1] 809.3 (653.0, 1002.9)[2ccc] [38]	-
MTHFR 677 TT + *SLC19A1 AA* genotype	716.2 (345.4, 2027.2)[1] 822.1 (667.9, 1012.1)[2] [42]	1252.2 (601.4, 2693.7)[1] 1237.0 (981.5, 1559.0)[2aaa] [28]	910.5 (425.9, 2950.5)[1] 1009.7 (820.4, 1242.6)[2b] [36]	797.5 (393.3, 2033.3)[1] 871.3 (676.3, 1122.4)[2ccc] [31]	-
p value	0.250	0.518	0.402	0.104	-

***Table 7**. Maternal one carbon metabolism status according to maternal combination of both genotypes.*

	<12GW	15GW	24-27GW	34GW	Labour
Plasma total homocysteine (µmol/L)					
MTHFR 677 CC +	5.1 (4.2, 7.0)[1]	4.3 (3.7, 5.3)[1]	4.7 (3.4, 6.2)[1]	5.1 (3.8, 7.1)[1]	6.1 (3.8, 8.9)[1]
SLC19A1 GG	5.4 (5.1, 5.6)[2]	4.4 (4.2, 4.6)[2aaa]	4.6 (4.4, 4.9)[2$#]	5.1 (4.8, 5.5)[2$$$# ccc]	6.0 (5.6, 6.5)[2$##ddd]
genotype	[78]	[43]	[68]	[62]	[64]
MTHFR 677 CC +	5.1 (4.0, 7.1)[1]	4.4 (3.5, 5.5)[1]	4.4 (3.6, 6.0)[1]	5.0 (3.8, 6.8)[1]	5.9 (4.1, 9.0)[1]
SLC19A1 GA	5.2 (5.0, 5.4)[2]	4.4 (4.2, 4.6)[2aaa]	4.5 (4.3, 4.7)[2]	5.2 (4.9, 5.5)[2ccc]	5.9 (5.6, 6.4)[2ddd]
genotype	[110]	[67]	[96]	[88]	[90]
MTHFR 677 CC +	5.2 (3.8, 6.4)[1]	4.7 (3.3, 5.8)[1]	4.7 (3.4, 5.7)[1]	4.9 (3.6, 7.1)[1]	5.8 (4.7, 7.9)[1]
SLC19A1 AA	5.0 (4.8, 5.3)[2]	4.6 (4.3, 4.9)[2aa]	4.5 (4.3, 4.8)[2]	4.9 (4.6, 5.3)[2ccc]	6.0 (5.6, 6.3)[2ddd]
genotype	[60]	[38]	[53]	[52]	[52]
MTHFR 677 CT +	5.0 (3.9, 7.0)[1]	4.3 (3.2, 6.0)[1]	4.6 (3.4, 6.6)[1]	5.2 (3.7, 7.9)[1]	6.1 (4.4, 8.9)[1]
SLC19A1 GG	5.1 (4.9, 5.4)[2]	4.4 (4.1, 4.6)[2aaa]	4.7 (4.4, 4.9)[2bb]	5.3 (5.0, 5.7)[2ccc]	6.2 (5.9, 6.7)[2ddd]
genotype	[85]	[55]	[76]	[75]	[70]
MTHFR 677 CT +	5.3 (4.0, 7.2)[1]	4.4 (3.4, 5.8)[1]	4.6 (3.4, 6.3)[1]	5.1 (3.8, 6.8)[1]	6.0 (4.3, 8.0)[1]
SLC19A1 GA	5.3 (5.1, 5.5)[2]	4.4 (4.3, 4.6)[2aaa]	4.5 (4.4, 4.7)[2]	5.1 (4.9, 5.3)[2ccc]	6.0 (5.7, 6.3)[2ddd]
genotype	[175]	[104]	[155]	[143]	[149]
MTHFR 677 CT +	5.4 (3.9, 7.0)[1]	4.5 (3.5, 5.9)[1]	4.8 (3.5, 6.3)[1]	5.3 (3.6, 7.1)[1]	6.0 (4.3, 9.5)[1]
SLC19A1 AA	5.3 (5.0, 5.5)[2]	4.5 (4.3, 4.8)[2aaa]	4.7 (4.4, 5.0)[2]	5.2 (4.9, 5.5)[2ccc]	6.1 (5.7, 6.6)[2ddd]
genotype	[92]	[52]	[75]	[78]	[68]
MTHFR 677 TT +	5.8 (4.5, 10.0)[1]	5.2 (3.8, 7.0)[1]	5.3 (4.0, 8.2)[1]	5.8 (4.3, 9.9)[1]	6.9 (4.8, 14.2)[1]
SLC19A1 GG	6.0 (5.5, 6.6)[2*††ʃ]	5.0 (4.6, 5.5)[2aaa]	5.4 (4.9, 6.0)[2^^**†≠≠]	6.1 (5.5, 6.8)[2†≠]	7.0 (6.0, 8.1)[2d]
genotype	[35]	[25]	[31]	[32]	[29]
MTHFR 677 TT +	5.8 (4.4, 9.1)[1]	4.7 (4.0, 6.0)[1]	4.9 (3.9, 6.9)[1]	5.6 (3.8, 7.9)[1]	6.4 (4.8, 10.5)[1]
SLC19A1 GA	5.9 (5.5, 6.4)[2§ϕ ϕ∞]	4.8 (4.4, 5.2)[2aaa]	5.2 (4.8, 5.7)[2£§}]	5.8 (5.2, 6.5)[2]	6.9 (6.2, 7.7)[2ddd]
genotype	[46]	[31]	[42]	[39]	[36]

***Table 7**. Maternal one carbon metabolism status according to maternal combination of both genotypes.*

	<12GW	**15GW**	**24-27GW**	**34GW**	**Labour**
MTHFR 677 TT +	5.4 (3.7, 8.1)[1]	4.9 (3.2, 6.2)[1]	4.8 (3.0, 6.8)[1]	5.3 (3.4, 7.4)[1]	6.4 (4.0, 10.2)[1]
SLC19A1 AA	5.5 (5.1, 6.0)[2]	4.7 (4.2, 5.1)[2aa]	4.6 (4.2, 5.2)[2]	5.3 (4.8, 6.0)[2]	6.4 (5.7, 7.3)[2ddd]
genotype	[42]	[28]	[36]	[32]	[30]
p value	<0.001	0.069	0.002	0.006	0.050

Abbreviations: GW, gestational week; MTHFR, methylenetetrahydrofolate reductase; SLC19A1, solute carrier family 19 member 1 (reduced folate carrier). [1]Median (P_{10}, P_{90}). 2 Geometric mean (95% confidence interval). Geometric means were compared between time points using ANOVA for repeated measures and between genotypes using ANOVA (post hoc Bonferroni p value are shown). [*]P<0.05, [**]p<0.01 TT/GG compared to CC/GA genotype. [†]p<0.05, [††]p<0.01 TT/GG compared to CC/AA genotype. [ƒ]p<0.05 TT/GG compared to CT/GG genotype. [^^]p<0.01 TT/GG compared to CC/GG. [ǂ]p<0.05, [ǂǂ]p<0.05 TT/GG compaed to CT/GA genotype. [§]p<0.05 TT/GA compared to CC/GA genotype. [Φ]p<0.05, [ΦΦ]p<0.01 TT/GA compared to CC/AA genotype. [ᵒᵒ]P<0.05 TT/GA compared to CT/GG. [£]TT/GA compared to CC/GG genotype. [}]P<0.05 TT/GA compared to CT/GA genotype. [$]p<0.05, [$$$]p<0.001 CC/GG compared to CC/GA genotype. [#]p<0.05, [##]p<0.01 CC/GG compared to CC/AA genotype. [a]p<0.05, [aa]p<0.01, [aaa]p<0.001 compared with <12 GW, [b]p<0.05, [bb]p<0.01, [bbb]p <0.001 compared with 15GW, [c]p <0.05, [cc]p <0.01, [ccc]p <0.001 compared with 24-27GW, [d]p <0.05, [dd]p <0.01, [ddd]p <0.001 compared with 34GW.

There was a significant interaction between folic acid supplementation and plasma and red blood cell folate status according to *MTHFR* 677 C>T and *SLC19A1* 80G>A genotypes. People with the *MTHFR* 677 TT genotype had lower plasma folate concentrations than people with the CC and CT variants, even though they were taking folic acid supplements.

Folate and tHcy status according to genotypes in fathers and cords are reported in *Tables 8* and *9*. Paternal tHcy was higher in the *MTHFR* 677 TT genotype compared to CC and CT genotypes. It did not differ among the *SLC19A1* 80 G>A genotypes. THcy was highest for the *MTHFR* 677 TT *+ SLC19A1* 80 AA and *MTHFR* 677 TT *+ SLC19A1* 80 GA genotype combinations (*Table 8*). Cord tHcy also differs among maternal *MTHFR* 677 C>T genotypes, with the highest concentration for the TT genotype (*Table 9*).

Table 8. *Paternal one carbon metabolism status according to MTHFR 677C>T, SLC19A1 80 G>A and the combination of both genotypes.*

Genotype	Plasma folate (nmol/L)	Erythrocyte folate (nmol/L)	Plasma B12 (pmol/L)	Plasma homocysteine (µmol/L)
***MTHFR 677 C>T* genotype**				
CC	9.2 (4.5, 17.0)[1]	474.2 (192.5, 806.1)[1]	371.2 (291.5, 519.4)[1]	9.3 (7.3, 12.0)[1]
	8.9 (8.2, 9.7)[2] [136]	431.7 (393.7, 473.5)[2] [128]	379.9 (362.2, 398.6)[2] [136]	9.3 (9.0, 9.7)[2] [136]
CT	8.9 (4.8, 17.2)[1]	461.0 (233.8, 804.9)[1]	374.5 (273.2, 578.6)[1]	9.4 (7.5, 12.7)[1]
	9.1 (8.4, 9.7)[2] [189]	439.8 (409.9, 471.9)[2] [183]	385.5 (369.4, 402.2)[2] [188]	9.7 (9.4, 10.0)[2] [189]
TT	7.1 (4.1, 18.1)[1]	396.9 (146.0, 782.2)[1]	336.9 (248.2, 586.9)[1]	10.9 (8.0, 19.4)[1]
	7.8 (6.7, 9.1)[2] [53]	343.5 (290.0, 406.8)[2] *†† [50]	356.9 (328.9, 387.3)[2] [53]	12.3 (10.8, 13.9)[2]*** ††† [53]
p value	0.156	0.010	0.235	<0.001
***SLC19A1 80 G>A* genotype**				
GG	8.7 (4.7, 16.8)[1]	417.4 (174.4, 714.4)[1]	378.5 (268.2, 617.2)[1]	9.5 (7.6, 12.7)[1]
	8.8 (8.0, 9.7)[2] [104]	400.0 (360.8, 443.4)[2] [99]	394.9 (369.3, 422.4)[2] [103]	9.8 (9.4, 10.2)[2] [104]
GA	9.0 (4.2, 16.5)[1]	481.3 (213.9, 844.0)[1]	356.1 (283.5, 526.4)[1]	9.4 (7.4, 13.7)[1]
	8.7 (8.1, 9.5)[2] [162]	449.2 (413.2, 488.3)[2] [154]	368.9 (354.4, 384.0)[2] [162]	9.9 (9.4, 10.3)[2] [162]
AA	8.9 (4.6, 18.6)[1]	455.9 (185.5, 734.9)[1]	384.7 (261.4, 574.2)[1]	9.6 (7.5, 15.4)[1]
	9.0 (8.2, 9.9)[2] [109]	412.3 (373.2, 455.4)[2] [105]	381.8 (362.1, 402.7)[2] [109]	10.0 (9.5, 10.7)[2] [109]
p value	0.880	0.179	0.173	0.770

***Table 8**. Paternal one carbon metabolism status according to MTHFR 677 C>T, SLC19A1 80 G>A and the combination of both genotypes.*

Genotype	Plasma folate (nmol/L)	Erythrocyte folate (nmol/L)	Plasma B12 (pmol/L)	Plasma homocysteine (µmol/L)
***MTHFR 677 C>T + SLC19A1 80 G>A* genotype**				
CC-GG	8.6	463.9	349.0	8.5
	(4.4, 16.6)[1]	(227.4, 843.0)[1]	(291.5, 544.5)[1]	(7.1, 12.4)[1]
	8.6	431.1	388.7	9.1
	(7.3, 10.2)[2]	(362.2, 513.3)[2]	(346.6, 435.9)[2]	(8.4, 9.8)[2]
	[37]	[36]	[37]	[37]
CC-GA	9.1	396.2	378.7	9.3
	(4.2, 16.5)[1]	(167.6, 745.1)[1]	(296.5, 521.2)[1]	(7.4, 11.9)[1]
	8.7	415.8	378.0	9.5
	(7.6, 9.9)[2]	(358.5, 482.3)[2]	(355.4, 402.0)[2]	(8.9, 10.0)[2]
	[65]	[60]	[65]	[65]
CC-AA	9.6	534.2	379.5	9.6
	(4.9, 18.2)[1]	(225.3, 748.4)[1]	(276.4, 580.9)[1]	(6.9, 12.4)[1]
	9.6	469.1	376.1	9.4
	(8.2, 11.2)[2]	(400.2, 549.8)[2]	(342.5, 412.9)[2]	(8.7, 10.1)[2]
	[35]	[33]	[35]	[35]
CT-GG	8.8	377.7	391.1	9.9
	(5.0, 18.6)[1]	(183.4, 672.3)[1]	(261.9, 650.8)[1]	(8.1, 12.9)[1]
	9.0	412.7	405.7	10.2
	(7.8, 10.3)[2]	(361.5, 471.2)[2]	(367.3, 448.1)[2]	(9.7, 10.8)[2]
	[50]	[49]	[49]	[50]
CT-GA	9.5	486.0	342.6	9.4
	(4.3, 17.4)[1]	(247.1, 848.9)[1]	(272.5, 572.9)[1]	(7.4, 13.4)[1]
	9.1	480.8	363.9	9.6
	(8.2, 10.2)[2]	(430.8, 536.6.5)[2]	(342.6, 386.5)[2]	(9.1, 10.1)[2]
	[83]	[80]	[83]	[83]
CT-AA	8.7	464.4	401.6	9.0
	(5.2, 17.4)[1]	(224.0, 728.9)[1]	(309.8, 564.0)[1]	(7.4, 13.0)[1]
	9.1	427.1	406.1	9.4
	(7.9, 10.5)[2]	(374.4, 487.4)[2]	(378.5, 435.7)[2]	(8.8, 10.0)[2]
	[54]	[52]	[54]	[54]
TT-GG	7.8	307.6	364.0	9.3
	(4.3, 19.2)[1]	(134.2, 710.8)[1]	(232.6, 634.2)[1]	(6.9, 16.4)[1]
	8.5	295.5	378.3	10.0
	(6.4, 11.5)[2]	(205.8, 424.4)[2]	(316.0, 452.9)[2]	(8.6, 11.7)[2]
	[17]	[14]	[17]	[17]

***Table 8**. Paternal one carbon metabolism status according to MTHFR 677 C>T, SLC19A1 80 G>A and the combination of both genotypes.*

Genotype	Plasma folate (nmol/L)	Erythrocyte folate (nmol/L)	Plasma B12 (pmol/L)	Plasma homocysteine (µmol/L)
TT-GA	6.5 (3.7, 14.3)[1]	481.8 (248.5, 849.5)[1]	389.0 (282.1, 502.9)[1]	11.3 (9.2, 45.0)[1]
	7.1 (5.6, 9.0)[2] [16]	456.7 (357.1, 584.1)[2] [16]	367.3 (329.7, 409.1)[2] [16]	13.5 (10.3, 17.8)[2] $^{\phi\phi\phi}$ [16]
TT-AA	7.5 (2.9, 19.0)[1]	308.4 (116.4, 784.1)[1]	311.3 (248.2, 588.4)[1]	12.2 (7.8, 30.0)[1]
	7.7 (5.8, 10.4)[2] [20]	303.8 (228.1, 404.6)[2] §§ [20]	332.0 (287.3, 383.6)[2] [20]	13.5 (10.8, 16.8)[2] $^{\int\int\int}$ [20]
p value	0.647	0.006	0.142	<0.001

Abbreviations: *MTHFR, methylenetetrahydrofolate reductase; SLC19A1, solute carrier family 19 member 1 (reduced folate carrier).* [1] Median (P_{10}, P_{90}). [2] Geometric mean (95% confidence interval) [n]. Geometric means were compared between genotypes using ANOVA, with post hoc Bonferroni correction for multiple comparisons. *P<0.05, ***p<0.001 TT compared to CC genotype. ††p<0.01 †††p<0.001 TT compared to CT genotype. §§p<0.01 TT/AA compared to CT/GA genotype. $^{\int\int\int}$p<0.001 TT/AA compared to other genotypes except TT/GA. $^{\phi\phi\phi}$p<0.001 TT/GA compared to other genotypes except TT/AA.

Table 9. *Cord one carbon metabolism status according to MTHFR 677 C>T, SLC19A1 80 G>A and the combination of both genotypes.*

Genotype	Plasma folate (nmol/L)	Plasma B12 (pmol/L)	Plasma homocysteine (µmol/L)
***MTHFR 677 C>T* genotype**			
CC	23.5 (11.3, 53.5)[1]	322.4 (121.2, 677.7)[1]	4.5 (3.1, 6.6)[1]
	24.2 (22.2, 26.3)[2]	314.6 (284.1, 348.3)[2]	4.5 (4.3, 4.7)[2]
	[186]	[180]	[186]
CT	27.0 (13.1, 56.9)[1]	351.7 (138.9, 637.2)[1]	4.9 (3.3, 6.8)[1]
	26.6 (24.7, 28.5)[2]	326.9 (303.1, 352.5)[2]	4.8 (4.6, 5.0)[2]
	[238]	[231]	[238]
TT	28.1 (11.9, 55.6)[1]	336.1 (155.5, 640.8)[1]	5.4 (3.6, 7.8)[1]
	26.9 (24.0, 30.1)[2]	311.3 (273.2, 354.8)[2]	5.3 (4.9, 5.6)[2]***
	[97]	[93]	[97]
p value	0.168	0.754	0.001
***SLC19A1 80 G>A* genotype**			
GG	26.1 (12.7, 54.4)[1]	348.6 (185.1, 660.7)[1]	4.9 (3.3, 7.0)[1]
	25.7 (23.5, 28.1)[2]	329.1 (297.4, 364.3)[2]	4.9 (4.6, 5.2)[2]
	[134]	[132]	[134]
GA	26.1 (12.2, 57.8)[1]	356.5 (120.1, 621.5)[1]	4.7 (3.4, 6.5)[1]
	26.0 (24.1, 28.1)[2]	320.6 (294.0, 349.7)[2]	4.8 (4.6, 4.9)[2]
	[241]	[232]	[241]
AA	25.8 (11.7, 52.8)[1]	337.1 (125.7, 679.9)[1]	4.8 (3.0, 6.9)[1]
	25.4 (23.2, 27.8)[2]	315.1 (284.3, 349.3)[2]	4.7 (4.5, 5.0)[2]
	[142]	[136]	[142]
p value	0.913	0.852	0.633
***MTHFR 677 C>T* + *SLC19A1 80 G>A* genotype**			
CC-GG	27.0 (13.0, 58.5)[1]	334.4 (156.8, 660.3)[1]	4.8 (3.6, 6.6)[1]
	27.0 (23.2, 31.5)[2]	321.3 (264.2, 390.9)[2]	4.8 (4.4, 5.1)[2]
	[54]	[53]	[54]
CC-GA	23.2 (10.7, 47.3)[1]	351.2 (161.6, 723.2)[1]	4.4 (3.1, 6.6)[1]
	23.6 (20.8, 26.8)[2]	303.9 (258.1, 357.9)[2]	4.4 (4.1, 4.7)[2]
	[85]	[83]	[85]
CC-AA	20.5 (10.5, 53.3)[1]	351.2 (163.1, 731.7)[1]	4.3 (3.1, 6.5)[1]
	22.0 (18.2, 26.6)[2]	335.2 (283.5, 396.2)[2]	4.4 (4.0, 4.9)[2]
	[46]	[43]	[46]
CT-GG	24.7 (14.2, 46.1)[1]	344.8 (177.2, 557.4)[1]	5.1 (3.1, 7.4)[1]
	24.6 (21.8, 27.7)[2]	330.0 (290.8, 374.4)[2]	4.9 (4.5, 5.4)[2]
	[56]	[55]	[56]

***Table 9**. Cord one carbon metabolism status according to MTHFR 677 C>T, SLC19A1 80 G>A and the combination of both genotypes.*

Genotype	Plasma folate (nmol/L)	Plasma B12 (pmol/L)	Plasma homocysteine (µmol/L)
CT-GA	28.4 (12.6, 67.0)[1]	382.8 (133.8, 641.4)[1]	4.8 (3.6, 6.4)[1]
	28.3 (25.0, 32.0)[2]	337.0 (297.1, 382.3)[2]	4.8 (4.6, 5.1)[2]
	[107]	[103]	[107]
CT-AA	27.7 (13.2, 50.4)[1]	329.8 (123.4, 642.5)[1]	4.8 (2.9, 6.9)[1]
	26.0 (23.3, 29.1)[2]	317.2 (276.2, 364.2)[2]	4.7 (4.4, 5.2)[2]
	[71]	[69]	[71]
TT-GG	27.6 (8.9, 53.6)[1]	338.4 (161.1, 711.6)[1]	5.1 (3.7, 8.4)[1]
	25.5 (19.7, 33.1)[2]	345.0 (272.0, 437.5)[2]	5.1 (4.3, 6.0)[2]
	[24]	[24]	[24]
TT-GA	25.2 (11.8, 48.7)[1]	334.5 (134.7, 613.1)[1]	5.3 (3.5, 7.8)[1]
	25.4 (21.6, 30.0)[2]	310.6 (259.9, 371.0)[2] †	5.3 (4.8, 5.8)[2]
	[47]	[44]	[47]
TT-AA	30.2 (15.3, 65.7)[1]	327.0 (55.0, 722.0)[1]	5.7 (3.0, 8.1)[1]
	30.7 (24.8, 38.0)[2]	277.1 (198.7, 386.4)[2]	5.4 (4.7, 6.1)[2]
	[25]	[24]	[25]
p value	0.180	0.917	0.025

Abbreviations: *MTHFR, methylenetetrahydrofolate reductase; SLC19A1, solute carrier family 19 member 1.* [1] Median (P_{10}, P_{90}). [2] Geometric mean (95% confidence interval) [n]. Geometric means were compared between genotypes using ANOVA, with post hoc Bonferroni correction for multiple comparisons. ***p<0.001 TT compared to CC genotype. †p<0.05 TT/GA compared to CC/GA genotype.

The prevalences of the pregnancy outcomes investigated in the study are reported in *Table 10*.

Table 10. Prevalences of *the pregnancy outcomes included in the study.*[1]

Outcomes	%	N
Pathological Doppler of uterine artery flow at 20GW[2]	19.2 (16.3, 22.5)	117/609
Gestational Hypertension[3]	11.8 (9.5, 14.6)	75/635
Intrauterine Growth Retardation[4]	8.8 (6.9, 11.3)	56/726

[1] All data shown are percentages (95% confidence interval).
[2] Defined as the mean of pulsatility index of right and left uterine arteries ≥P95 or the presence of a bilateral notch in the uterine artery waveforms.
[3] Defined as systolic blood pressure ≥140 mmHg and diastolic blood pressure ≥90 mmHg.
[4] According to the Spanish Obstetrics and Gynaecology guidelines.

In *Table 11* the prevalence of pregnancy outcomes studied (pathological doppler, gestational hypertension and IUGR) according to maternal and paternal *MTHFR* 677 C>T and *SLC19A1* 80 G>A genotypes and the combination of both are reported. Regarding the *MTHFR* 677 C>T genotype alone, there was no difference in prevalence of any of the adverse outcomes among the maternal or paternal genotypes. In the case of the *SLC19A1* 80 G>A genotype, intrauterine growth retardation was more prevalent in the paternal homozygote variant genotype (AA). No differences in prevalence in any of the outcomes was observed among the maternal genotypes and the combination of both paternal genotypes.

Table 11. *Pregnancy outcomes prevalence according to maternal and paternal MTHFR 677 C>T, SLC19A1 80 G>A and the combination of both genotypes[1].*

	Pathological Doppler[2]		Gestational hypertension[3]		IUGR[4]	
	Maternal	**Paternal**	**Maternal**	**Paternal**	**Maternal**	**Paternal**
***MTHFR* 677 C>T genotype**						
CC	21.1 (16.0, 27.3) [42/199]	15.2 (10.0, 22.2) [20/132]	12.8 (8.9, 18.1) [26/203]	8.9 (5.2, 14.9) [12/135]	8.2 (5.3, 12.4) [19/233]	8.6 (5.0, 14.5) [12/139]
CT	20.7 (16.3, 26.0) [56/270]	18.8 (13.8, 25.1) [34/181]	30.1 (22.1, 39.5) [31/308]	12.8 (8.6, 18.6) [22/172]	7.0 (4.8, 10.2) [24/342]	5.2 (2.9, 9.4) [10/191]
TT	17.5 (11.3, 25.9) [18/103]	24.5 (14.6, 38.1) [12/49]	12.8 (7.5, 21.0) [12/94]	12.0 (5.6, 23.8) [6/50]	10.4 (6.1, 17.4) [12/115]	9.3 (4.0, 19.9) [5/54]
***SLC19A1* 80 G>A genotype**						
GG	20.1 (14.6, 27.2) [31/154]	13.0 (7.8, 21.0) [13/100]	11.8 (7.7, 17.7) [19/161]	7.8 (4.0, 14.7) [8/102]	8.1 (4.9, 12.9) [15/186]	6.6 (3.2, 13.0) [7/106]
GA	19.6 (15.3, 24.8) [52/265]	18.0 (12.7, 24.9) [27/150]	10.1 (7.1, 14.1) [29/287]	10.1 (6.2, 16.0) [15/148]	7.9 (5.4, 11.4) [25/317]	3.1 (1.3, 7.0) [5/163]
AA	21.2 (15.4, 28.4) [32/151]	21.8 (15.1, 30.4) [24/110]	13.5 (9.0, 19.7) [21/156]	16.3 (10.5, 24.6) [17/104]	8.2 (5.0, 13.0) [15/184]	13.4 (8.3, 20.9)** [15/112]

	Pathological Doppler[2]		Gestational hypertension[3]		IUGR[4]	
	Maternal	**Paternal**	**Maternal**	**Paternal**	**Maternal**	**Paternal**
***MTHFR* 677 C>T + *SLC19A1* 80 G>A genotype**						
CC-GG	13.1 (6.8, 23.8) [8/61]	11.1 (4.4, 25.3) [4/36]	10.6 (5.2, 20.3) [7/66]	5.6 (1.5, 18.1) [2/36]	5.5 (2.2, 13.3) [4/73]	10.5 (4.2, 24.1) [4/38]
CC-GA	24.4 (16.7, 34.2) [22/90]	17.7 (10.2, 29.0) [11/62]	11.0 (6.1, 19.1) [10/91]	9.2 (4.3, 18.7) [6/65]	6.8 (3.3, 13.4) [7/103]	4.5 (1.5, 12.4) [3/67]
CC-AA	25.0 (14.9, 38.8) [12/48]	14.3 (6.3, 29.4) [5/35]	19.6 (10.7, 33.2) [9/46]	11.4 (4.5, 26.0) [4/35]	14.0 (7.3, 25.3) [8/57]	14.3 (6.3, 29.4) [5/35]
CT-GG	25.0 (16.0, 36.8) [16/64]	16.3 (8.5, 29.0) [8/49]	12.7 (6.8, 22.4) [9/71]	10.4 (4.5, 22.2) [5/48]	7.5 (3.5, 15.4) [6/80]	4.0 (1.1, 13.5) [2/50]
CT-GA	19.1 (13.4, 26.5) [26/136]	15.8 (9.3, 25.6) [12/76]	8.3 (4.9, 13.7) [13/156]	11.3 (5.8, 20.7) [8/71]	8.2 (4.9, 13.3) [14/171]	1.2 (0.2, 6.6) [1/82]
CT-AA	19.1 (11.5, 30.0) [13/68]	21.8 (12.9, 34.4) [12/55]	11.3 (6.0, 20.0) [9/80]	17.6 (9.6, 30.3) [9/51]	4.5 (1.8, 11.1) [4/88]	12.3 (6.1, 23.2) [7/57]
TT-GG	24.1 (12.2, 42.1) [7/29]	6.7 (1.2, 29.8) [1/15]	12.5 (4.3, 31.0) [3/24]	5.6 (1.0, 25.8) [1/18]	15.2 (6.7, 30.9) [5/33]	5.6 (1.0, 25.8) [1/18]
TT-GA	10.3 (4.1, 23.6) [4/39]	28.6 (11.7, 54.6) [4/14]	15.0 (7.1, 29.1) [6/40]	7.1 (1.3, 31.5) [1/14]	9.3 (3.7, 21.6) [4/43]	6.3 (1.1, 28.3) [1/16]
TT-AA	20.0 (10.0, 35.9) [7/35]	35.0 (18.1, 56.7) [7/20]	10.0 (3.5, 25.6) [3/30]	22.2 (9.0, 45.2) [4/18]	7.7 (2.7, 20.3) [3/39]	15.0 (5.2, 36.0) [3/20]

Abbreviations: *MTHFR, methylenetetrahydrofolate reductase; SLC19A1, solute carrier family 19 member 1.* [1]All data shown are percentages (95% confidence interval). [2] Defined as the mean of pulsatility index of right and left uterine arteries ≥P95 or the presence of a bilateral notch. [3] Defined as systolic blood pressure ≥140 mmHg and diastolic blood pressure ≥90 mmHg. [4] According to the Spanish Obstetrics and Gynaecology guidelines. Prevalence of the outcomes among the genotypes for each polymorphism were compared by the chi-square test. **P<0.01 TT compared to CC genotype.

4.2. One carbon metabolism and uterine artery pulsatility index

Maternal *MTHFR* 677C>T genotype, uterine artery resistance (pulsatility index) and impaired placentation (pathological doppler measurements of uterine artery flow and waveforms).

We investigated the association between maternal *MTHFR* 677C>T genotype and pulsatility index of the uterine arteries at 20 GW (dependent variable natural log transformed mean of the right and left uterine artery pulsatility index) using multiple linear regression analysis. The models included *SLC19A1* 80G>A genotype, and red blood cell folate and plasma cobalamin concentrations (at <12 GW and 15 GW, in separate models). They were further adjusted for smoking habit during pregnancy, socioeconomic status, age, 1st trimester BMI, parity and gestational age in weeks and days at the time of the doppler measurement. None of the models were significant (data not shown). Impaired placentation is characterised as pathological when the mean uterine artery pulsatility index is elevated (≥P95) and/ or by the presence of bilateral notches in the uterine artery waveforms analysed by Doppler echography. Analysis of the association between maternal *MTHFR* genotype and pathological placentation using multiple logistic regression analysis in which the dependent variable was pathological Doppler outcome, and adjusting the models for the same confounding factors as described for uterine artery pulsatility index, also showed that there was no association.

Maternal tHcy status and pulsatility index of uterine arteries

We studied the association between early pregnancy tHcy at <12 and 15 GW, in separate models) and pulsatility index of uterine arteries using multiple linear regression analysis. The dependent variable was natural log transformed mean right

and left uterine artery pulsatility index. THcy was included in the models and they were adjusted for smoking during the first trimester only versus never, smoking throughout pregnancy versus never, mid and high versus low socioeconomic status, maternal age, 1st trimester BMI, parity (previous pregnancy <20 GW versus none) and gestational weeks at doppler measurement. The models were not significant. We repeated the analysis using the variable elevated tHcy status during early pregnancy (≥P90). While the model was not significant, the association between tHcy ≥P90 and pulsatility index was positive with a beta coefficient of 0.075 (P= 0.059). We then investigated whether elevated early pregnancy tHcy was associated with impaired placentation (pathological measurement by Doppler echography at 20 GW) using multiple logistic regression analysis. The categorical tHcy variable (≥P90 tHcy in early pregnancy versus <P90) replaced the genotypes, red blood cell folate and plasma cobalamin variables described in the models in the previous section at <12 and at 15 GW. The models were adjusted for smoking habit, socioeconomic status, BMI, age, parity and gestational age at doppler measurement. There was no association between elevated early pregnancy tHcy status and impaired placentation.

Paternal genotype and uterine artery pulsatility index

The association between paternal *MTHFR* 677C>T genotype and uterine artery pulsatility index was studied using multiple linear regression analysis. We performed the same maternal models, as at <12 and 15 GW, adding paternal *MTHFR* 677C>T, red blood cell folate concentration, age and smoking habit. The results are shown in *Table 12*.

***Table 12**. Predictors (Beta coefficients) of pulsatility index of uterine arteries according to paternal MTHFR 677 C>T genotype.*

		< 12 GW	**15 GW**
Model 1 (maternal, non adjusted)	Model, R^2, n	0.004, 310	-0.010, 186
	Maternal *MTHFR* 677TT genotype	0.027	-0.102
Model 2 (maternal, adjusted)	Model, R^2, n	0.024, 310$^{\#}$	0.068, 186*
	Maternal *MTHFR* 677TT genotype	0.011	-0.083
	Maternal smoking throughout pregnancy vs never	-0.088	-0.154*
	Maternal BMI	0.005	0.207**
Model 3 (maternal adjusted and paternal factors)	Model, R^2, n	0.057, 310**	0.149, 186***
	Maternal *MTHFR* 677TT genotype	0.007	-0.088
	Maternal smoking throughout pregnancy vs never	-0.130*	-0.168*
	Maternal BMI	-0.011	0.179*
	Paternal *MTHFR* 677CT genotype	0.161**	0.205**
	Paternal *MTHFR* 677TT genotype	0.131*	0.226**
	Paternal age	0.126	0.084
	Paternal smoking	0.109†	0.167*

Abbreviations: *MTHFR, methylenetetrahydrofolate reductase; BMI, body mass index; GW, gestational weeks. E*xcluded 2 outliers. Multiple linear regression analysis. Dependent variable, natural log transformed mean of the uterine artery pulsatility index of uterine arteries (PI). Independent variable for Model 1: maternal *MTHFR* 677 CT and TT versus *MTHFR* 677 CC *genotype*, maternal *SLC19A1* 80 GA and AA versus 80 GG genotype, low tertile red blood cell folate (<= 724.33 nmol/L) and low tertile plasma cobalamin (<= 315.45 pmol/L) concentration at the corresponding time points of pregnancy for each model. Model 2: same variables as model 1 as well as smoking during the first trimester and throughout pregnancy versus never, mid and high versus low socioeconomic status, maternal age (y), 1st trimester BMI and parity (previous pregnancy versus none). Model 3 (paternal factors): same variables as model 2 as well as paternal *MTHFR* 677 CT and TT versus *MTHFR* 677CC genotype, red blood cell folate levels, age and smoking. *p<0.05, **p<0.01. $^{\#}$P= 0.086, †P= 0.067

The results show a positive association between paternal *MTHFR* 677 CT and TT vs CC genotypes and pulsatility index of uterine arteries in all models. Paternal smoking habit was also positively associated with uterine artery pulsatility index at 20 GW. As we observed before, maternal genotype was not associated. Maternal BMI was positively associated and smoking throughout pregnancy versus never was negatively associated with uterine artery pulsatility index.

Paternal tHcy status and uterine artery pulsatility index

The association between paternal tHcy and mean left and right uterine artery pulsatility index was investigated using multiple linear regression analysis. The dependent variable was the natural log transformed mean pulsatility index. We built on the maternal models by adding paternal tHcy concentrations, age and smoking habit. Paternal tHcy was positively associated with pulsatility index at <12 and 15 GW. There was no association between maternal tHcy and pulsatility index.

When we repeated the analysis including elevated tHcy (≥P90 versus <P90) at <12 GW and elevated paternal tHcy , the model was also significant (n= 364, Adjusted R^2 =0.044, P= 0.005) and the results indicate a positive association between elevated paternal tHcy and increased uterine artery pulsatility index (paternal tHcy ≥P90 Beta coefficient= 0.151, P= 0.004). Paternal smoking compared to nonsmoking was also associated with increased uterine artery pulsatility index (Beta coefficient= 0.102, P= 0.060).

Paternal genotype and pathological Doppler measurement of uterine arteries at 20 GW

We studied the association between paternal *MTHFR* 677C>T genotype and risk of pathological Doppler measurement of uterine arteries at 20 GW using multiple logistic regression analysis. We added the paternal factors *MTHFR* 677C>T genotype, age, smoking habit and red blood cell folate concentration to the same models used for the mothers, at <12 and 15 GW. The first model was not significant. Results from the model at 15 GW are shown.

***Table 13**. Associations between paternal MTHFR 677C>T genotype and pathological Doppler measurement of the uterine arteries at 20 GW.*

	N	R^2	Maternal TT vs CC	Paternal CT vs CC	Paternal TT vs CC	Paternal smoking
Model 1 (maternal, non adjusted)	191	0.130*	0	-	-	-
Model 2 (maternal, adjusted)	191	0.178#	0	-	-	-
Model 3 (maternal adjusted and paternal factors)	192	0.358***	0	4.0 (1.3, 12.6)	7.1 (1.6, 32.8)	3.2 (1.3, 7.9)

Abbreviations: *MTHFR*, *methylenetetrahydrofolate reductase*. Multiple logistic regression analysis was used. Nagelkerke R^2; OR (95% CI) for pathological Doppler measurement of the uterine arteries at 20 GW in mothers with *MTHFR* 677 CC vs CT and *MTHFR* 677 CC vs TT genotype are shown. Model 1: (basic model) comparing *MTHFR* 677 CC vs CT and *MTHFR* 677 CC vs TT and *SLC19A1* 80GG vs GA and *SLC19A1* 80GG vs AA maternal genotypes. Model 2: Included the same variables as model 1 as well as lifestyle factors such as parity (previous pregnancy versus none), maternal age (y), 1st trimester BMI, smoking during pregnancy, mid and high versus low socioeconomic status, gestational age at doppler measurement, low tertile red blood cell folate (<= 724.33 nmol/L) and low tertile plasma cobalamin (<= 315.45 pmol/L) concentration at 15 GW. Model 3: Included the same variables as model 2 as well as paternal *MTHFR* 677 CC vs CT and *MTHFR* 677 CC vs TT genotype, paternal red blood cell levels, age and paternal smoking habit. *p<0.05, **p<0.01. #P=0.051.

There is a clear association between paternal factors and increased risk of pathological Doppler assessment of the uterine arteries at 20 GW. Paternal *MTHFR* 677 CT and TT were associated with four and seven times, respectively, more risk of pregnancy affected by impaired placentation compared to fathers with the *MTHFR* 677 CC genotype. Paternal smoking also conveyed a threefold greater risk. Paternal red blood cell folate had a small but significant protective effect against impaired placentation OR (95% CI) 0.99 (0.99, 0.99).

Paternal tHcy status and pathological Doppler measurement of the uterine arteries at 20 GW

We investigated whether elevated paternal tHcy status is associated with risk of pathological Doppler measurement of the uterine arteries at 20 GW. The categorical maternal tHcy variable (≥P90 tHcy in early pregnancy versus <P90) replaced genotypes, red blood cell folate and plasma cobalamin variables in each model, described in the previous section for <12 and 15 GW. We included elevated paternal tHcy status (≥P90) and smoking habit in both models. The results are shown below in *Table 14*.

***Table 14**. Associations between paternal tHcy levels and pathological Doppler measurement of the uterine arteries at 20 GW.*

	N	R^2	Maternal tHcy ≥ P90	Paternal tHcy ≥ P90	Paternal smoking
Model 1 (maternal, non adjusted)	370	0.001	1.3 (0.6, 2.9)	-	-
Model 2 (maternal, adjusted)	370	0.045	1.3 (0.5, 3.0)	-	-
Model 3 (maternal adjusted and paternal factors)	370	0.089*	1.3 (0.5, 3.1)	2.7 (1.2, 6.0)	1.9 (1.0, 3.3)

Abbreviations: *tHcy*, *plasma total homocysteine.* Multiple logistic regression analysis was used. Nagelkerke R^2; OR (95% CI) for pathological Doppler measurement of the uterine arteries at 20 GW in mothers with tHcy levels ≥ P90 at early pregnancy versus other percentiles. P90 tHcy cut offs (µmol/L) were <12GW 7.11, 15 GW 5.85, 34 GW 7.25, labour 8.52. Model 1: (basic model) comparing tHcy levels ≥P90 at early pregnancy versus other percentiles. Model 2: Included the same variables as model 1 as well as lifestyle factors such as parity (previous pregnancy versus none), maternal age (y), 1st trimester BMI, smoking during pregnancy, mid and high versus low socioeconomic status, and gestational age (w) at doppler measurement. Model 3: Included the same variables as model 2 as well as paternal tHcy levels ≥P90 (≥ 14.1 µmol/L). *p<0.05, **p<0.01.

Elevated paternal homocysteine levels and smoking habit were associated with increased risk of pathological Doppler measurement of uterine arteries at 20 GW. Maternal tHcy was not associated.

4.3. One carbon metabolism and birthweight

Association between maternal genotype and birthweight

We investigated the association between maternal *MTHFR* 677C>T genotype and birthweight using multiple linear regression analysis. 3 models were designed to adjust for maternal folate and cobalamin status at different times of pregnancy (first trimester, mid-pregnancy, late pregnancy). We included the interaction term *MTHFR* 677C>T genotype * smoking habit during pregnancy in the model. This was significant in the 1st trimester (P=0.024). Therefore, we proceeded to carry out stratified analyses according to smoking habit (never smoking or smoking at some point of pregnancy). We excluded participants with gestational diabetes from these analyses. The results are shown in *Table 15*.

***Table 15**. Association between maternal genotype and birthweight, according to smoking habit during pregnancy (Beta coefficients).*

		1st trimester	34 GW	Labour
Non smokers				
Basic model	Model, R^2, *n*	0.001, 427	-0.005, 388	-0.002, 344
	MTHFR 677TT genotype	0.0004	0.042	0.017
	SLC19A1 80GA genotype	-0.051	0.005	-0.00003
	Low tertile RBCF	0.095†	-0.050	-0.028
Complete model	Model, R^2, *n*	0.298,427***	0.283, 388***	0.271, 344***
	MTHFR 677TT genotype	-0.018	0.025	0.026
	SLC19A1 80GA genotype	0.022	0.016	0.020
	Low tertile RBCF	0.064	-0.081§	-0.069
Smokers at some point of pregnancy				
Basic model	Model, R^2, *n*	0.060, 161*	0.010,146	-0.017,123
	MTHFR 677TT genotype	0.083	0.053	0.040
	SLC19A1 80GA genotype	0.169‡	0.210*	-0.053
	Low tertile RBCF	-0.229**	-0.024	-0.020
Complete model	Model, R^2, *n*	0.350, 161***	0.261, 146***	0.282, 123***
	MTHFR 677TT genotype	0.186*	0.161¶	0.076
	SLC19A1 80GA genotype	0.102	0.161¥	0.003
	Low tertile RBCF	-0.175*	0.125	-0.069

Abbreviations: *MTHFR*, *methylenetetrahydrofolate reductase*; *SLC19A1, solute carrier family 19 member 1; RBCF*, *red blood cell folate; GW, gestational weeks*. Birth weight was the dependent variable in all models. Basic model: *MTHFR* 677CT versus 677CC genotype, *MTHFR* 677TT versus 677CC genotype, *SLC19A1* 80GA versus 80GG genotype, *SLC19A1* 80AA versus 80GG genotype, low tertile RBCF: ≤708 nmol/L at <12 GW and ≤761 nmol/L at 34 GW, low tertile B12: ≤ 315 pmol/L at <12 GW, ≤216 pmol/L at 34 GW and ≤199 pmol/L at labour concentrations versus the middle and high. Complete model: Smoking during the 1st trimester versus never, smoking throughout pregnancy versus never, maternal age (y), 1st trimester BMI, 3rd trimester haemoglobin <11 g/dL, parity (previous pregnancy versus none), gestational weeks at birth (w), foetal sex. †P=0.056, ‡P=0.080; §P=0.068; ¶P=0.054, ¥P=0.079.

The early pregnancy model showed that the *MTHFR* 677TT genotype was not associated with birth weight in non smokers but was positively associated with birth weight in smokers. A positive association between the *SLC19A1* 80GA genotype and birth weight was also observed in the basic model but weakened in the adjusted model. Low tertile red blood cell folate concentration was inversely associated with birthweight in smokers only. Both genotype – birth weight associations were confirmed in the mid-pregnancy model.

Maternal red blood cell folate status and cord plasma folate status according to maternal smoking habit and genotype are shown in *Table 16*. Red blood cell folate status was lower in smokers at <12, 24-27 and 34 GW than in non smokers. Red blood cell folate status was lower in non-smoking mothers with the *MTHFR* 677TT genotype compared to those with the CC and CT genotype at <12, 24-27 and 34 GW. However, there was no difference in red blood cell folate status between genotypes in smoking mothers. Cord folate status did not differ among genotypes in any of the mothers or between smokers and non smokers.

***Table 16**. Maternal red blood cell folate and cord plasma folate status according to smoking habit and MTHFR 677 C>T genotype.*

	<12 GW	15 GW	24-27 GW	34 GW	Cord
Non smokers	981 (936, 1027) [546]	1283 (1213, 1357) [313]	1184 (1131, 1241) [475]	1054 (960, 1158) [457]	26.2 (24.7, 27.7) [428]
CC	1047 (970, 1131) [167]	1311 (1311, 1311) [96]	1251 (1159, 1350) [150]	1028 (957, 1103) [135]	25.8 (25.7, 25.9) [133]
CT	979 (979, 979) [257]	1312 (1312, 1312) [155]	1169 (1168, 1169) [224]	1029 (1029, 1029) [220]	24.7 (24.6, 24.8) [208]
TT	880† (773, 1001) [85]	1245 (1070, 1449) [59]	1030† (905, 1173) [74]	853$^{\dagger\int}$ (732, 995) [72]	27.6 (27.5, 27.8) [69]
Smokers	853** (788, 924) [191]	1165 (1074, 1265) [133]	1057** (981, 1141) [176]	853*** (783, 929) [159]	25.4 (23.3, 27.8) [149]
CC	917 (916, 917) [67]	1231 (1231, 1231) [50]	1092 (1092, 1092) [62]	873 (873, 873) [59]	24.4 (24.2, 24.5) [58]
CT	822 (822, 822) [85]	1156 (1155, 1156) [59]	1038.1 (1037.9, 1038.2) [77]	864 (864, 864) [71]	25.2 (25.1, 25.4) [65]
TT	787 (787, 787) [34]	1061 (1061, 1061) [24]	994 (994, 994) [33]	789 (788, 789) [29]	28.0 (27.8, 28.1) [25]

Abbreviations: *MTHFR, methylenetetrahydrofolate reductase; GW, Gestational weeks*. Geometric mean (95% confidence interval) [n]. Geometric means were compared between groups (non smokers versus smokers) and between genotypes using ANOVA, with post hoc Bonferroni correction for multiple comparisons. *P<0.05, **p<0.01 ***p<0.001 smokers compared to non smokers. †p<0.05, ††p<0.01 TT compared to CC genotype. $^{\int}$p<0.05, TT compared to CT genotype.

Maternal tHcy levels predicting birthweight

Applying the same stratification for smoking during pregnancy as in the *MTHFR* 677C>T models, we also studied the association between early and late pregnancy tHcy and birthweight, using multiple linear regression analysis. For early pregnancy we tested the association between maternal tHcy ≥P90 (7.1 µmol/L at <12 GW or 5.9 µmol/L at 15 GW) versus <P90 with birthweight. In late pregnancy at (34 GW) and at labour we tested tHcy as a continuous variable. The tHcy variables replaced the genotypes and red blood cell folate and plasma cobalamin variables in each model. All the models were significant and the adjusted R^2 ranged from 0.288 to 0.448. The results were confirmed when limited to the pregnancies in which the father participated in the study. These results are reported together with the fathers in the section on paternal tHcy and birth weight below (*Table 18)*. In non-smokers, late pregnancy tHcy was positively associated with birthweight.

Paternal genotype predicting birthweight

We then investigated the association between paternal *MTHFR* 677C>T genotype and birthweight building on the same multiple linear regression model shown for the mothers by adding a final model including paternal *MTHFR* 677C>T genotype, plasma cobalamin and red blood cell folate concentrations. The results are shown in *Table 17*.

***Table 17**. Predictors, including paternal factors, of birth weight according to maternal smoking habit.*

		1st trimester	34 GW	Labour
Non smokers				
Adjusted model (maternal factors)	Model, R^2, *n*	0.230, 236***	0.268, 214***	0.237, 194***
	Maternal *MTHFR* 677TT genotype	-0.011	0.061	-0.070
	Maternal *SLC19A1* 80GA genotype	-0.074	-0.028	-0.028
	Maternal low tertile RBCF	0.070	-0.080	-0.058
	Maternal low tertile B12	0.003	0.111‡	0.109
Complete model (paternal factors)	Model, R^2, *n*	0.238, 236***	0.264, 214***	0.228, 194***
	Maternal *MTHFR* 677TT genotype	-0.023	0.059	-0.075
	Maternal *SLC19A1* 80GA	-0.079	-0.038	-0.036
	Maternal low tertile RBCF	0.080	-0.0075	-0.064
	Maternal low tertile B12	0.005	0.106	0.107
	Paternal *MTHFR* 677TT genotype	0.039	0.048	0.043
	Paternal RBCF	0.114	0.050	0.004
	Paternal plasma B12	0.025†	-0.012	-0.023
Smokers				
Adjusted model (maternal factors)	Model, R^2, *n*	0.422, 66**	0.402, 60***	0.429, 51**
	Maternal *MTHFR* 677TT genotype	0.185	0.163	0.118

***Table 17**. Predictors, including paternal factors, of birth weight according to maternal smoking habit.*

		1st trimester	34 GW	Labour
Complete model (paternal factors)	Maternal *SLC19A1* 80GA genotype	0.093	0.240	-0.019
	Maternal low tertile RBCF	-0.171	-0.004	-0.040
	Maternal low tertile B12	0.037	0.067	0.085
	Model, R^2, *n*	0.418, 66**	0.398, 60**	0.457, 51***
	Maternal *MTHFR* 677TT genotype	0.213	0.221	0.201
	Maternal *SLC19A1* 80GA	0.083	0.252	0.002
	Maternal low tertile RBCF	-0.172	-0.012	-0.052
	Maternal low tertile B12	0.035	0.268	0.169
	Paternal *MTHFR* 677TT genotype	-0.059	0.0003	-0.083
	Paternal RBCF	0.010	0.025	-0.069
	Paternal plasma B12	0.203†	0.241#	0.307*

Abbreviations: *MTHFR*, *methylenetetrahydrofolate reductase*; *SLC19A1, solute carrier family 19 member 1; RBCF,* red blood cell folate; *GW, gestational weeks*. Birth weight was the dependent variable in all models. Basic model: *MTHFR* 677CT versus 677CC genotype, *MTHFR* 677TT versus 677CC genotype, *SLC19A1* 80GA versus 80GG genotype, *SLC19A1* 80AA versus 80GG genotype, low tertile red blood cell folate (<= 724.33 nmol/L) and low tertile plasma cobalamin (<= 315.45 pmol/L) concentrations versus the middle and high tertiles at the corresponding time points of pregnancy for each model. Second model: Smoking during the 1st trimester versus never, smoking throughout pregnancy versus never, maternal age (y), 1st trimester BMI, 3rd trimester haemoglobin <11 g/dL, parity (previous pregnancy versus none), gestational weeks at birth (w), foetal sex. Complete model (paternal factors): paternal *MTHFR* 677CT versus 677CC genotype, *MTHFR* 677TT versus 677CC genotype, paternal cobalamin and red blood cell folate status. Beta coefficient values are shown. *p<0.05, **p>0.01, ***p<0.001. †p=0.073, ‡p=0.074, #p=0.063.

Paternal cobalamin status was positively associated with birth weight. Paternal *MTHFR* 677 C>T genotype and red blood cell folate concentration were not associated with birth weight.

Paternal tHcy levels predicting birthweight

Continuing with the same analysis that we performed with the mothers, we also studied the association between elevated paternal tHcy (≥P90, 14.1 µmol/L, versus <P90) and birthweight using multiple linear regression analysis. We also applied the same stratification for smoking during pregnancy as in the *MTHFR* 677C>T models. The tHcy variable replaced the genotypes and red blood cell folate and plasma cobalamin variables in each model. Results are shown in *Table 18*.

***Table 18**. The associations between maternal and paternal tHcy and birth weight, according to maternal smoking habit.*

		1st trimester	34 GW	Labour
Non smokers				
Adjusted model (maternal factors)	Model, R^2, *n*	0.255, 289***	0.295, 263***	0.240, 274***
	Maternal tHcy	-0.057	0.193***	0.129*
Complete model (paternal factors)	Model, R^2, *n*	0.254, 289***	0.297, 263***	0.271, 263***
	Maternal tHcy	-0.054	0.193***	0.129*
	Paternal tHcy	0.043	0.067	-0.006
Smokers				
Basic model (maternal factors)	Model, R^2, *n*	0.399, 75***	0.367, 65***	0.425, 67***
	Maternal tHcy	-0.025	-0.040	-0.040
Complete model (paternal factors)	Model, R^2, *n*	0.444, 75***	0.443, 69***	0.450, 67***
	Maternal tHcy	-0.047	-0.033	-0.039
	Paternal tHcy	-0.234*	-0.295**	0.010

Abbreviations: *tHcy, plasma total homocysteine; GW, gestational weeks.* Multiple linear regression analysis was performed with birth weight as the dependent variable in all models. Basic model: maternal tHcy at each corresponding time point of pregnancy (early pregnancy, P90: <12GW ≥ 7.11 µmol/L or 15 GW ≥ 5.85 µmol/L 34 GW and labour, direct tHcy concentrations). Second model: same as basic model + adjusting covariables including mid and high versus low socioeconomic status, maternal age (y), 1st trimester BMI, 3rd trimester haemoglobin <11 g/dL, parity (previous pregnancy versus none), gestational weeks at birth (w), foetal sex. Complete model (paternal factors): same as second model + paternal tHcy ≥P90 (≥ 14.1 µmol/L). Beta coefficient values are shown. *p<0.05, **p>0.01, ***p<0.001.

Paternal tHcy concentrations were inversely associated with birthweight in smoking mothers only, both in the early pregnancy and 34 GW models.

Maternal tHcy status during pregnancy and probability of intrauterine growth retardation

We investigated tHcy status during pregnancy as a predictor of IUGR, using multiple logistic regression analyses. First, we tested the categorical tHcy variable (≥ P90 tHcy in early pregnancy versus < P90), as a potential biomarker for IUGR, in models adjusted for lifestyle factors such as parity, maternal age, BMI, smoking habit, socioeconomic status, anaemia and gestational diabetes. Because folic acid supplement use was highest during the first trimester, maternal tHcy category may have changed as pregnancy progressed. Therefore, we repeated the same analysis with two different variables to account for changes in tHcy category (≥P90 tHcy versus tHcy <P90 in early pregnancy or ≥P90 tHcy at some point of pregnancy versus tHcy always <P90). Results are shown in *Table 19*.

***Table 19**. Associations between elevated maternal plasma homocysteine and intrauterine growth retardation*.

	N	R^2	Plasma tHcy	BMI	Smoking throughout pregnancy vs never
tHcy ≥P90 vs tHcy <P90 in early pregnancy					
Model 1 (non adjusted)	590	0.003	0.6 (0.2, 2.1)	-	-
Model 2 (adjusted)	590	0.091**	0.6 (0.2, 2.0)	0.9 (0.8, 0.9)	2.5 (1.2, 5.0)
tHcy ≥P90 at some point of pregnancy versus low tHcy <P90					
Model 1 (non adjusted)	586	0.002	1.3 (0.6, 2.6)	-	-
Model 2 (adjusted)	586	0.094**	1.2 (0.6, 2.5)	0.9 (0.8, 0.9)	2.5 (1.2, 5.0)

Abbreviations: *BMI, Body Mass Index; tHcy, plasma total homocysteine.* Multiple logistic regression analysis was used. Nagelkerke R^2; OR (95% CI) for IUGR. Mothers with high plasma homocysteine levels at early pregnancy and at some point of pregnancy versus low tHcy are shown. P90 tHcy cut offs (μmol/L) were <12 GW 7.11, 15 GW 5.85, 34 GW 7.25, labour 8.52. Model 1: (basic model) comparing ≥P90 tHcy maternal levels at first extraction versus other percentiles or comparing ≥P90 tHcy maternal levels at some point of pregnancy vs always <P90 tHcy. Model 2: Included the same variables as model 1 as well as parity (previous pregnancy ≥ 20 GW versus none), maternal age (y), 1st trimester BMI, smoking during pregnancy, mid and high versus low socioeconomic status, 3rd trimester haemoglobin <11 g/dL and gestational diabetes. *p<0.05, **p<0.01.

The results showed no association between plasma homocysteine and IUGR at early or at some other point of pregnancy. However, we observed a tendency, but not significant, for a higher probability of developing IUGR when tHcy is elevated at some point of pregnancy compared with always low (<P90). There was a clear association between smoking throughout pregnancy and increased risk of IUGR.

We then assessed plasma folate and red blood cell folate status according to tHcy status divided in four categories at different pregnancy points; tHcy levels always <P90 (<7.1 µmol/L at <12 GW, <5.9 µmol/L at 15 GW, <7.2 µmol/L at 34 GW, < 8.5µmol/L at labour) , tHcy levels low at 1st trimester (< 7.1 µmol/L), tHcy levels high at 1st trimester (≥ 7.11 µmol/L) and tHcy levels always high (≥P90 7.1 µmol/L, 7.2 µmol/L at 34 GW, 8.5 µmol/L at labour). The results are shown in *Figure 10*.

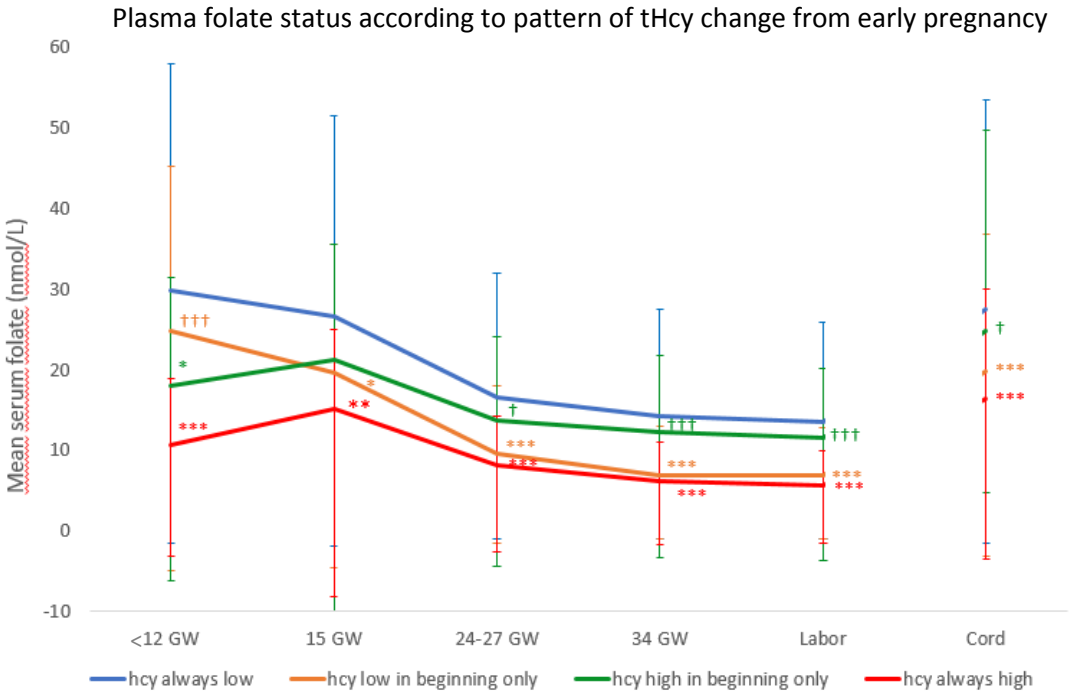

Figure 10. Mean Plasma folate throughout pregnancy and in the cord according to tHcy category. Blue: thcy always low (<12GW n=577, 15GW n=355, 24-27GW n=540, 34GW n=507, labour n=489 and cord n=464). Orange: thcy low in beginning only (<12GW n=64, 15GW n=46, 24-27GW n=60, 34GW n=58, labour n=59 and cord n=55). Green: thcy high in beginning only (<12GW n=39, 15GW n=23, 24-27GW n=32, 34GW n=35, labour n=32 and cord n=31). Red: thcy always high (<12GW n=25, 15GW n=16, 24-27GW n=24, 34GW n=23, labour n=21 and cord n=20). Means (dots) and 95% CI (bars) are reported. Student's unpaired t-test comparing differences between tHcy categories: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to tHcy always low (blue line); $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ compared to tHcy always high (red line).

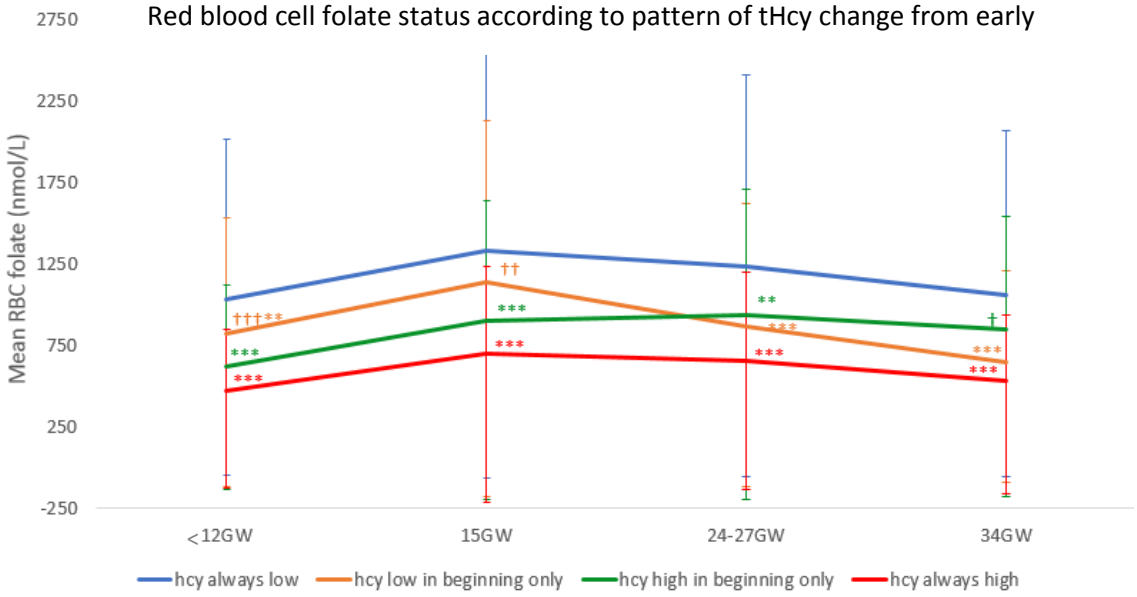

Figure 11. Mean Red blood cell folate throughout pregnancy according to tHcy category. Blue: thcy always low (<12GW n=565, 15GW n=354, 24-27GW n=429 and 34GW n=495). Orange: thcy low in beginning only (<12GW n=63, 15GW n=46, 24-27GW n=58 and 34GW n=57). Green: thcy high in beginning only (<12GW n=39, 15GW n=22, 24-27GW n=32 and 34GW n=35). Red: thcy always high (<12GW n=24, 15GW n=16, 24-27GW n=23 and 34GW n=20). Means (dots) and 95% CI (bars) are reported. Student's unpaired t-test comparing differences between tHcy categories: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to tHcy always low (blue line); $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ compared to tHcy always high (red line).

Participants with low tHcy throughout pregnancy had the best plasma and red blood cell folate status throughout pregnancy and those with high tHcy throughout pregnancy always had the lowest plasma and red blood cell folate status (*Figures 10* and *11*). In those that changed tHcy category as pregnancy progressed, folate status also changed. At <12 GW plasma folate status was similar between women with low tHcy in early pregnancy only and those with low tHcy throughout pregnancy. On the contrary, at <12 GW red blood cell folate levels are significantly higher in the group with low tHcy throughout pregnancy compared to those with low tHcy in the beginning only. At 15GW

plasma folate levels from participants with low tHcy in the beginning only are lower than those with always low tHcy concentrations. However, red blood cell folate levels do not differ between the groups (tHcy low in the beginning only versus tHcy levels always low) at 15 GW. From 24-27 GW throughout the rest of pregnancy, plasma folate status was consistently lower in the women that had started pregnancy with low tHcy status but then went on to belong to the high tHcy status group. From 24-27 GW to the end of pregnancy, participants with high tHcy levels at the beginning of pregnancy only, had higher red blood cell folate status than people with low tHcy levels in early pregnancy only.

Paternal genotype and intrauterine growth retardation

We also investigated the association between paternal *MTHFR* 677C>T genotype and probability of IUGR using multiple logistic regression analysis. We added the paternal *MTHFR* 677C>T genotype (reference CC genotype), plasma cobalamin and red blood cell folate concentrations to the complete maternal model with maternal *MTHFR* 677C>T genotype, red blood cell folate and plasma cobalamin at 1st and 3rd trimester, adjusted for lifestyle factors and complications such as anaemia and gestational diabetes. None of the models were significant and are not shown. Nevertheless, the relationship between the paternal *MTHFR* 677TT genotype compared to CC was as follows: in the 1st trimester model (n= 304) OR (95% CI) 1.1 (0.3, 4.1), in the 34 GW model (n= 272) OR (95% CI) 1.8 (0.4, 7.5) and in the Labour model (n= 242) OR (95% CI) 1.4 (0.4, 2.5).

Paternal tHcy status and intrauterine growth retardation

We built on the multiple logistic regression models, previously described for the mothers, to investigate the association between elevate paternal tHcy (≥P90 versus the other percentiles) and probability of IUGR. We included the variable maternal tHcy ≥P90 at some point of pregnancy versus always <P90, I the model. The results are reported in *Table 20*.

***Table 20**. Associations between elevated (≥P90 versus <P90) maternal and paternal tHcy and intrauterine growth retardation*.

	N	R^2	Maternal tHcy ≥P90 vs <P90	BMI	Smoking throughout pregnancy vs never	Paternal tHcy ≥P90 vs <P90
Model 1 (maternal, non adjusted)	390	0.008	1.7 (0.7, 4.3)	-	-	-
Model 2 (maternal adjusted)	390	0.081	1.3 (0.5, 3.4)	0.9 (0.8, 1.0)	1.5 (0.5, 4.7)	-
Model 3 (maternal adjusted and paternal factors)	390	0.123[#]	1.4 (0.5, 3.9)	0.9 (0.7, 1.0)	1.8 (0.6, 5.7)	4.5 (1.7, 12.8)

Abbreviations: *BMI, Body Mass Index; tHcy, total plasma homocysteine*. Multiple logistic regression analysis was used. Nagelkerke R^2; OR (95% CI) for intrauterine growth retardation in babies born to mothers with high tHcy status (≥P90[1], 7.1 µmol/L at <12 GW, 5.85 at 15 GW, 7.25 at 34 GW, 8.52 at labour) are shown. Model 1: (basic model) comparing ≥P90 tHcy maternal levels at some point of pregnancy vs <P90 tHcy throughout pregnancy. Model 2: Included the same variables as model 1 as well as parity (previous pregnancy ≥20 GW versus none), maternal age (y), 1st trimester BMI, smoking during pregnancy, mid and high versus low socioeconomic status, 3rd trimester haemoglobin <11 g/dL and gestational diabetes. Model 3: Included the same variables as model 2 as well as paternal tHcy (≥14.1 µmol/L, ≥P90 vs <P90). [#]P=0.06.

Elevated paternal tHcy was associated with a greater probability of an IUGR-affected pregnancy.

4.4. One carbon metabolism and gestational hypertension

Maternal genotype and gestational hypertension

We investigated the association between maternal *MTHFR* 677C>T genotype and risk of developing gestational hypertension using multiple logistic regression analysis. Pregnancies affected by gestational diabetes or intrauterine growth retardation were excluded from the analysis. Two models were designed: including *SLC19A1* 80G>A genotype and including red blood cell folate and plasma cobalamin concentrations at <12 or at 15 GW. We also adjusted the analysis for smoking habit, socioeconomic status, BMI, age and previous pregnancy. The adjusted R^2 of the first complete model at <12 GW (n= 346) was 0.155 (p=0.019). There was no significant association between maternal genotype and gestational hypertension. BMI was a risk factor predicting gestational hypertension with an OR (95% CI) 1.2 (1.1, 1.2). Results from the second model, at 15 GW are shown in *Table 21*.

Table 21. *Associations between maternal MTHFR 677C>T genotype and gestational* hypertension.

	N	R^2	TT vs CC	BMI
Model 1 (non adjusted)	346	0.022	2.4 (0.9, 6.6)	-
Model 2 (adjusted)	346	0.155*	2.9 (1.0, 8.4)	1.2 (1.0, 1.3)

Abbreviations: *MTHFR*, *methylenetetrahydrofolate reductase*; *BMI, Body Mass Index*. Multiple logistic regression analysis was used. Nagelkerke R^2; OR (95% CI) for gestational Hypertension in mothers with *MTHFR* 677 CC *vs* CT and TT genotype are shown. Model 1: (basic model) comparing *MTHFR* 677 CC genotype vs TT or CT and *SLC19A1* 80 AA vs GA or GG genotypes. Model 2: Included the same variables as model 1 as well as lifestyle factors such as previous pregnancies, age, BMI, smoking during pregnancy, low versus mid-high socioeconomic status, low tertile red blood cell folate (<= 724.33 nmol/L) and low tertile plasma cobalamin (<= 315.45 pmol/L) concentration at the corresponding time points of pregnancy for each model. **p<0.01, *p<0.05.

We observed an increased risk of developing gestational hypertension in mothers with the *MTHFR* 677 TT compared to CC genotype. BMI was also a predicting factor.

Maternal tHcy status and gestational hypertension

We also studied the association between early and late pregnancy tHcy and the risk of gestational hypertension, using multiple logistic regression analysis, again excluding pregnancies affected by intrauterine growth retardation and gestational diabetes. The categorical tHcy variable (≥P90 tHcy in early pregnancy versus < P90) replaced the genotypes and red blood cell folate and plasma cobalamin variables in each model, at <12 and 15 GW. Models were adjusted for smoking habit, socioeconomic status, BMI, age and parity. Elevated maternal tHcy levels in early pregnancy was not associated with increased risk of gestational hypertension. In the adjusted model, BMI was a risk factor for gestational hypertension with an OR (95% CI) 1.1 (1.1, 1.2) and having been pregnant previously was protective 0.56 (0.32, 0.96).

Paternal genotype and gestational hypertension

We investigated the association between paternal *MTHFR* 677C>T genotype and risk of developing gestational hypertension using multiple logistic regression analysis. To the same models used for the mothers, at <12 and 15 GW, we added paternal *MTHFR* 677C>T genotype, smoking habit, age and red blood cell folate concentration. None of the models were significant.

Paternal tHcy status and gestational hypertension

As we did with mothers, we also studied the association between paternal tHcy levels and the risk of gestational hypertension. The categorical tHcy variable (≥ P90 tHcy in early pregnancy versus < P90) replaced, genotypes, red blood cell folate and plasma cobalamin variables in each model, at <12 and 15 GW. We included elevated paternal tHcy levels (≥P90), age and smoking habit as paternal factors in both models. The adjusted R^2 of the model was 0.129 (p=0.013). Neither elevated paternal tHcy or smoking were associated with risk of gestational hypertension.

DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
GENETIC AND METABOLIC ALTERATIONS IN MATERNAL AND PATERNAL ONE CARBON METABOLISM AND DEVELOPMENT OF PREGNANCY COMPLICATIONS OF PLACENTAL ORIGIN
Júlia Haro Barceló

5. Discussion

5.1. General findings

Our study population consists of a pregnancy cohort in which participants were recruited from their prenatal visits in the hospitals before 12GW. Most women reported planning their pregnancy. However, only 36% took folic acid supplements before conception. At their first prenatal visit, all women were routinely recommended to take folic acid supplements until the end of the first trimester. They adhered very well to this advice, and only 5.8% reported not taking any folic acid supplements during the first trimester. Their age ranged from 26 to 39 years, most had a mid-high socioeconomic status and 25.7% of women smoked during pregnancy, 9.4% during the first trimester only and 16.3% throughout pregnancy. 19.2% of the mothers had impaired placentation indicated by pathological Doppler measurements of uterine artery flow at 20GW, 11.8% developed gestational hypertension and 8.8% intrauterine growth retardation. The percentage of pregnancies affected by these complications in other cohorts ranges between 2-17% for gestational hypertension (240) and 10-15% for IUGR (241).

Paternal participation in the study was 66.1%. There were 3 main factors affecting paternal recruitment. Firstly, maternal consent was required to contact the fathers and 5.8% declined to provide the contact details. Secondly, we could not establish contact with 1% of the fathers. Thirdly 33.9% of the fathers allegated fear of needles, no interest or incompatible work schedule as the reason for declining the invitation. Only 6.8% of the fathers reported taking folic acid-containing supplements. Their age ranged between 29 to 41 years, most had a mid-high socioeconomic status and 36.7% of the men smoked before and during pregnancy.

Folate status

Median plasma folate during the first trimester of pregnancy was relatively high (28.9 nmol/L) and then declined as pregnancy progressed. On the other hand, median red blood cell folate status increased between the first trimester (936.4 nmol/L) and 15 GW (1266 nmol/L), before gradually declining into the 3rd trimester. The decline in folate status occurred earlier in plasma folate compared to RBC folate because plasma folate reflects very recent intake (actual status) and is sensitive to folic acid supplementation, whereas RBC folate concentrations reflect both the long-term average of intake over the life span of RBC and folate stores in the liver (242). In addition, plasma folate as well as other nutrients circulating concentrations are affected by haemodilution due to the increase on plasma volume (243).

50.1% of participants were close to plasma folate deficiency according to WHO criteria (≤ 10 nmol/L (8)) and 30.2% according to the old criteria (≤ 7 nmol/L (7)) at labour. The prevalence of folate deficiency depends on the cut off applied. We applied that of Chanarin et al (≤ 7 nmol/L (7)), in *Figure* 7 and *Figure 8*, because it is the one that was used until the WHO in 2008 recommended changing it to ≤ 10 nmol/L for folate deficiency (8). These recommendations in both cases are applicable outside of pregnancy. In the absence of an established cut off for pregnancy, we consider it more precise to apply the previous (pre-mandatory fortification) cut off because our participants were not exposed to mandatory folic acid fortification and taking into the account that plasma micronutrient concentrations are lower due to haemodilution and increased glomerular filtration rate. Nevertheless, we highlight the need to establish a cut off for pregnant women.

Globally, red blood cell folate status was not deficient. The advantage of measuring RBC folate during pregnancy is that it is not subject to the same

physiological changes as plasma folate concentration. Only 0.13% of the mothers started pregnancy with red blood cell folate deficiency (<340 nmol/L (8)) and few cases were deficient by 34 GW (0.5%). Nevertheless, 51.8% did not meet the recommended red blood cell folate status of 906 nmol/L for NTDs prevention. We can expect participants to have good plasma folate status during the 1st trimester while they were taking folic acid supplements. The decline in plasma folate status started when folic acid supplement use was largely stopped, after 15 GW. Red blood cell folate concentrations also increased following the period of high folic acid supplement use. It did not fall until the 3rd trimester. There are other factors affecting also the drop in folate status during pregnancy when folate requirements are increased because of elevated folate utilization and catabolism (244). The preparation for the demands of rapid fetal growth that occur late in pregnancy may double nutrient requirement to protect fetal development (245). The increased demand on folate may lead to the mobilization of folate maternal reserves ending sometimes in the depletion of those reserves. Our group has previously shown that low folate status enhances the inverse association between betaine and tHcy in late pregnancy, reflecting increased betaine remethylation to homocysteine when folate availability becomes limited (246). Haemodilution can be another factor involved in the decrease of folate concentrations. Maternal plasma volume expansion from 10 GW throughout pregnancy and serum albumin decrease, lead to a reduction in nutrient concentration in the circulation during the same period (243). In addition, changes in renal function during normal pregnancy due to increased glomerular filtration, increased kidney plasmatic flow and decreased kidney vascular resistance (247) may contribute to the excess of plasma folate elimination leading to a drop in folate status (248).

We observed that smoking affected maternal red blood cell folate status during pregnancy. It was lower in smokers than in non smokers. Comparing red blood cell folate status between these two groups allows us to see the long term effects of smoking on folate status, as well as a measurement of folate status unaffected by haemodilution. We did not have red blood cell folate determinations from the cord, but cord plasma folate concentration is unaffected by haemodilution. Despite the difference in folate status between the smoking and non-smoking mothers, plasma folate status in the cord did not differ. During pregnancy, folate transport to the foetus is enhanced by additional mechanisms for folate uptake and transport including folate receptor α expression by the placenta (249). If folate status is impaired in smoking mothers, there may be a compensatory effect to ensure the supply of folate to the fetus by increasing cellular folate uptake, as described in animal models. Hye Won Kim et al (2011) showed that folate receptor α expression in the placenta was higher (by 130%) in rats fed with a deficient folic acid diet compared to the folic acid supplemented group (250).

The median of paternal plasma folate was close to folate deficiency (8.8 nmol/L) and the median of red blood cell folate level was 456.8 nmol/L. 61% of the fathers had folate deficiency according to WHO criteria (plasma folate ≤ 10 nmol/L (8)), and 35.6% according to the old criteria (7). This percentage is similar to that observed in the mothers at the end of pregnancy. The relatively high prevalence of folate deficiency in this population, compared to others like the population from the United States described by Bailey et al (251) with a low prevalence (< 1%) of folate deficiency (< 7 nmol/L), is likely due to poor dietary intake of folic acid in the form of fortified foods. There is no mandatory fortification of flour with food folic acid in Spain and, even though voluntarily fortified food products are available (breakfast cereals, cookies, milk, milkshakes, yogurts, canned juices, etc), consumption of these

is generally lower than in other countries. In addition, fathers no recommendations are in place to take folic acid supplements before conception.

On the other hand, current trends are towards a dietary pattern increasingly further removed from the original Mediterranean diet in our general population, resulting in less vegetable and fruit intake (252). However, it is well known that dietary folate is relatively ineffective at increasing folate status compared with folic acid supplements and fortified food (44).

We hypothesise that the lower folate status observed in the fathers compared to the mothers is due to the lack of targeted recommendations on folic acid supplementation in this group, to improve folate status. We previously reported, in a study of our adult population between 1998-2002, that in the absence of folic acid supplementation, 18.8% of women and men have folate deficiency. Furthermore, folate status is relatively low with geometric mean of plasma folate concentrations of 11.5 nmol/L and red blood cell folate of 810 nmol/L (9).

Homocysteine status

The pattern of change in tHcy is similar to that which our group first described in 2002 (100). Folic acid supplementation influences tHcy, lowering its concentrations (99, 253). The extensive use of supplements by the mothers in our cohort may be one of the reasons why tHcy was low during the first trimester and fell by 15 GW and remained low at 27-27 GW. There was an increase in tHcy levels from 34 GW to the end of pregnancy. Independently of folate status, hormonal changes occurring during pregnancy and the physiologic role of homocysteine in the preparation of labour lead to a return

towards nonpregnancy concentrations of tHcy in late pregnancy (35, 99). Previously, in a non supplemented cohort, our group observed higher tHcy levels in early pregnancy (geometric mean of 6.48 µmol/L) than those observed in the present population (100). The median tHcy in the fathers, of 9.6 µmol/L, is higher than the mothers, as expected due to the difference in tHcy between men and women (138, 254), the fact that tHcy was measured during pregnancy in women, and likely also because folic acid supplement use was prevalent in the women (99, 253).

MTHFR 677 C>T and *SLC19A1* 80G>A genotype

The homozygote variant genotypes of these two polymorphisms are quite prevalent in our pregnancy cohort, with 17.0% of the women with the *MTHFR* 677 TT genotype and 27.2% with the *SLC19A1* 80AA genotype. The TT + AA genotype combination s was observed in 6.0% of the women. Among the fathers, 14.1% had the *MTHFR* 677 TT genotype, 28.9% the *SLC19A1* 80AA genotype and 5.3% the combination of TT + AA. Our group previously reported the *MTHFR* 677TT genotype to be present in 18.1% of adults in a representative sample of our local population (9) and 11.8% of newborns in Spain, data from hospitals of the National Health Service (15), lower than the prevalence in our cohort. This prevalence is also higher than in other countries such as Italy, France or The Netherlands (15).

The maternal *MTHFR* 677TT genotype does not affect plasma folate concentrations during pregnancy in our population. In contrast, red blood cell folate concentrations were lower in the *MTHFR* 677TT compared to CC genotypes in early, mid and late pregnancy and also compared to CT genotypes in late pregnancy. However, the stratified analysis according to smoking habit during pregnancy showed no differences in red blood cell folate

levels between CC and CT compared to TT genotypes in smokers. There were no differences in cord plasma folate among the genotypes in either group. This might be explained by a compensatory effect in smoking mothers, commented before, necessary to ensure adequate supply of folate to the foetus. Despite the lack of difference in folate status, higher tHcy levels in the mothers with the TT genotype were observed compared to CC or CT genotypes from 15 GW to the end of pregnancy. This suggests that folate status is still in fact impaired in the TT mothers during pregnancy. It is possible that any compensation in maternal folate status in these mothers, is prioritised for the foetus before the mother. Regarding the *SLC19A1* 80G>A polymorphism, plasma folate, red blood cell folate and tHcy concentration did not differ between genotypes. In a previous study our group reported that compared with the CC and GG genotypes for *MTHFR* 677 and *SLC19A1* 80 genotypes respectively, people with the TT or AA alleles had lower plasma folate and RBCF plasma levels (9). We may not have observed the effect of these polymorphisms on folate status due to extensive folic acid supplement use. Nevertheless, the effects of the polymorphism on folate reserves (red blood cell folate) and the functional folate status biomarker (tHcy) were evident. Also, plasma variables are affected by the physiological effects of pregnancy including haemodilution (243), increased glomerular filtration rate (247) and foetal growth requirements (244). Determinations of folate status in clinical laboratories are largely based on plasma folate concentrations and these may not accurately reflect true underlying status due to these reasons.

Fathers with the *MTHFR* 677TT genotype had lower red blood cell folate concentrations and higher tHcy compared to the other genotypes. Some studies have previously reported the relation between the *MTHFR* 677TT polymorphism and the increase in total plasma homocysteine (40, 255). Our group have recently shown that the *MTHFR* 677TT genotype was the most

important determinant of tHcy in men and that the association was stronger in smokers (139). Plasma folate concentrations also tended to be lower in fathers with the TT genotype. In the case of the *SLC19A1* 80G>A polymorphism, no significant differences in one carbon metabolism status between genotypes were observed.

5.2. One Carbon Metabolism and uterine artery pulsatility index and impaired placentation

Relation between genetics and uterine artery pulsatility index and impaired placentation

We explored whether the *MTHFR* 677 C>T polymorphism influences placentation, assessed by uterine artery pulsatility index and Doppler wave forms at 20 GW. We did not find any association between the maternal genotype and placentation. Abnormal uterine artery Doppler measurement is commonly used in the screening of pregnant women to detect increased resistance to flow in the uterine arteries due to impaired placentation. Poor perfusion indicated by increased uterine artery resistance is associated with adverse pregnancy outcomes (167). The homozygote variant *MTHFR* 677TT genotype has been associated with elevated tHcy and decreased serum folate concentrations (14) and is considered a genetic risk factor for some obstetric complications such as IUGR (256) and preeclampsia (257). Out of pregnancy, *MTHFR* 677TT genotype has been also related with increased risk of venous thrombosis (258), due to the alterations in arterial structure and function caused by elevated homocysteine concentrations. In that way, this polymorphism may affect uterine artery resistance also, and increase the risk of developing pregnancy outcomes.

Stonek et al (2008) obtained the same results as ours in a prospective study where they measured the uterine artery pulsatility index values and unilateral or bilateral diastolic notches at 12 and at 22 GW of 1995 singleton pregnant women. No statistical differences between mean uterine artery Doppler values and bilateral or unilateral notch in any *MTHFR* genotype were found, showing that the *MTHFR* C677T polymorphism does not influence Doppler flow measurements (259). In the same line, Driul et al (2005) in a case control study with 103 preeclamptic women found no association between the

polymorphism and uterine artery flow. The frequency of homozygosity for the *MTHFR* mutation in the group of women with abnormal Doppler measurement and preeclampsia was 21.7% while it was 10.3% in the group with normal pregnancies with no significant differences (260).

Contrarily, more recently Akdemir et al (2018) among 71 patients with recurrent pregnancy loss, observed that the uterine artery impedance was higher in the group with multiple thrombophilic mutations (*MTHFR* C677T among them) compared with the group who had a single thrombophilic mutation (261). Even though their study group are not comparable with our population, because they include complicated pregnancies, and they measured the combination of some polymorphisms, this result may suggest that the implication of *MTHFR* C677T alone is not strong enough to lead to an adverse pregnancy outcomes As Stoneak et al have proposed, may be it is not reasonable to speculate that *MTHFR* genotype influences Doppler flow measurements, because in their results none of the three *MTHFR* genotypes showed a significant association with Doppler measurement (259). However, it is important to consider that environmental factors such as folate status and smoking can modify the effect of the *MTHFR* 677TT polymorphism (289, 290). We have studied those factors in detail, especially RBC folate status in the context of smoking. We have observed no differences between *MTHFR* 677 C>T genotypes among smoking mothers, and cord folate status did not differ among genotypes in any of the mothers or between smokers and nonsmokers. The high prevalence of folic acid supplement use during the first trimester (more than 90% of our mothers) leading to good folate status, may hide the effect of the *MTHFR* 677TT polymorphism. May be for those reasons in the studies commented before, as well as in our study, we do not observe an association between maternal genotype and uterine artery pulsatility index and impaired placentation. Or may be further investigation is needed to have strong evidence.

On the other hand, paternal *MTHFR* 677 CT and TT genotypes were associated with impaired placentation compared with the CC genotype. This was confirmed by their association with increased resistance in the uterine arteries and increased probability of pathological Doppler assessment of the uterine arteries at 20 GW.

Uterine artery perfusion during pregnancy involves the maternal-placental-foetal unit. Trophoblast invasion of the uterine arteries is the basis for successful placentation and during placentation for establishing the maternal-placental-foetal unit (259). As commented in the introduction, trophoblast invasion of the uterine arteries converts them from their spiral, high resistance, non-pregnant form to their tubular, low-resistance pregnant form. If this conversion is incomplete, placentation is impaired and placental perfusion is reduced leading to increased risk of severe pregnancy outcomes. Doppler sonography of the uterine arteries is an established screening method to detect impaired uterine artery flow.

There is a strong paternal genetic component in trophoblast formation and function. If we consider that certain combinations of maternal and fetal genotypes may impede proper placentation and trophoblast invasion (264), paternal genes can also affect placentation, but is rarely considered. With the results obtained, we propose that paternal genotype is crucial for a successful trophoblast invasion. To the best of our knowledge, this is the first study to report this and further investigation should replicate this association as well as exploring other genotypes.

We found that paternal smoking was a predictive factor of uterine artery pulsatility index and tripled the risk of impaired placentation. Paternal smoking is a common factor studied in the investigation of the possible implication of the father in pregnancy complications. In 1994, Martinez showed that the number of cigarettes smoked by the father was associated

with lower birthweight in children with non-smoking mothers (265). Three years later, Horta et al (1997) confirmed that women whose partner smoked were at higher risk of having a child with IUGR [OR 95%CI 1.3 (1.1, 1.7)] (266). It has been hypothesized that mutations in the paternal germ line caused by the tobacco, can later cause mutations in the offspring and impact directly on the health of the newborn (267). Further support of this hypothesis is provided by *in vitro* evidence that cigarette smoking increases oxidative DNA damage in human sperm cells and causes mutations in germ cells (268). However, the results are still unclear and further investigation is needed to clarify the correlation between paternal smoking habit and perinatal outcomes.

Relation between tHcy and uterine artery pulsatility index and impaired placentation

Maternal tHcy status was not associated with placentation (either pulsatility index or pathological Doppler measurement of uterine arteries at 20 GW). Poor placentation, measured by Doppler uterine flow, is an underlying cause of preeclampsia (269) and other complications related with abnormal trophoblastic invasion. Some of the hypothesized factors leading to abnormal trophoblast invasion are disruptions of endothelial junctional proteins (270), subendothelial changes in uterine arteries (271) and generalized vascular complications. Elevated total homocysteine levels have been shown to have a deleterious effect on the vascular endothelium (272) and have been linked with various pregnancy complications (273).

If tHcy is elevated the risk of some pregnancy complications caused by endothelial dysfunction leading to modulation of the arterial resistance to flow, we would expect to find an association between elevated tHcy and Doppler parameters. However, homocysteine plasma determinations are

affected by folic acid supplementation and that might mask any association between tHcy and impaired placentation. In our cohort, maternal tHcy was normal during the first trimester when they are being supplemented and their levels rise when supplementation stops.

Few studies have investigated this. Yu C et al (2004) in a multicentre study with 683 healthy pregnant women investigated the association between maternal plasma homocysteine concentrations (mid-trimester) and preeclampsia, in women with abnormal uterine artery Doppler outcome (22-24 GW). Differences in maternal tHcy concentrations were not significant between women with abnormal uterine artery Doppler and controls. Furthermore, they did not report any difference in maternal tHcy levels in those with an adverse pregnancy outcome compared to those with uncomplicated pregnancies (190). This finding is in the same line as ours, even though their samples were collected at mid trimester and were nonfasting. More recently, Maged et al (2017) evaluated the role of maternal tHcy concentrations (15-19 GW) and uterine artery Doppler (18-22 GW) as a possible predictor of pregnancy complications related to poor placentation in 453 women. They observed that tHcy levels and uterine artery resistance index were significantly higher in preeclampsia, IUGR and other complications groups when compared to the uncomplicated group. Also, the percentages of participants with uterine artery notch (unilateral or bilateral) were significantly higher in the group with complications compared with the control one (274).

Many other studies have investigated tHcy levels and uterine artery doppler separately, as possible predictors for pregnancy complications. In addition, the use of both parameters has been proposed to improve the sensitivity of prediction of pregnancy complications relative to the use of each one alone (274). The lack of association between maternal homocysteine plasma concentrations and pathological Doppler measurement of uterine arteries in our study may be because our cut off point for P90 levels of homocysteine was

not high enough to cause endothelial disfunction. Low levels of homocysteine in the highest percentile may be due to folic acid supplementation in our participants. As mentioned previously, women that temporarily took folic acid supplements in the first trimester may only have had transitory "low" tHcy and in that sense were misclassified as belonging to the low tHcy group. We do not know if their tHcy was high during the early stages of placentation and before they started to take the supplements. It would be unethical to test this in humans because the recommendation is for women to take folic acid supplements before and during early pregnancy, but it might be interesting to test in animal studies. Mingda HAN et al (2012) demonstrated in a mouse model that exposure to elevated serum homocysteine levels (by injection) during embryonic day 6.75 have a moderate effect on altered embryonic blood flow and placental anomalies including IUGR. They observed a mis expressed NMM-IIs, a cytoskeletal protein family associated with cell migration, in mouse placentas (275). Xu X et al (2016) also studied the effect of elevated homocysteine concentrations (injections of 50mg/g/day on embryonic day 7.5) in pregnant mice. Those animals showed preeclampsia like symptoms such as higher systolic blood pressure and proteinuria in late pregnancy and decreased expression levels of vascular endothelial growth factor in the placenta compared with control mice (276).

On the other hand, elevated paternal tHcy was positively associated with uterine artery pulsatility index and showed a threefold increase in risk of developing pathological Doppler measurement of uterine arteries at 20 GW. This association confirmed what we might have expected to observe in mothers in the absence of folic acid supplementation. Paternal tHcy was not affected by folic acid supplementation because few of them took supplements before conception. In general, paternal folate status was lower than in the mothers and low, as previously reported by our group in a population study

(9) in the absence of mandatory folic acid fortification. In this context, maybe the potential effect of tHcy increasing uterine artery flow resistance is detectable. It is possible that tHcy is related with pathological Doppler via endothelial dysfunction, as previous non-pregnant animal and human studies have described (272). We also hypothesise that in cases where the mother develops pregnancy complications, but with no apparent underlying risk factors, paternal factors can be the precursors of these complications. Is has been shown that fathers born from a complicated pregnancy have more risk of fathering a pregnancy with adverse outcomes (17). As we observe in our results, while maternal factors seem to have no apparent relation with pathological doppler measurement, paternal homocysteine levels were associated with it. More investigation will be necessary to answer this question, especially focused on paternal factors which have been scarcely considered to date.

5.3. One Carbon Metabolism and birthweight and intrauterine growth retardation

Relation between genetics and birthweight and intrauterine growth retardation

There was an interaction between the *MTHFR* 677 C>T genotype and smoking in their association with birth weight. In the stratified analysis by smoking pattern, the *MTHFR* 677TT genotype was only associated with birthweight in smoking mothers. Compared to the CC reference genotype, the TT genotype was associated with increased birth weight. We would expect to see a negative effect of smoking, a long established risk factor for low birth weight (277) in all genotypes. This unexpected result might be explained by the increase of placental folate receptors in situations of impaired folate status as commented before (250). The only evidence we have of this is the fact that cord plasma folate status was unaffected by either maternal smoking habit or *MTHFR* 677C>T genotype, despite the fact that maternal folate status was affected by each of these factors. Thamar Ayo Yila et al (2012) observed the effect that we were expecting. In their study with 1784 native Japanese mother-child pairs, the *MTHFR* 677 T allele was associated with lower birth weight in the offspring of active smokers. Contrary to our results, they also found a protective effect of the CT genotype against low birth weight only in the absence of smoke (278). A limitation of stratified analysis is the reduced sample size in the sub-groups. However, it is unlikely that the unexpected effect that we observed is due to low sample size in the TT genotype group because it was confirmed in early and mid-pregnancy and despite lack of significance in late pregnancy, the positive association was maintained. Our study did not set out to investigate the effect of smoking on birth weight, but rather to control for it as an established confounding factor. We do not interpret the TT genotype in smoking mothers as a protective factor for birth

weight in the offspring but hypothesise that the observed association is related with a potential compensatory mechanism in mothers with two factors adversely affecting birth weight. Low early pregnancy red blood cell folate status was inversely associated with birth weight in smokers only. This early pregnancy measurement of red blood cell folate was a true reflection of folate status, before first trimester supplement use affected red blood cell folate status. In countries with mandatory folic acid fortification or high intake of voluntarily fortified foods and higher prevalence of folic acid supplement use, it is possible that the negative association between low early pregnancy maternal folate status and birth weight might be missed.

In several studies the *MTHFR* 677TT genotype in the mother has been associated with low birth weight in the offspring. In a metanalysis published in 2017, including 25 studies, Han Wu et al concluded that the maternal *MTHFR* 677TT genotype was associated with increased probability of low birthweight, OR (95%CI) 2.3 (1.4, 3.5). They proposed that the identification of the maternal *MTHFR* 677TT genotype may play a key role for primary prevention of low birth weight (12). Lee et al (2013) in a Mendelian randomization analysis to investigate the causal relation between maternal tHcy levels, as represented by *MTHFR* C677T genotype, with birth weight of the offspring, found that birthweight was significantly lower in mothers with the TT genotype compared to the CC and CT genotypes. They also observed a dose-response association with homocysteine concentrations for each additional T allele, and birth weight decreased as maternal tHcy at 24-28 GW increased (p=0.06) (279). Those results may explain the association between this polymorphism and birthweight of the newborn. Reduced enzyme activity due to the *MTHFR* C677T polymorphism, increases plasma homocysteine levels (13). This in turn may lead to oxidative stress, endothelial damage and placental thrombosis (280). All these conditions might be associated with

impaired flow of uterine arteries, inadequate trophoblast invasion and poor pregnancy outcomes (281). On the other hand, other reports observed no association between *MTHFR* 677C>T genotype and risk of IUGR. Infante-Rivarde et al, reported twice the absence of association between thrombophilic polymorphisms and intrauterine grow restriction. In 2002 in a case-control study including 493 newborns with IUGR, they reported no increased risk of IUGR among mothers with the *MTHFR* 677TT genotype (282). With a subgroup of the same cohort, in 2005 they conducted a family study including 250 case trios (affected newborn, mother and father). They tested whether there was excess transmission of the *MTHFR* C677T and A1298C haplotypes (CA, TA, CC, TC) to affected offspring and they observe that transmission and haplotype frequencies were not different between case and controls groups (283). Their results are in line with ours, however, their study was in the presence of mandatory folic acid fortification and paternal genotype was performed from buccal swabs. Giovanni Larciprete et al (2007) reported similar findings in a case control study with 284 pregnant women in Rome. Even though *MTHFR* C677T genotypes were more prevalent than other thrombophilia mutations included in the study, they did not observe an increased incidence of adverse pregnancy outcomes in subjects with the *MTHFR* TT genotype (284). Interestingly, they did not have cases with *MTHFR* mutations combined with hyperhomocysteinemia. Folate supplementation of their population may mask a potential association between the polymorphism and IUGR or other adverse pregnancy outcomes. It has been proposed that variations in the relative contribution of disease alleles in different populations might explain the discrepant results of previous studies on *MTHFR* and pregnancy complications (284). This could be another reason for the absence of association between the *MTHFR* 677 C>T polymorphism and IUGR in our study. In addition, an increase of IUGR cases in our population would increase our statistical power and possibility of observing the association.

Also, an increase in paternal participation would improve sample size for observing an association between the paternal polymorphism and IUGR. Regarding a paternal effect, we found few studies investigating paternal thrombophilic mutations as a possible risk factor for IUGR. Several are focused on other paternal factors such as age, ethnicity, weight and height and other polymorphisms.

Low red blood cell folate status was inversely associated with birth weight in the mothers that smoked at some point of pregnancy. Low folate status has previously been reported to be related with adverse pregnancy outcomes, including birth weight (285). Exposure to tobacco smoke during pregnancy is a well-known risk factor for adverse perinatal outcomes as many studies have previously described (286). The first correlation between smoking and fetal adverse outcomes was proposed in 1957 by Simpson (287) who pointed out how the neonates of smoking mothers weighed 200g less on average than neonates of non-smoking mothers. Furthermore, smoking has also been shown to be associated with low RBC folate (288). We saw the inverse association between low RBCF levels and birth weight in smoking mothers but not in non smoking mothers. This may be because smoking mothers also had lower RBC folate status. Or maybe because smoking has a direct negative effect on birthweight mediated by hypoxia, as smoking reduces the oxygen binding capacity and oxygen supply is essential for placental and fetal growth (289). Another possible explanation is that nicotine may lead to vasoconstriction in the maternal circulation with subsequent reduced blood flow to the placenta and the fetus (290).

We also observed that paternal cobalamin status was positively associated with birthweight. Maternal inadequate or deficient vitamin B12 status has been previously associated with higher risk of low birthweight (264, 270). Despite not finding previous studies focused on paternal B12 status, there are

studies suggesting that maternal and paternal micronutrient intake, including B vitamins involved in one carbon metabolism, which are essential for DNA methylation, can influence the programming of the offspring's epigenome influencing neonatal outcomes (228). Following this evidence, we hypothesize that paternal B12 status can influence the programming of genes involved in fetal growth and subsequent offspring's birthweight.

Relation between tHcy and birthweight and intrauterine growth retardation

Previously our group reported that elevated tHcy at 8 GW is associated with increased probability of birth weight percentile <P25 in the offspring (35). That study was carried out between 1992-1996, before prenatal supplementation with folic acid was routinely recommended in Spain. In this study what we have observed is a positive association between late pregnancy tHcy (34 GW and at labour) and birthweight only in non-smoking mothers. In normal pregnancy, tHcy increases at the end of pregnancy (292). Homocysteine can be methylated to form methionine required for cell proliferation and gene expression essential for fetal growth (293). In addition, *in vitro* studies show that homocysteine enhances spontaneous contractions of myometrium derived from pregnant women (294). The increase in tHcy late in uncomplicated pregnancies may suggest a physiologic role for homocysteine in the preparation for labour.

The Reus- Tarragona Birth Cohort study set out to investigate whether elevated first trimester tHcy is an early pregnancy biomarker for IUGR in the foetus. There was no association between elevated tHcy (≥P90) in the first trimester and increased risk of IUGR. The early pregnancy blood sample was collected later in the RTBC study (up until 12 GW) compared to the previous

one (at 8 GW). Geometric mean tHcy (6.48 µmol/L) was higher in the previous study than in the RTBC study (5.3 µmol/L). Later blood sample collection as well as folic acid supplement use in the RTBC are two factors associated with lower tHcy, compared to the previous study.

What we observe in this thesis is a possible masking of elevated plasma homocysteine levels due to the transitory effect of folic acid supplementation. In a non-fortified country like Spain, women are recommended to take folic acid supplementation 3 months before pregnancy. As we mentioned before, 93.8% of the women in the RTBC reported use of folic acid supplements but the timing and dose was variable. In the majority of cases this supplementation was during the first trimester only. When participants stopped the oral supplement use, their folate status fell. tHcy is sensitive to folic acid supplement use so the first trimester measurement may be more indicative of recent folic acid supplement use, rather than long term 1CM status. This is contrary to the previous study in our group where tHcy at 8 GW was likely to reflect true underlying 1CM metabolic status.

McNulty et al (2013) showed in a randomized controlled trial that continued folic acid supplementation during the second and third trimester prevented the elevation in homocysteine concentrations by 36 GW that otherwise occur with the progression of pregnancy (295). More recently, Shazia H Chaudhry et al (2019) reported that one of the factors significantly associated with lower homocysteine concentration was folic acid supplementation in the Ottawa and Kingston Birth Cohort (296). Maybe for this reason we cannot see the negative effect of elevated tHcy levels as previously observed by our group when the folic acid supplementation was not extensive as it is now. However, it has been proven that when women stop folic acid supplement use during pregnancy their tHcy levels increase again. In a trial performed with German women, 500µg of folic acid were administered for 8 weeks, and when they stopped the supplementation homocysteine and folate returned to baseline

levels (297). Our group have previously described that continued supplement use beyond the first trimester was associated with lower tHcy from 24 to 27 GW compared with non use (92). In the RTBC women, supplementation in early pregnancy may mask the real status of tHcy in women with apparently normal levels but that return to their true tHcy status when they stop folic acid supplementation. In those cases, they return to their original status and became part of the group with increased risk. The true normal tHcy mothers would be those with tHcy consistently <P90 throughout pregnancy. Therefore, in a setting where mandatory folic acid fortification is absent and folate status is only temporarily enhanced for the duration of folic acid supplement use, first trimester tHcy appears not to be a useful biomarker of IUGR for all women and consequently the possible risk of developing IUGR may not be reflected. We did observe in the RTBC study that women with early pregnancy tHcy <P90 but with tHcy ≥P90 later in pregnancy, had a tendency for higher risk of IUGR. Another difference between the RTBC study and the previous one by our group is in the endpoint. Previously it was birth weight <P25 whereas in the RTBC study it was IUGR (≤P10). Other studies have demonstrated the association between elevated homocysteine levels and IUGR. The Generation R cohort in The Netherlands (2012), based on early pregnancy (13 GW) with a higher sample size (n=5805), reported that the risk of delivering an SGA infant was higher in women with homocysteine concentrations in the highest quintile (≥8.3 µmol/L) compared with women in the reference group (adjusted OR 1.68; p=0.006) (298). In India, the Pune Maternal Nutrition Study form Yajnik et al reported that mothers with higher plasma homocysteine concentrations delivered earlier and had lighter babies (36). They have a cohort of 526 women who took folic supplements from 18 GW. Some studies report no association between tHcy status and IUGR. Rosario d'Anna et al (2004) failed to demonstrate any difference in serum homocysteine levels at early pregnancy (16 GW) among women who later developed IUGR (299). The

population studied had no mandatory folic acid food fortification policy, like ours (Italy), and they only included non-smoking participants. Another study performed in Canada during early pregnancy (<20 GW) observed that high tHcy was not significantly associated with having an SGA infant (273). Those results were similar to ours but in the presence of mandatory folic acid food fortification and a larger cohort of 2016 women. Hogg et al (2000) in a study with prenatal zinc treatment, did not report differences between the zinc and placebo groups in the mean plasma homocysteine level in late pregnancy (26-37GW), and also no difference in tHcy levels between pregnancies complicated by IUGR and those that resulted in normal foetal growth (300). Although the study was performed with a non-comparable population, they were African American women, the results were in line with ours.

Elevated paternal tHcy was inversely associated with birth weight in the babies born to mothers that smoked during pregnancy and increased the probability of IUGR. We did not stratify the IUGR models by maternal smoking status because this would have reduced our statistical power due to the low number of IUGR cases. However, we did adjust for maternal smoking and the negative association between elevated paternal tHcy and foetal growth was confirmed in these models. Elevated paternal tHcy has been previously associated with higher risk of NTDs (207) and recurrent pregnancy loss (109). But, as far as we know, no research has been focused on paternal homocysteine concentrations and the pregnancy complications studied in this thesis. We hypothesise that the link between homocysteine, other micronutrients and metabolites from the 1C metabolic network and DNA methylation could be involved. Coppedè et al (2019) described an inverse correlation between plasma homocysteine and DNA methyltransferase 1 methylation levels. DNMT1 is responsible for the maintenance of DNA methylation patterns during cell division. The folate metabolic pathway may be relevant in the

development of disorders characterized by altered DNA methylation (301). Alterations in paternal germ cells or spermatozoids DNA methylation may transfer disease predisposition to the second generation according to the intergenerational inheritance effect (302).

5.4. One Carbon Metabolism and Gestational Hypertension

Relation between genetics and Gestational hypertension

We found that women with *MTHFR* 677 TT versus CC genotype had almost 3 times more risk of developing gestational hypertension. Our findings were largely in line with other studies and metanalysis confirming the relationship between the variants of that polymorphism and gestational hypertension. The *MTHFR* 677 C>T polymorphism has been previously associated with hypertension and stroke outside of pregnancy (303). Our group recently reported that the TT genotype is associated with increased risk of hypertension in adults under 50 years of age (139). There are also several studies that have investigated the association between the polymorphism and gestational hypertension.

Hernández-Díaz et al (2005) studied U.S. and Canadian white women with nonmalformed infants and found that the T allele was present int 69% of women with gestational hypertension versus 57% of control women. Compared with 677CC homozygotes, the OR (95% CI) of gestational hypertension for women who were 677 TT/CT was 1.9 (0.9, 4.0) (304). Stronger associations were found in two metanalysis. Kosmas et al (2004) across 23 comparisons (3169 hypertensive women and 3044 controls) found that having the T allele (TT or CT) increased the OR (95% CI) of gestational hypertension by 1.2 (1.0, 1.4) in patients with diastolic hypertension > 110mmHg. No association was seen in patients with less severe diastolic hypertension. They conclude that the C677T polymorphism increased the risk of severe hypertension but not the risk for milder hypertension (305). More recently, Yang et al (2014) confirmed also that the C677T polymorphism was

significantly associated with gestational hypertension with an OR (95% CI) 1.2 (1.1, 1.3) (306).

However, some results are controversial. An underestimated effect of the polymorphism may come from the variance of the polymorphism according to ethnicity. When Yang et al stratified the analysis by ethnicity they observed significant association among East Asian and Caucasian, but not among Latinos, Black Africans and Indians (306). The use of folic acid supplementation during pregnancy can also mask the effect. Hernández-Díaz et al suggest that the association between *MTHFR* C677T polymorphism and gestational hypertension disappeared when they restrict the analyses to women supplemented with folic acid during the first five months of gestation (304). In the case of our study, we did not observe an association between the genotype and gestational hypertension when we adjusted for folate status when it was highest during pregnancy, during the peak of folic acid supplement use during the first trimester. We were strict in excluding pregnancies affected by gestational diabetes and intrauterine growth retardation from our analysis. While these pregnancy complications and underlying conditions can occur simultaneously, with gestational hypertension, in an initial analysis, exploring potential mechanisms, we excluded them. On the other hand, an inconsistent definition of gestational hypertension or prenatal control of the same can also be bias for the results. We strictly adhered to the definition of gestational hypertension, so that we did not misclassify participants with underlying hypertension as having hypertension induced by pregnancy. We only included pregnancies that had complete prenatal control of blood pressure (consisting of a measurement before and after 20 GW), in our analysis.

On the contrary, paternal genotype was not associated with increased risk of gestational hypertension. We did not find in our literature search, any other

study of the association between paternal *MTHFR* 677 C>T genotype and gestational hypertension. Nonetheless, Hernández-Díaz et al (2005) suggest a possible effect of the offspring genotype in the risk of gestational hypertension because they found an association between fetal 677TT/CT genotype and maternal risk of gestational hypertension [0R (95%CI) 2.4 (1.1, 5.0)] (304). As foetal genotype is determined 50% from father's genotype, we propose that paternal genotype can influence maternal risk of developing gestational hypertension. Other studies have suggested that paternal imprinted genes in the fetus contribute to increase pregnant woman's risk to develop preeclampsia (307). It makes sense if we consider that preeclampsia results from dysfunctional placentation and the placenta has the same genotype as the fetus.

We found that maternal BMI was also a risk factor for gestational hypertension. We expected this because BMI is a well known risk factor for hypertension and gestational hypertension. Previous studies have also reported the same association (308, 309, 310). In addition, is well known that have had previous pregnancies reduces the risk to develop gestational hypertension compared to nulliparous women (311).

Relation between tHcy and Gestational hypertension

We found no association between maternal and paternal tHcy levels and the risk of gestational hypertension. Some studies showed that elevated tHcy is a risk factor of developing hypertension outside of pregnancy (312, 313). Our group recently reported that elevated tHcy was only associated with increased risk of hypertension in adults older than 50 years (139) and it is unclear that homocysteine is causally involved. Furthermore, a potential association is

harder to see between tHcy and gestational hypertension because tHcy concentration is greatly affected during pregnancy by physiological and endocrinological processes (100). There have been few studies of such an association. Studies examining maternal tHcy levels at early pregnancy, like ours, have demonstrated a relationship between elevated tHcy and preeclampsia but they did not find the same association with gestational hypertension. In addition, we did not find studies examining the contribution of paternal homocysteine levels to the risk of gestational hypertension.

In a prospective cohort study, Dodds et al reported that there was no significant association between tHcy concentration before 20 GW and the risk of developing gestational hypertension (273). Sun et al (2017) in a retrospective cohort study with 147 confirmed cases of gestational hypertension failed to find a significant difference between tHcy concentrations in the gestational hypertension and control groups (240). They only found a significant association in the group of severe preeclampsia compared to controls with an OR (95% CI) of 1.1 (1.1, 1.2). Cotter et al (2003) studied 71 cases of non-severe preeclampsia and 142 controls in Dublin, taking blood samples at early pregnancy. Participants with plasma homocysteine values >7.8 µmol/L (upper third) had significantly increased risk of developing preeclampsia [OR 95%CI 4.1 (1.4, 12.6)] (85).

Prenatal tHcy may not be a good biomarker for gestational hypertension, but it is relevant for detecting preeclampsia. Normally, elevated tHcy concentrations are related with severe disease status leading to a higher damage of the structure of blood vessels. It has been suggested that low concentrations of tHcy may even affect the endothelium slightly and play a subtle role in occurrence of lowest disease stages (240) like gestational hypertension, making this relationship almost undetectable. In our study population, mothers are mostly folic acid supplemented at early pregnancy. This could be a reason why our tHcy ≥P90 group have tHcy levels too low to

cause the endothelial dysfunction and the following rise of blood pressure. Lastly, another possibility lies in the difference between the pathological processes involved in gestational hypertension and preeclampsia, justified by the higher increase on tHcy concentrations in preeclampsia than in gestational hypertension (240).

Further investigation is needed to establish whether there is an association between paternal factors related to one carbon metabolism and gestational hypertension or preeclampsia. This knowledge would inform preventive preconception policies including fathers.

5.5. General discussion

Pregnancy complications derived from impaired placentation, including gestational hypertension and IUGR are the main causes of perinatal morbidity and mortality and a risk for different diseases in the newborn (314). Even though there are some established genetic and environmental factors that contribute to these adverse outcomes, they can appear in women who were healthy before pregnancy, without a history of complications themselves or in their family (315). The risk factors are usually studied in the context of the maternal-foetal-placental unit, without considering paternal factors (19). However, maternal risk factors alone may not explain the cause of the complication in many cases. In this thesis, we have proposed some paternal factors as contributors to the development of pregnancy complications. As important adverse pregnancy outcomes can trace their origin to the placenta, paternal factors affecting placentation may influence the risk of adverse pregnancy outcome. The results of the Reus Tarragona Birth Cohort reported in this thesis show that paternal *MTHFR* C677T genotype and elevated tHcy levels are associated with impaired placentation and lower birthweight. In addition, we observed that in some cases maternal factors were not associated as a risk factor for those adverse outcomes. Nevertheless, this applied to elevated tHcy during early pregnancy and the *MTHFR* 677TT genotype and it is possible that their underlying associations with adverse outcomes were not detected due to the protective effect of folic acid supplementation during the first trimester of pregnancy in 94.2% of the mothers, and from preconception in about a 36% of them. Previous studies have also focused on the important role of paternal genetic and nutritional status on the success of placenta and fetal development. In a study with male rats exposed to a high-fat diet and females on a control diet, Sheau-Fang et al (2010) have shown that the offspring had early onset of impaired insulin

secretion and glucose tolerance, as well as altered gene expression in pancreatic islets (316). Bielawski et al (2002) in a study performed also in rats, observed that paternal chronic alcohol intake decreased messenger RNA expression of cytosine methyltransferase in sperm, which could result in hypomethylated DNA which is then passed on to the offspring (21). In the same line, Lundi et al (2017) have observed that mice exposed to both a folate deficient diet and a high dose of folic acid supplementation have male descendants (F1) with lower sperm counts which leads to increased postnatal death in the offspring (F2) (189). Therefore, it is also important to consider that paternal lifestyle, apart from genetics, can play a role in pregnancy complications and fetal programming. We consider that it will be important to include paternal factors in the focus of interest to learn more about their implication in complications developed during pregnancy that we cannot explain only with maternal factors. In a context without mandatory folic acid fortification like Spain, where 61% of the men adult have folic acid deficiency, it would be interesting to establish a protocol of folic acid supplementation in fertile man planning to have a baby to avoid pregnancy complications if scientific evidences support it.

Strengths and Limitations

The principal strength of our study is the time on data collection. The prospective design from early pregnancy allows as to have clinical, obstetric and lifestyle data from the first trimester of pregnancy and a blood sample before 12 GW with a confirmed viable foetus. It's very difficult to obtain first trimester data because it depends on the timely initiation of prenatal care by the mother. Most studies have data from the mid or final trimesters of pregnancy. We also have the participation of the fathers, which is difficult to

obtain because they are not visited as part of prenatal care and they have to agree to come voluntarily and especially for the interview and blood sample. Many are uninterested for work reasons or don't want to come to the hospital to give a blood sample. In the fathers, the absence of folic acid food fortification in Spain and the very low use of folic acid supplementation use, allowed us to study the effect of the *MTHFR* C677T polymorphism in 1C metabolism and pregnancy complications without the masking effect of folic acid.

One of the limitations of this thesis is that observational studies do not allow to assume causality effect in the associations studied. However, we adjusted for potential confounding factors. Over 60% of the fathers participated, but it is possible that these were more motivated for research and possibly healthier. However, this did prevent us from testing our hypothesis as shown by the novel associations we observed. Another limitation is our participant recruitment. We could not access participants having the prenatal check-up outside the hospital, before 12 GW. Participants visiting the hospital for the prenatal care were mostly people with more risk of developing pregnancy complications and some hospital / university staff.

Future perspectives

Further investigation will be useful to replicate the results regarding paternal involvement in pregnancy adverse outcomes. Our study reports ground breaking results about paternal *MTHFR* C677T genotype and 1C metabolism influence on pregnancy complications. More studies focused on this topic would inform future recommendations or protocols. If the father is associated with pregnancy complications that we cannot explain by maternal risk factors and thus not covered by primary and secondary prevention measures, the

associated complications likely emerge when it is too late. We could focus on recommendations for the father at preconception. Recommendations on diet and healthy lifestyle or folic acid supplement use in the father might contribute to reducing the incidence of pregnancy complications. In addition, further investigation will be useful to know the mechanisms by which paternal factors are affecting those adverse outcomes. Epigenetic factors have been proposed as a possible mechanism (206). Micronutrients involved in one carbon metabolism can contribute to DNA methylation and affect foetal programming (228).

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Júlia Haro Barceló

CONCLUSIONS

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6. Conclusions

Main objective

To investigate the association between genetic, nutritional and metabolic components of the maternal and paternal 1C metabolic network and the probability of impaired placentation, intrauterine growth retardation and gestational hypertension.

Specific objectives

- To compare the likelihood of impaired placentation, intrauterine growth retardation or gestational hypertension between the maternal or paternal *MTHFR* 677C>T *(*TT *versus* CC*,* CT *versus* CC*)* and *SLC19A1* 80 G>A *(*AA *versus* GG*,* AG *versus* GG*)* genotypes*.*

  Maternal *MTHFR* 677 C>T genotype was not associated with impaired placentation.

  Fathers with *MTHFR* 677 CT and TT genotypes have fourfold and sevenfold, respectively, more probability of impaired placentation compared to the CC genotype.

  Maternal *MTHFR* 677 TT genotype was positively associated with birthweight only in smokers.

  Maternal and paternal genotypes were not associated with increased risk of IUGR.

Mothers with the *MTHFR* TT genotype were threefold more likely to develop gestational hypertension compared to mothers with the CC genotype.

Paternal genotypes were not associated with increased risk of gestational hypertension.

Maternal and paternal *SLC19A1* 80 G>A genotypes were not associated with increased risk of any of the three pathologies.

- To investigate the association between elevated early pregnancy maternal tHcy, elevated paternal tHcy and the probability of impaired placentation diagnosed by pathological Doppler of uterine arteries at 20 gestational weeks, intrauterine growth retardation or gestational hypertension.

Elevated first trimester maternal tHcy was not associated with impaired placentation.

Pregnancies from fathers with elevated tHcy have threefold increased risk of impaired placentation compared to fathers with normal tHcy.

Elevated maternal tHcy at late pregnancy was positively associated with birthweight in non smokers.

Elevated first trimester maternal tHcy, as a standalone measurement, was not associated with increased risk of intrauterine growth retardation.

Elevated paternal tHcy was associated with a nearly fivefold greater probability of an IUGR-affected pregnancy.

No association between maternal and paternal tHcy concentrations and the probability of gestational hypertension were found.

- To explore whether the associations between the genotypes and the three pathologies is modulated by the status in the 1C metabolism nutrients.

Maternal red blood cell folate status is affected by the *MTHFR* 677 C>T genotype. Mothers with the TT genotype have lower RBC folate compared with the CC and CT genotype at 12, 24-27 and 34 GW.

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SCIENTIFIC AND ACADEMIC CONTRIBUTIONS

UNIVERSITAT ROVIRA I VIRGILI
GENETIC AND METABOLIC ALTERATIONS IN MATERNAL AND PATERNAL ONE CARBON METABOLISM AND DEVELOPMENT OF PREGNANCY COMPLICATIONS OF PLACENTAL ORIGIN
Júlia Haro Barceló

Scientific and academic contributions and other merits

Articles

Cavallé-Busquets P, Inglès-Puig M, Fernandez-Ballart JD, Haro-Barceló J, Rojas-Gómez A, Ramos-Rodriguez C, Ballesteros M, Meyer K, Ueland PM, Murphy MM. Moderately elevated first trimester fasting plasma total homocysteine is associated with increased probability of miscarriage. The Reus-Tarragona Birth Cohort Study. *Biochimie*. 2020;173:62-67. doi:10.1016/j.biochi.2020.01.008

Haro-Barceló J, Cavallé-Busquets P, Fernandez-Ballart J, Ramos-Rodriguez C, Rojas-Gomez A, Roigé J, Ballesteros M, Ueland PM, Murphy MM. The contribution of paternal folate metabolism to placentation and fetal size. In preparation to be published in the *American Journal of Obstetrics and Gynecology*.

Haro-Barceló J, Rodriguez-Ramos C, Rojas A, Murphy MM, Fernandez-Ballar J. The contribution of paternal factors to pregnancy complications of placental origin: systematic review. In preparation. Registred in PROPSPERO ID: 148864.

International conference contributions

Conference: 11th International Conference on One Carbon Metabolism, B Vitamins and Homocysteine. Arhus (Denmark), 2017.
Authors: Ornosa-Martin G, Solé-Navais P, Cavallé-Busquets P, Fernandez-Ballart JD, Haro-Barceló J, Ballesteros M, Ueland PM, Meyer K, Murphy MM.

Title: Early pregnancy folate and cobalamin status and overweight and obesity in children at 7.5 years.

Format: Poster.

Conference: Federation of American Societies for Experimental Biology (FASEB). Nova Scotia (Canada), 2018.

Authors: Júlia Haro-Barceló, Alejandra Rojas-Gomez, Gemma Ornosa-Martin, Pol Sole-Navais, Monica Ballesteros, Montserrat Ingles-Puig, Per M. Ueland, Klaus Meyer, Joan D. Fernandez-Ballart, Pere Cavallé-Busquets, Michelle M. Murphy.

Title: Associations between maternal and paternal 1C metabolism and mid-childhood growth and health in the Reus-Tarragona Birth Cohort Study.

Format: Poster.

Conference: Federation of American Societies for Experimental Biology (FASEB). Nova Scotia (Canada), 2018.

Authors: Alejandra Rojas-Gomez, Júlia Haro-Barceló, Gemma Ornosa-Martin, Pol Sole-Navais, Monica Ballesteros, Montserrat Ingles-Puig, Per M. Ueland, Klaus Meyer, Joan D. Fernandez-Ballart, Pere Cavallé-Busquets, Michelle M. Murphy.

Title: Associations between prenatal 1C metabolism and mid-childhood metabolic profile in the Reus-Tarragona Birth Cohort Study.

Format: Poster.

Conference: 12th International Conference on One Carbon Metabolism, B Vitamins and Homocysteine. Montbrió del Camp (Spain), 2019.

Authors: Júlia Haro-Barceló, Alejandra Rojas-Gomez, Carla Ramos-Rodríguez, Gemma Ornosa-Martin, Pol Sole-Navais, Monica Ballesteros, Montserrat

Ingles-Puig, Per M. Ueland, Luis Masana, Mercedes Heras, Joan D. Fernandez-Ballart, Pere Cavallé-Busquets, Michelle M. Murphy.

Title: Genetic and metabolic alterations in paternal one carbon metabolism and development of pregnancy complications of placental origin.

Format: Poster.

Conference: 12th International Conference on One Carbon Metabolism, B Vitamins and Homocysteine. Montbrió del Camp (Spain), 2019.

Authors: Alejandra Rojas-Gomez, Júlia Haro-Barceló, Carla Ramos-Rodríguez, Gemma Ornosa-Martin, Pol Sole-Navais, Monica Ballesteros, Montserrat Ingles-Puig, Per M. Ueland, Luis Masana, Mercedes Heras, Joan D. Fernandez-Ballart, Pere Cavallé-Busquets, Michelle M. Murphy.

Title: Associations between prenatal plasma homocysteine and metabolic score at mid-childhood.

Format: Poster.

Conference: 12th International Conference on One Carbon Metabolism, B Vitamins and Homocysteine. Montbrió del Camp (Spain), 2019.

Authors: Montserrat Inglès-Puig, Joan Fernandez-Ballart, Pere Cavallé-Busquets, Júlia Haro-Barceló, Mónica Ballesteros, Alejandra Rojas-Gómez, Carla Ramos-Rodriguez, Per M Ueland, Klaus Meyer, Michelle M Murphy.

Title: Moderately elevated 1st trimester fasting plasma total homocysteine is associated with increased probability of miscarriage. A prospective cohort study.

Format: Poster.

Conference: Virtual FASEB. The Folic Acid, Vitamin B12 and One-Carbon Metabolism Conference. 2020

Authors: Michelle M. Murphy, Júlia Haro-Barceló, Luis Adolfo Santos, Alejandra Rojas-Gomez, Carla Ramos-Rodríguez, Joan D. Fernandez-Ballart, Marta Herrero, Pol Solé-Navais, Monica Ballesteros, Pere Cavallé-Busquets.
Title: Preconception and first trimester folic acid supplement use and folate status. The Reus Tarragona Birth Cohort.
Format: Poster.

Teaching and academic activities

Medicine Grade in the Faculty of Medicine and Health Sciences of Universitat Rovira i Virgili (approximately 60 hours/ year; 2016-2019)
- Research and Documentation Bases (70%)

- General Epidemiology (30%)

Other contributions

- Active role in the fieldwork of the pregnancy and follow-up phases of the Reus-Tarragona Birth Cohort.

Involvement in scientific projects

Title: Genetic and metabolic alternations in maternal and paternal one carbon metabolism related with pregnancy complications of placental origin.
Principal Investigator: Michelle Murphy.
Duration: 3 years.
REF: 16/00506
Funding source: Instituto de Salud Carlos III/ Fondo FEDER.

APPENDICES

Nom..... *Data ....*

QÜESTIONARI DE FREQÜÈNCIA DE CONSUM ALIMENTARI

INSTRUCCIONS PER OMPLIR-LO

Procuri contestar tranquil·lament aquest qüestionari. Prengui's el temps que consideri necessari. En el cas de tenir algun dubte contacti amb nosaltres per correu electrònic: silvia.fernandez@urv.cat o per telèfon: 977 759379 (Equip NUTCIR). Aquest qüestionari pregunta per la freqüència en la que consumia, de forma **habitual,** determinats aliments, **durant els 6 mesos previs a l'embaràs de la seva parella.**

La freqüència de consum s'ha d'especificar als quadres de la dreta del llistat d'aliments d'aquest qüestionari. Per a cada aliment del llistat ha d'apuntar el **número de vegades** que el consumia. Per exemple :

- Si el consumia **tots els dies de la setmana**, posi un 7 a la columna "**A LA SETMANA".**
- Si el consumia **alguna vegada a la setmana,** posi les vegades: 1-2-3-4-5 o 6 a la columna "**A LA SETMANA".** Pensi sempre en sumar el consum de totes les menjades del dia (esmorzar, dinar, berenar, sopar o altres). Per exemple, si prenia llet per esmorzar cada dia i per sopar alguna vegada a la setmana: 7 + 4 = 11 vegades a la setmana.
- Si consumia l'aliment **alguna vegada al mes**, posi les vegades: 1 ó 2 ó 3 etc. en la columna **"AL MES".**
- Si no el consumia **mai** o gairebé mai, deixi la casella en blanc, sense posar res.

Exemple: Un home esmorzava habitualment un got de llet (7 vegades) amb magdalenes (7 vegades), per sopar a vegades prenia llet (4 vegades) i a vegades prenia iogurt de postres (3 vegades). A més, menjava peix algunes vegades a la setmana per dinar (2 vegades) i altres vegades per sopar (4 vegades). De llegums en consumia alguna vegada al mes (aproximadament 3 vegades). I no menjava mai formatge de règim.

El consum l'apuntaria de la següent manera:

LLISTAT D'ALIMENTS	QUANTES VEGADES MENJAVA...?	
	A LA SETMANA	**AL MES**
Llet	*11*	
Iogurt	*3*	
Coc ràpid, magdalenes..	*7*	
...		
Peix	*6*	
...		
Llegum		*3*
Formatge de règim		

QÜESTIONARI DE FREQÜÈNCIA DE CONSUM ALIMENTARI

LLISTAT D'ALIMENTS	QUANTES VEGADES MENJAVA...?	
	A LA SETMANA	**AL MES**
Llet		
Iogurt		
Xocolata: bombons, "Kit-Kat" "Mars"...		
Cereals inflats d'esmorzar ("Corn-Flakes" "Kellog's")		
Galetes tipus "maria"		
Galetes amb xocolata, crema...		
Magdalenes, coc ràpid,...		
Ensaïmada, Donut, croissant...		

	A LA SETMANA	**AL MES**
Amanida: Enciam, tomàquet, escarola...		
Mongetes verdes, bledes, o espinacs		
Verdures de guarnició: albergínia, carbassó, xampinyons...		
Patates al forn, fregides o bullides		
Llegums: llenties, cigrons, fesols ...		
Arròs blanc, paella		
Pasta: fideus, macarrons, espaguetis...		
Sopes i cremes		

	A LA SETMANA	**AL MES**
Ous		
Pollastre o gall d'indi		
Vedella, porc, xai (bistec, empanada...)		
Carn picada: llonganissa, hamburguesa		
Peix blanc: lluç, mero...		
Peix blau: sardines, tonyina, salmó...		
Marisc: musclos, gambes, llagostins, pop, calamars...		
Croquetes, empanadilles, pizza		
Pa (en entrepans, a les menjades...)		

	A LA SETMANA	**AL MES**
Pernil salat, dolç, embotits		
Formatge fresc (Burgos,...) o baix en calories		
Formatges: curats o semicurats, cremosos		

	A LA SETMANA	**AL MES**
Fruites cítriques: Taronja, mandarina		
Altres fruites: Poma, pera, préssec, albercoc, plàtan		
Fruites en conserva (en almívar...)		
Sucs de fruita natural		
Sucs de fruita comercial		
Fruits secs: cacauets, avellanes, ametlles		
Postres làctics: natilles, flam, mató		
Pastissos de crema o xocolata		
Bosses d'aperitiu ("chips", "chetos", "fritos"...)		
Llaminadures: gominoles, caramels...		
Gelats		

LLISTAT D'ALIMENTS	QUANTES VEGADES MENJAVA...?	
	A LA SETMANA	**AL MES**
Begudes ensucrades ("coca-cola", "Fanta"...)		
Begudes baixes en calories (coca-cola light...)		
Vi, sangria		
Cervesa		
Cervesa sense alcohol		
Begudes destil·lades: (Whisky, ginebra, conyac,...)		

Indiqui amb una X la resposta que vulgui assenyalar:

1.- Han canviat els seus hàbits alimentaris des dels 6 mesos previs a l'embaràs de la seva parella fins ara?

Sí__ No__

En cas que la resposta sigui afirmativa, esculli el tipus de dieta que fa actualment:

- ☐ Dieta baixa en calories (reducció de pes)
- ☐ Dieta hipercalòrica
- ☐ Dieta hiperproteica
- ☐ Dieta per a hipercolesterolèmia
- ☐ Dieta per a diabètics
- ☐ Dieta baixa en sal
- ☐ Altres _____

2.- A taula, s'afegia sal a les menjades?

Mai__ Alguna vegada__ Freqüentment __ Gairebé sempre __

3.- Com definiria la seva gana d'aquells 6 mesos?

Molta __ Força __ Normal __ Poca __ Gens__

4.- Quin tipus de llet prenia habitualment?:

Sencera __ Semidescremada __ Descremada __

5.- Quin tipus de iogurt consumia habitualment?

a) Natural ___	b) Natural descremat ___
c) De sabors ___	d) De sabors descremat ___
e) Amb trossets de fruita__ ___	f) Amb trossets de truites descremat ___

6.- Quin tipus de pa menjava habitualment?: Blanc __ Integral __

7.- Es posava tomàquet i oli als entrepans?:

Sempre__ Habitualment__ Alguna vegada__ Gairebé mai__

Nom..... Data ....

ENQUESTA SOBRE HÀBITS I ESTIL DE VIDA

Per diferents motius, els hàbits nutricionals i l'estil de vida del pare poden tenir una clara incidència en el procés de la reproducció. Si us plau, contesti sincerament aquestes preguntes per ajudar-nos a valorar la influència que podien haver tingut en el desenvolupament de l'embaràs de la seva parella.

Completi aquesta informació ***(pensant en els 6 mesos previs a l'embaràs de la seva parella):***

Data de naixement: _____

Pes: _____ kg

Alçada: _____ cm

Antecedents mèdics: _____

_____

Grups ABO i Rh: _____

1. Ús de suplements vitamínics

Va prendre suplements vitamínics durant els 6 mesos anteriors a l'embaràs de la seva parella? ☐ Sí ☐ No

Només en cas que sí:

- Quin? _____

- Quantes vegades a la setmana el prenia?

  ☐ Cada dia
  ☐ Alguns dies (1-3 vegades)
  ☐ La majoria dels dies (4-6 vegades)

- Quantes pastilles/sobres/ampolles prenia cada dia? _____

2. Esmorzar

	Sí	No
Tenia el costum d'esmorzar?	☐	☐
Esmorzava cereals inflats habitualment (ex. Kelloggs / Nestlé etc)?	☐	☐
Prenia cafè amb cafeïna?	☐	☐
Prenia cafè descafeïnat?	☐	☐

3. Tabac :

Era fumador passiu (exposat al fum de tabac habitualment, a casa o a la feina)?

☐ Sí ☐ No

Era fumador actiu abans que la seva parella es quedés embarassada?

☐ Sí ☐ No

	0 cigs/dia	1-5 cigs/dia	6-10 cigs/dia	> 10 cigs/dia
Quants cigarrets fumava els 6 mesos abans de l'embaràs de la seva parella?	☐	☐	☐	☐

Si era fumador actiu:

☐ Va mantenir el seu hàbit durant tot l'embaràs

☐ Va abandonar totalment el seu hàbit durant tot l'embaràs

☐ Va reduir el seu consum de tabac durant l'embaràs

4. Alcohol:

	Mai /Ocasionalment	< 3 cops /setmana	Cada dia com aperitiu i/o amb els àpats	> 7 copes /setmana
Als 6 mesos anteriors a l'embaràs de la seva parella, amb quina freqüència bevia alcohol ?	☐	☐	☐	☐

Ha pres algun tipus de substància tòxica en els 5 anys anteriors a l'embaràs de la seva parella (marihuana, cocaïna, heroïna, etc...)?

☐ Sí ☐ No

En el cas que sí, especifiqui quines: _____

	Mai	Ocasionalment	Regularment
Als 6 mesos anteriors a l'embaràs de la seva parella, amb quina freqüència prenia substàncies tòxiques ?	☐	☐	☐

5. Activitat física:

a) De què treballava? _____

b) Com definiria el treball d'aquell moment?

- Bàsicament d'estar assegut i caminar poc (estudiant, docent, conductor de vehicles, dependent, administratiu, ...) ☐
- Caminar força però sense fer esforç vigorós (pagès, fàbrica, carter...) ☐
- Bàsicament de molta activitat física (esportista) ☐

c) Quina activitat feia en el seu temps lliure?

- Lectura, televisió i activitats que no requereixin activitat física important. ☐
- Caminar, anar amb bicicleta, jardineria (no s'inclou el transport d'anar i tornar del treball). ☐
- Córrer, esquiar, gimnàstica, jocs amb pilota o esports vigorosos regularment. ☐
- Entrenament esportiu regular per competició. ☐

Quants cops per setmana realitzava les activitats de la pregunta anterior? _____

6. Ingressos anuals nets totals a la llar, en aquell moment:

- Nombre de persones que formaven la unitat familiar**:** _____

- Ingressos totals:

Exemple, si la dona tenia un sou de 20000 €, l'home un de 18000€ i hi havia un avi que vivia amb la família i rebia una pensió de 6000 €

< 9000 €	>9000 € - 19000 €	>19000 € - 25000 €	>25000 € - 35000 €	> 35000 €
☐	☐	☐	☐	■

≤ 9000 €	>9000 € - 19000 €	>19000 € - 25000 €	>25000 € - 35000 €	> 35000 €
☐	☐	☐	☐	☐

Anoti qualsevol dubte o aclariment relacionat amb aquesta enquesta:

Moltes gràcies per la teva participació.

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Research paper

Moderately elevated first trimester fasting plasma total homocysteine is associated with increased probability of miscarriage. The Reus-Tarragona Birth Cohort Study

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ABSTRACT

The association between elevated early pregnancy fasting plasma total homocysteine (tHcy) and miscarriage risk was investigated prospectively in participants (n = 544) from the Reus-Tarragona Birth Cohort study. Pregnancy was confirmed before 12 gestational weeks (GW) by ultrasound scan and a fasting blood sample collected. Pregnancies with complications other than miscarriages were excluded. Miscarriages were diagnosed by ultrasound scan and gestational age at the time of miscarriage estimated by embryo size, where possible. Cases in which blood samples were collected more than a week after the miscarriage, or the miscarriage was of known cause, were excluded.

Fasting plasma folate, vitamin B_{12}, tHcy, cotinine (biomarker of smoking), red blood cell (RBC) folate, *MTHFR* 677C > T (rs1801133) and *SLC19A1* 80G>A (rs1051266) genotypes were determined.

The exposed group consisted of participants with first trimester tHcy $\geq P_{90}$ (7.1 μmol/L) (n = 57) and unexposed of those with tHcy < P_{90} (n = 487). Adherence to folic acid supplement recommendations, plasma folate, plasma vitamin B_{12}, RBC folate and prevalence of optimal RBC folate status ($\geq$ 906 μmol/L) were lower in the exposed compared to unexposed group. The prevalences of the *MTHFR* 677 TT genotype and miscarriage were higher in the exposed group. The relative risks (95% CI) of pregnancy ending in miscarriage were 2.5 (1.1, 5.7) and 2.1 (1.0, 4.5) for participants in the high tHcy and suboptimal RBC folate groups (compared to the reference groups) respectively. Adherence to folic acid supplement recommendations was positively associated, while the *MTHFR* 677 TT versus CC genotype and smoking versus non-smoking were negatively associated, with RBC folate status.

1. Introduction

1.1. Background

Moderately elevated fasting plasma total homocysteine (tHcy) has been associated with various pregnancy complications or adverse outcomes such as neural tube defects [1], preeclampsia [2] or low birth weight [3,4], among others [5]. Homocysteine metabolism is regulated by gene-nutrient interactions and depends on dietary B-group vitamins: folate, vitamin B_{12}, pyridoxine, and riboflavin as well as choline and betaine. The *MTHFR* 677C > T (rs1801133) and *SLC19A1* 180G > A (rs1051266) polymorphisms affect the role of folate in homocysteine metabolism and folate transport, respectively. They have negative effects on folate status and are associated with elevated tHcy [6].

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1.2. Evidence to date regarding tHcy and miscarriage/pregnancy loss

Numerous studies have investigated the association between tHcy and recurrent pregnancy loss or miscarriage. However, early pregnancy tHcy determinations from the index pregnancy in which miscarriage is clinically diagnosed are difficult to obtain. Most studies compared tHcy, measured after the affected pregnancies have ended, between women with a history of recurrent miscarriage versus normal pregnancy. Some of these studies reported higher tHcy in women with a history of miscarriage compared to normal pregnancy [7–10] and that the probability of history of miscarriage was increased with increasing tHcy concentration [7]. However, in this latter study, vitamin B12 deficiency prevalence was high among the miscarriage cases but low in the controls. Other studies did not observe any differences in tHcy between women with history of pregnancy loss compared to normal pregnancies [11–14]. The disparity in results between the aforementioned studies may be due to various reasons. None of them measured tHcy before the clinical diagnosis of miscarriage in the affected pregnancy. tHcy levels decrease during pregnancy [15] so nonpregnant measurements may not accurately reflect even early pregnancy concentrations. Furthermore, following the pregnancy loss women were taking folic acid supplements in many studies in preparation for the next pregnancy, thus affecting their tHcy. Studies with a reliable measurement of the exposure of interest prior to the miscarriage are lacking. In addition to the limitations regarding the exposure measurements, endpoints based on a clinical diagnosis of miscarriage are also scarce. This is relevant because efforts to differentiate between miscarriages likely resulting from other factors unrelated to tHcy, such as foetal chromosomal abnormalities or maternal infection, are warranted. A prospective study from preconception throughout pregnancy in which conception and pregnancy loss were monitored by urinary hCG concentrations, concluded that elevated tHcy at preconception ($\geq$12.4 μmol/L) did not increase the relative risk of early pregnancy loss [16]. Miscarriage causes are not assessed in this study of pregnancy loss before 6 GW. Another prospective study, from the first prenatal visit, of 100 pregnancies measured tHcy in blood samples collected between 4 and 16 GW. No difference in tHcy was observed between the women that went on to miscarry and those that had a normal pregnancy outcome [17]. It is not clear whether the statistical power was sufficient (there were only 12 miscarriages), the timing of sample collection covered a range of 12 weeks which affects tHcy and no information was provided regarding the timing, cause or type of miscarriage. Impaired vitamin B_{12} status was associated with a higher probability of miscarriage in that same study. A large French study reported higher tHcy in samples collected following hospitalization for miscarriage in the index pregnancy compared to elective pregnancy termination controls of similar gestational age [18]. The blood samples in cases and controls were collected soon after the events and detailed information regarding miscarriage diagnosis and exclusion of cases due to known causes is provided.

1.3. Hypothesis and aims

We hypothesised that moderately elevated early pregnancy tHcy is a potential biomarker of idiopathic first trimester miscarriage risk.

The aim of this study was to investigate, prospectively, the association between moderately elevated early pregnancy tHcy and the risk of first trimester miscarriage in the Reus-Tarragona Birth Cohort.

2. Materials and methods

2.1. Study participants

Women attending their first prenatal clinic at the University hospitals Sant Joan Reus and Joan XXIII Tarragona between 2005 and 2016, with confirmed pregnancy of less than 12 GW, were invited to participate in the Reus Tarragona Birth Cohort (RTBC) study. The study was approved by the Hospitals' Research and ethical committees and signed informed consent following the guidelines of the Declaration of Helsinki was obtained from all participants.

2.2. Blood sample collection

Fasting blood samples were collected at < 12 GW, 15 GW, 24–27 GW, 34 GW and nonfasting samples at labour. For the purposes of the present report, only the first blood sample will be considered. Participants that developed pregnancy complications other than miscarriage (preeclampsia, intrauterine growth retardation, gestational hypertension, among others) were excluded (n = 75) from this report. A total of 544 pregnancies were included. Samples were stored at −80 °C in the Pere Virgili Health Research Institute (IISPV) biobank until analysis. Clinical, nutritional and lifestyle data were recorded and plasma folate and RBC folate (microbiological assay with *Lactobacillus casei*) [19], plasma vitamin B_{12} (microbiological assay with *Lactobacillus leichmannii*) [20] and homocysteine (tHcy) and cotinine concentrations were determined by liquid-tandem mass spectrometry [21]. The *MTHFR* 677C>T (rs1801133) and *SLC19A1* 180G>A (rs1051266) genotypes were determined by matrix-assisted laser desorption/ionization/time-of-flight MS [22]. (Bevital; www.bevital.no). Data regarding smoking habits was collected from three different sources including interrogation by the investigating team (questionnaire), plasma cotinine determinations and from the prenatal history (recorded by the clinicians).

2.3. Pregnancy confirmation and miscarriage diagnosis

Between 11 and 13 + 6 GW, pregnancy was confirmed by ultrasound scan. The majority of the miscarriages were first trimester spontaneous "missed" abortions diagnosed on detection of no foetal heartbeat by ultrasound scan at 12 GW. The remaining miscarriages were in course and diagnosed on referral from the emergency room when the clinical symptoms were manifested. Ultrasound scans revealing absence of foetal heartbeat or empty yolk sac were diagnosed as miscarriage. Gestational age at the time of miscarriage was estimated, where possible, from the crown-rump length or biparietal diameter of the embryo. Cases of miscarriages occurring more than 7 days before blood sample collection, were excluded from the study.

2.4. Statistical analysis

Participants were classified as exposed to moderately elevated first trimester tHcy ($\geq P_{90}$: 7.1 μmol/L), n = 57, or unexposed ($< P_{90}$), n = 487. Smokers were identified based on plasma cotinine concentration $\geq$ 10 ng/ml and/or confirmation of smoking habit by questionnaire or on interrogation by the clinicians during the prenatal check-ups. Quantitative variables with non-normal distributions were natural log transformed for the application of parametric statistical tests. Means between groups were compared using ANOVA and proportions using the Chi-square test. We fitted a Cox regression model to calculate the relative risk (RR) of miscarriage associated with moderately elevated tHcy. The model was

adjusted for maternal age and smoking habit (active smoking versus non-smoking during pregnancy). Similarly, another Cox regression model was fitted to determine the RR of miscarriage associated with suboptimal RBC folate status during the first trimester of pregnancy. Predictors of tHcy and RBC folate status were assessed using multiple linear regression analysis and multiple logistics regression analysis respectively. IBM-SPSS software was used for all statistical tests. Significance level was set at $p < 0.05$.

3. Results

3.1. Cases included

Of the miscarriage cases, nine were excluded for the following reasons: chorioamnionitis (n = 3), antiphospholipid syndrome (n = 1), myoma (n = 1), trisomy 18 (n = 1), late miscarriage, > 18 GW (n = 2), missing information (n = 1). The 32 miscarriages occurring before 18 GW and of unknown cause were included in the analysis.

3.2. Participant characteristics according to first trimester tHcy category

Participant characteristics are described in Table 1. *SLC19A1* 80G>A genotypes, smoking habits, parity, maternal age and body mass index did not differ between the exposed and unexposed groups. Adherence to folic acid supplement recommendations of 400 μg/d was high in both groups but higher in the group with tHcy < P_{90}. Plasma vitamin B_{12} status, plasma and RBC folate status were lower and the *MTHFR* 677 TT genotype prevalence higher in the exposed versus unexposed to moderately elevated tHcy group. The WHO recommends a RBC folate status of 906 nmoL/L or more to prevent neural tube defects [23]. A higher proportion of participants in the high tHcy group had RBC folate concentrations below this recommendation and the proportion of early pregnancy miscarriage was higher in the exposed (high tHcy) than the unexposed group.

3.3. Exposure to first trimester tHcy and risk of miscarriage

The association between first trimester tHcy status and risk of miscarriage is reported in Table 2. Participants with tHcy at or above the 90th percentile (7.1 μmol/L) had over twice the risk of having a miscarriage. Risk of miscarriage also increased with increasing maternal age. We assessed whether RBC folate status below 906 nmoL/L affects the risk of miscarriage (Table 2). Women with RBC cell folate status < 906 nmol/L were twice as likely to have a miscarriage compared to women with red blood cell folate ≥ 906 nmol/L, after adjusting for maternal age, parity and smoking.

3.4. Participant characteristics according to pregnancy outcome

The participant characteristics according to outcome (miscarriage or normal pregnancy) are reported in Table 3. Women with pregnancies that ended in miscarriage were older, adhered less to the recommendation to take 400 μg/d of folic acid in the form of supplements and more of them had suboptimal folate reserves (indicated by RBC folate concentration, showing folate reserves entering pregnancy) compared to women that went on to have normal pregnancy outcomes.

3.5. Factors predicting first trimester tHcy

The predictors of first trimester tHcy were assessed using multiple lineal regression analysis (Table 4). The strongest predictor was *MTHFR* 677 TT genotype, followed by plasma creatinine, parity and plasma folate. In a separate model in which first trimester plasma folate was replaced with RBC folate, the strongest predictor was RBC folate, followed by plasma creatinine, *MTHFR* 677 TT genotype and parity.

3.6. Factors influencing first trimester RBC folate status

The factors influencing the probability of having optimal RBC folate status in the first trimester of pregnancy were assessed using multiple logistic regression analysis (Table 5). Regular use of folic acid supplements at or above the recommended dose of 400 μg/d strongly

Table 1
Participant characteristics according to exposure to first trimester fasting plasma total homocysteine category.

	First trimester fasting plasma total homocysteine group		
	≥ P_{90}[1]	< P_{90}	Total
MTHFR 677 C > T genotype, %			
CC	23.6 [13/55][2]	35.2 [170/483]	34.0 [183/538]
CT	43.6 [24/55]	49.3 [238/483]	48.7 [262/538]
TT	32.7 [18/55]	15.5 [75/483]**	17.3 [93/538]
SLC19A1 80 G > A genotype, %			
GG	38.2 [21/55]	26.7 [128/480]	27.9 [149/535]
GA	38.2 [21/55]	46.5 [223/480]	45.6 [244/535]
AA	23.6 [13/55]	26.9 [129/480]	26.5 [142/535]
First trimester smoking, %	31.6 [18/57]	26.9 [131/487]	27.4 [149/544]
First trimester folic acid use, %[3]	89.1 [49/55]	94.5 [446/487]***	93.9 [495/527]
Multiparity, %	47.4 [27/57]	54.2 [264/487]	54.4 [296/544]
Age (years), *mean (95% CI)*	32.4 (30.9, 33.9) [57]	32.1 (31.7, 32.5) [486]	32.2 (31.8, 32.5) [543]
BMI (kg/m2), *mean (95% CI)*	24.6 (23.2, 26.0) [55]	23.8 (23.4, 24.3) [464]	23.9 (23.5, 24.2) [516]
Plasma folate (nmol/L), *geometric mean (95% CI)*	14.4 (11.2, 18.4) [57]	26.7 (25.2, 28.4) [487]***	25.1 (23.6, 26.6) [544]
Plasma vitamin B_{12} (pmol/L), *geometric mean (95% CI)*	283 (261, 343) [57]	369 (358, 381) [487]**	363 (352, 373) [544]
tHcy (μmol/L), *geometric mean (95% CI)*	8.4 (8.0, 8.7) [57]	5.1 (5.0, 5.2) [487]***	5.3 (5.2, 5.4) [544]
RBC folate (nmol/L), *geometric mean (95% CI)*	556 (477, 647) [57]	954 (910, 1001) [474]***	901 (859, 945) [531]
RBC folate < 906 nmol/L, %	78.9 [45/57]	46.4 [220/474]***	49.9 [265/531]
Miscarriage, %	14.0 [8/57]	4.9 [24/487]*	5.9 [32/544]
Gestational week at miscarriage, *mean (95% CI)*	9.3 (7.8, 10.8) [7]	10.4 (9.1, 11.8) [16]	10.1 (9.1, 11.1) [23]

Abbreviations: tHcy: fasting plasma total homocysteine, RBC: red blood cell. [1]7.1 μmol/L, [2][n]. [3]≥ 400 μg/d. Statistical comparison between 2 groups, Chi square for proportions and ANOVA for quantitative variables: *p < 0.05, **p < 0.01, ***p < 0.001.

Table 2
Assessment of Relative risks of pregnancy ending in miscarriage according to early pregnancy fasting plasma total homocysteine (tHcy) and RBC folate status using Cox regression analysis. Abbreviations: tHcy: fasting plasma total homocysteine, RBC: red blood cell. [1]Relative risk; [2]measured at < 12 gestational weeks; [3]P_{90}: 7.1 μmol/L; [4]adjusted for parity (multiparous versus nulliparous) and for smoking versus nonsmoking during pregnancy.**p < 0.01.

	RR[1] (95% CI)	Deviance likelihood ratio, chi square	*n, df*
Unadjusted tHcy[2] model		17.2	544, 1**
tHcy ≥ P_{90} versus < P^3_{90}	2.85 (1.28, 6.34)		
Adjusted tHcy[2] model		17.5	543, 4**
tHcy ≥ P_{90} vs < P_{90}	2.52 (1.12, 5.68)		
Maternal age (y)	1.13 (1.04, 1.22)		
Unadjusted RBC folate[2] model[4]		2.6	531, 1
RBC folate	1.83 (0.87, 3.81)		
<906 versus ≥ 906 nmol/L			
Adjusted RBC folate[2] model[4]		15.0	530, 4**
RBC folate	2.11 (1.00, 4.45)		
<906 versus ≥ 906 nmol/L			
Maternal age (y)	1.15 (1.06, 1.24)		

Table 3
Participant characteristics according to pregnancy outcome.[1] [n], Abbreviations: tHcy: fasting plasma total homocysteine, RBC: red blood cell. Statistical comparison between 2 groups, Chi square for proportions and ANOVA for quantitative variables: *p < 0.05,**p < 0.01,***p < 0.001, #p = 0.067.

	Miscarriage	Normal pregnancy
	n = 32	n = 512
MTHFR 677 C > T genotype, %		
CC	36.7 [11/30][1]	33.8 [173/508]
CT	40.0 [12/30]	49.4 [253/508]
TT	23.3 [7/30]	16.8 [86/508]
SLC19A1 80 G > A genotype, %		
GG	30.0 [9/30]	27.7 [140/505]
GA	33.3 [10/30]	46.2 [234/505]
AA	36.7 [11/30]	25.9 [131/505]
First trimester smoking, %	21.9 [7/32]	28.0 [145/512]
First trimester folic acid supplement use, %	68.0 [17/25]	95.2 [478/502]***
Multiparity, %	50.0 [16/32]	53.7 [280/512]
Age (years), *mean (95% CI)*	34.6 (32.9, 36.3) [32]	32.0 (31.6, 32.4) [511]**
BMI (kg/m2), *mean (95% CI)*	23.5 (21.7, 25.3) [15]	23.9 (23.5, 24.3) [501]
Plasma folate (nmol/L), *geometric mean (95% CI)*	20.4 (14.7, 28.2) [32]	25.4 (23.9, 27.0) [512]*
Plasma vitamin B_{12} (pmol/L), *geometric mean (95% CI)*	359 (316, 408) [32]	363 (352, 374) [512]
tHcy (μmol/L), *geometric mean (95% CI)*	6.0 (5.4, 6.7) [32]	5.3 (5.2, 5.4) [512]**
RBC folate (nmol/L), *geometric mean (95% CI)*	837 (679, 1030) [31]	905 (861, 950) [500]
RBC folate < 906 nmol/L, %	64.5 [20/31]	49.0 [245/500]#

Table 4
Predictors of first trimester tHcy using multiple lineal regression analysis.

	Beta coefficient	Adjusted R square	n, *df*
Model 1		0.090***	504, 10
MTHFR 677 TT versus CC genotype	0.220***		
Plasma creatinine (μmol/L)	0.180***		
Parity (multiparous versus nulliparous)	−0.126**		
Plasma folate (nmol/L)	−0.109*		
Model 2		0.148***	491, 10
RBC folate (nmol/L)	−0.273***		
Plasma creatinine (μmol/L)	0.192***		
MTHFR 677 TT versus CC genotype	0.185***		
Parity (multiparous versus nulliparous)	−0.119*		

Abbreviations: RBC: red blood cell. The dependent variable in both models is ln tHcy. Both models were adjusted for maternal age, plasma B_{12}, smoking, *MTHFR* 677 CT versus CC genotype *SLC19A1* 80 AA vs GG and *SLC19A1* 80 GA vs GG genotypes. *p < 0.05, **p < 0.01, ***p < 0.001.

Table 5
Assessment of predictors of probability of optimal red blood cell folate status (≥ 906 μmol/L) using multiple logistic regression analysis. Model adjusted for maternal age, previous pregnancy, *MTHFR* 677 CT versus CC, *SLC19A1* 80 AA vs GG and *SLC19A1* 80 GA vs GG genotypes.***p < 0.001.

	OR (95% CI)	Nagelkerke R^2	n, *df*
Model		0.123***	505, 9
First trimester folic acid use (≥400 μg/d)	15.1 (3.5, 64.9)		
Smoking vs nonsmoking	0.53 (0.31, 0.91)		
MTHFR 677 TT vs CC genotype	0.56 (0.33, 0.94)		

increased the probability of having optimal RBC folate status. On the other hand, smoking versus nonsmoking and the *MTHFR* TT versus CC genotype were associated with 44% and 47% reductions, respectively, in the probability of having optimal RBC folate status.

4. Discussion

4.1. Principal findings

Elevated early pregnancy tHcy was associated with more than double the risk of having a miscarriage. First trimester RBC folate concentration < 906 nmol/L, indicative of suboptimal folate reserves entering pregnancy, was also associated with increased risk of miscarriage. Smoking had a negative effect on RBC folate status while folic acid supplement use at or above the recommended 400 μg/d had a protective effect.

4.2. Comparison with previous studies

Most previous studies determined tHcy after the miscarriage had occurred. A study that did collect blood samples between 4 and 12 GW reported no difference in tHcy between miscarriage cases and controls [16]. There were two important differences between that study and ours. Firstly, it only had 12 miscarriage cases. It is unclear whether it was sufficiently powered to detect a difference in tHcy between cases and controls, if it existed. Furthermore, RBC folate status in general was higher in that study than in ours. Secondly, no details regarding timing, types or potential causes of miscarriage were provided. To the best of our knowledge, the other study with tHcy measurements nearest to the miscarriage was in patients being treated for the miscarriage [17]. That study by Gris et al., was large and had blood samples and ultrasound confirmation of the miscarriage close to the time of the event. Miscarriages occurring late in pregnancy or due to chromosomal abnormalities or maternal infection were also excluded from that study. Our findings confirm their findings that miscarriage risk was increased with increasing tHcy concentrations. They reported a twofold increase in risk for tHcy $\geq$ 9.9 μmol/L. This effect size is similar to our observation regarding tHcy $\geq$ 7.1 μmol/L.

4.3. Interpretation

The mechanism for the association between tHcy and miscarriage warrants investigation. It is possible that elevated tHcy in our study is marking impaired folate status. The most important predictors of first trimester tHcy were RBC folate, followed by the *MTHFR* 677 TT genotype. We previously reported in a population study from the same region that adults with the *MTHFR* 677 TT genotype had lower folate status (both plasma and RBC folate) as well as higher tHcy than their CC or CT genotype counterparts [6]. We also observed in that same study that 18.8% of the participants had folate deficiency. In contrast to widespread folic acid use in the Reus Tarragona Birth Cohort, the population study did not include folic acid users. Nevertheless, there is no mandatory fortification with folic acid in Spain and most participants in the Reus Tarragona Birth Cohort did not initiate folic acid supplementation until they were pregnant [24]. Use of the recommended dose of folic acid supplements and plasma folate status were lower in cases than in controls and the percentage of cases with RBC folate below the threshold recommended by the WHO to prevent neural tube defect affected pregnancies, was higher in cases than in controls.

Impaired one carbon metabolism due to low folate status, the *MTHFR* 677C>T polymorphism or other polymorphisms affecting the role of folate or other nutrients in the one carbon metabolic network have been associated with adverse outcomes stemming from anomalies in early pregnancy [5]. It is possible that the physio pathological mechanism leading to embryo developmental abnormalities, impaired placentation and foetal growth is shared, at least in part, in pregnancies affected by suboptimal one carbon metabolism. Impaired chorionic vascularisation in spontaneous miscarriage tissue from women with history of recurrent pregnancy loss and with tHcy >18.3 μmol/L was reported in a study by Nelen et al. [25]. It is also possible that anomalies in DNA methylation and other epigenetic processes arising from impaired one carbon metabolism are involved. However, further research in this field is required to explore and establish the associations between early pregnancy folate status and tissue-specific outcomes, their impact and replication between studies.

4.4. Strengths and limitations

Strengths of this study were that pregnancy was confirmed by ultrasound scan and tHcy was determined before the miscarriage occurred. Few previously reported studies have achieved these measurements due to the difficulty in obtaining them. Late miscarriage cases (caused by infections or foetal developmental abnormalities) as well as miscarriages due to known causes such as chromosomal abnormalities were also excluded. Strictly, fasting blood samples and confirmation of pregnancy by ultrasound scan before 12 GW were required for eligibility to be included in the study. The study was designed to measure first trimester tHcy as a potential biomarker of adverse pregnancy outcome and blood samples were processed in strict adherence to protocol to prevent homocysteine release from blood cells [26].

Furthermore, RBC folate concentration was determined and is indicative of folate reserves during preconception and the start of pregnancy. It is spared the effects of haemodilution and the initial effects of folic acid supplement use (unlike plasma folate concentration and tHcy, which are sensitive to folic acid supplement use at the time of the blood draw).

Estimation of time of miscarriage in “missed” spontaneous abortions based on changes in transvaginal ultrasound measurements of crown-rump length or parietal circumference of the embryo are susceptible to error depending on time elapsed between cessation of foetal heartbeat and the performance of the scan. Gestational age based on reported date of the last menstrual period by the participants is also subject to error. However, we were able to minimise these errors due to the prospective nature of the study and recording of the timing of the blood samples and ultrasound scans. We stipulated that any miscarriage suspected to have occurred more than 7 days before the blood samples would be excluded.

4.5. Implications

This study shows that in the absence of mandatory fortification with folic acid, women not adhering to the recommended intake of 400 μg/d from folic acid supplements, are more likely not to meet the threshold RBC folate status proposed to offer protection against folate sensitive neural tube defects. This study shows that RBC folate status below this threshold also increases the risk of miscarriage. Smoking was negatively associated with RBC folate status. These results indicate that the message regarding the importance of periconception folic acid in the prevention of adverse pregnancy outcome needs to be reinforced, and especially in smokers.

5. Conclusions

Moderately elevated early pregnancy tHcy is associated with 2.5

times more risk of early miscarriage, of unknown cause. The results provide evidence to support the consideration of early pregnancy tHcy as a potential biomarker of adverse pregnancy outcome.

Author contributions

MMM, PC-B and JDF-B designed the research; PC-B, MI-P, MB were responsible for the clincial aspects of the study; JH-B, AR, CR-R, MMM were responsible for coordinating the field work of the study as well as data and sample collection, processing and biobanking; PMU and KM were responsible for the biochemical and genetic determinations carried out at Bevital AS; PC-B, MI-P, MMM, JF-B, JH-B, AR-G and CR-R analysed the data and wrote the manuscript; MMM and PC-B had primary responsibility for the final content.

Declaration of competing interest

None to declare.

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GENETIC AND METABOLIC ALTERATIONS IN MATERNAL AND PATERNAL ONE CARBON METABOLISM AND DEVELOPMENT OF PREGNANCY COMPLICATIONS OF PLACENTAL ORIGIN
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