PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES

Jose Maria Colomina Muela

UNIVERSITAT ROVIRA i VIRGILI

Prenatal one carbon metabolism-gene interactions, placenta trace element content and their effect on pregnancy outcomes

Jose Maria Colomina Muela

DOCTORAL THESIS

2017

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

Jose Maria Colomina Muela

Prenatal one carbon metabolism-gene interactions, placenta trace element content and their effect on pregnancy outcomes

Doctoral thesis

Thesis supervised by Dr. Michelle Murphy.

Department of Basic Medical Sciences

Reus

2017

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

UNIVERSITAT ROVIRA I VIRGILI

Unitat de Medicina Preventiva i Salut Pública
Departament de Ciències Mèdiques Bàsiques
Facultat de Medicina i Ciències de la Salut
Carrer Sant Llorenç, 21
43201 – Reus (977 759 306)

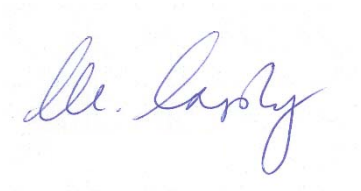
FAIG CONSTAR que aquest treball, titulat "**Prenatal one carbon metabolism-gene interactions, placenta trace element content and their effect on pregnancy outcomes**", que presenta el **Jose Maria Colomina Muela** per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de **Ciències Mèdiques Bàsiques** d'aquesta universitat.

HAGO CONSTAR que el presente trabajo, titulado "**Prenatal one carbon metabolism-gene interactions, placenta trace element content and their effect on pregnancy outcomes**", que presenta **Jose Maria Colomina Muela** para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento de **Ciencias Médicas Básicas** de esta universidad.

I STATE that the present study, entitled "**Prenatal one carbon metabolism-gene interactions, placenta trace element content and their effect on pregnancy outcomes**", presented by **Jose Maria Colomina Muela** for the award of the degree of Doctor, has been carried out under my supervision at the Department of **Basic Medical Sciences** of this university.

Reus, 3 d'abril de 2017
Reus, 3 de abril de 2017
Reus, 3rd April 2017

El/s director/s de la tesi doctoral
El/los director/es de la tesis doctoral
Doctoral Thesis Supervisor/s



Dr. Michelle Murphy

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

Acknowledgments

This work was supported by funding from:

- Instituto de Salud Carlos III. FIS: PI 10/00335
- Instituto de Salud Carlos III/ Fondo FEDER. FIS: 13/02500
- Centros de Investigación Biomédica en Red (CB07/03/2004)
- Comisión interministerial de Ciencia y Tecnología de España. SAF: 2005-05096
- Italfarmaco (2005-2008)
- AGAUR: 2009 SGR 1237; 2014 SGR 332
- Institut d'Investigació Sanitària Pere i Virgili: 2010/IISPV/21
- Universitat Rovira i Virgili: 2013PMF-PIPF-58

I must give my acknowledgements to all the people without whom this work would have not being possible. All of you, mentioned and unmentioned along the following lines, have contributed to this thesis and have the place in my heart you deserve.

To the staff of the Areas of Obstetrics and Gynaecology of the University Hospitals Sant Joan de Reus and Joan XXIII de Tarragona, especially to their heads Dr Pere Cavallé-Busquets and Dr Ramon Miralles.

To the staff of the emergency laboratory, and nurse assistants and nurses of the "Área de Extracciones" of the University Hospital Sant Joan de Reus. To the staff of the research laboratory of the University Hospital Joan XXIII de Tarragona, and of the Institut d'Investigació Sanitària Pere Virgili (IISPV) Biobank.

To the staff of the Laboratory of Toxicology and Environmental Health (IISPV, Universitat Rovira i Virgili) and Centres Científics i Tecnològics (Universitat de Barcelona), especially to Dr Martí Nadal. To the staff of the Department of Basic Medical Sciences (Universitat Rovira i Virgili), especially my PhD supervisor Dr Michelle Murphy.

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

Abbreviations

Abbreviation[1]	Definition, synonyms
1C	One carbon
AHCY	Adenosylhomocysteinase, S-adenosyl homocysteine hydrolase
AGA	Appropriate for gestational age
ALDH7A1	Betaine aldehyde dehydrogenase
ATP7A	ATPase copper transporting alpha, MNK
ATP7B	ATPase copper transporting beta, WND
B_2	Riboflavin
B_6	Pyridoxine
B_{12}	Cobalamin, cbl
BHMT	Betaine-homocysteine methyltransferase
BMI	Body mass index
CBS	Cystathionine-beta-synthase
CD320	Transcobalamin 2 receptor
CDP-choline	Cytidine diphosphate-choline
CGL	Cystathionine-gamma-lyase, CTH
CHDH	Choline dehydrogenase
CCiT-UB	Centres Científics i Tecnològics - Universitat de Barcelona
CVD	Cardiovascular disease
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DMG	Dimethylglycine
DMGDH	Dimethylglycine dehydrogenase
DNMTs	DNA methyltransferases
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine monophosphate
d	Day
dw	Dry weight
EC	Enzyme commission
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
FOLH1	Folate hydrolase 1, glutamate carboxypeptidase II, GCPII
FOLRs	Folate receptors
FPGS	Folypolyglutamate synthetase
GABA	Gamma-aminobutyric acid

GC-MS	Gas chromatography - mass spectrometry
GD	Gestational day
GGH	Gamma glutamyl hydrolase
GIF	Gastric intrinsic factor
GW	Gestational weeks
GWAS	Genome wide association study
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled plasma - mass spectrometry
ICP-OES	Inductively coupled plasma - optic emission spectrometry
IDL	Instrument detection limit
IUGR	Intrauterine growth restriction
LBW	Low birth weight
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
LOD	Limit of detection
MALDI-TOF MS	Matrix-assisted laser desorption/ionization mass spectrometry
MATs	Methionine adenosyltransferases
MDL	Method detection limit
MTs	Methyltransferases
MTHFDs	Formyltetrahydrofolate synthetases, methenyltetrahydrofolate cyclohydrolases, methylenetetrahydrofolate dehydrogenases
MTHFR	Methylenetetrahydrofolate reductase
MTR	Methionine synthase, 5-methyltetrahydrofolate-homocysteine methyltransferase
MTRR	Methionine synthase reductase, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase
NAD^+/NADH	Nicotinamide adenine dinucleotide
$NADP^+$/NADPH	Nicotinamide adenine dinucleotide phosphate
NHANES	National Health and Nutrition Examination Survey
NTDs	Neural tube defects
PEMT	Phosphatidylethanolamine methyltransferase
PTFE	Polytetrafluoroethylene
RBC	Red blood cell
RDA	Recommended dietary allowance
RTBC	Reus-Tarragona Birth Cohort
SAH	S-adenosyl homocysteine, AdoHcy
SAM	S-adenosyl methionine, AdoMet
SARDH	Sarcosine dehydrogenase

SGA	Small for gestational age
SHMTs	Serine hydroxymethyltransferases
SLC5A7	High affinity choline transporter 1, CHT1
SLC6A12	GABA/betaine transporter
SLC6A20	IMINO transporter
SLC11A2	Divalent cation transporter 1
SLC19A1	Reduced folate carrier, RCF
SLC22As	Organic cation transporters, OCTs/OCTNs
SLC30As	Zinc transporters ZNT
SLC31A1	Copper transporter 1, CTR1
SLC39As	Zinc transporters ZIP
SLC40A1	Ferroportin
SLC44As	Choline transporter-like proteins, CTLs
SLC46A1	Proton coupled folate transporter, PCFT
SNP	Single nucleotide polymorphism
SOD1	Cu/Zn superoxide dismutase, superoxide dismutase 1
SOD3	Extracellular superoxide dismutase, superoxide dismutase 3
TCN1	Haptocorrin
TCN2	Transcobalamin
THF	Tetrahydrofolate
tHcy	Total homocysteine concentration
TYMS	Thymidylate synthetase
UL	Tolerable upper intake level
URV	Universitat Rovira i Virgili
WHO	World health organization
wk	Weeks
ww	Wet weight
y	Years
ZTT	Zinc tolerance test

[1] Protein abbreviations are written in bold.

Genetic polymorphism nomenclature

Polymorphism	Synonym	Reference SNP	Definition
MTHFR c.665C>T	*MTHFR* 677C>T	rs1801133	Methylenetetrahydrofolate reductase c.665C>T
BHMT c.716G>A	*BHMT* 742G>A	rs3733890	Betaine-homocysteine methyltransferase c.716G>A
SLC19A1 c.80G>A	*SLC19A1* 80G>A	rs1051266	Reduced folate carrier c.80G>A
MTRR c.66A>G	*MTRR* 66A>G	rs1801394	Methionine synthase reductase c.66A>G

List of figures

List of tables

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

List of publications resulting from the thesis

- Colomina JM, Cavallé-Busquets P, Fernàndez-Roig S, Solé-Navais P, Fernandez-Ballart JD, Ballesteros M, Ueland PM, Meyer K, Murphy MM.
  Maternal Folate Status and the BHMT c.716G>A Polymorphism Affect the Betaine Dimethylglycine Pathway during Pregnancy.
  Nutrients 2016, 8(10), 621; doi:10.3390/nu8100621. PMID: 27735840
  ISSN 2072-6643

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

Table of contents

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

Abstract

One carbon (1C) metabolism is highly important in foetal development, maternal-placental-foetal unit development and function, and optimal pregnancy outcome. The folate and methionine cycles, betaine-homocysteine methyltransferase (BHMT) pathway and related reactions of 1C unit transfer are key components of the 1C metabolic network. These pathways interact and their activity may be modulated by status in the nutrients that regulate them. Nutrients involved in 1C metabolism (including amino acids, osmolytes, B vitamins, nitrogenous bases, phospholipids and methyl donors) are essential for cell proliferation, metabolism, regulation and differentiation. The trace elements, zinc, copper, selenium and iron are also essential. Enzymes and other proteins containing these trace elements are involved in processes such as oxygen transport, respiratory chain, metal detoxification and oxidative stress. The effects of frequent 1C metabolism genetic polymorphisms on 1C metabolism components or whether they are modulated by folate are not clearly understood, especially during pregnancy. There is much interest in the effect of folate because mandatory fortification of flour with folic acid has been implemented in numerous countries but not European countries, among others. Placenta functions include nutrient and metabolite transport, defence against toxicity, as well as endocrine functions. The role of the above trace elements in the placenta is unclear and evidence on determining factors of their concentrations in the placenta is inconsistent. No previous study has investigated whether 1C metabolism affects these.

The aims of the thesis are to investigate:

1) the effects of frequent maternal 1C metabolism polymorphisms (methylenetetrahydrofolate reductase *MTHFR* c.665C>T, betaine-homocysteine methyltransferase *BHMT* c.716G>A, reduced folate carrier *SLC19A1* c.80G>A and methionine synthase reductase *MTRR* c.66A>G) on related metabolite concentrations in blood during pregnancy and whether these effects are modulated by folate status.

Abstract

2) whether lifestyle, obstetrical and demographic parameters and 1C metabolism affect placental concentrations of zinc, copper, selenium and iron.

3) whether these polymorphisms or placenta trace element concentrations are associated with adverse pregnancy outcomes such as miscarriage and intrauterine growth restriction (IUGR), or preterm delivery and IUGR.

The study was based on 617 pregnancies from the Reus-Tarragona Birth Cohort (RTBC), a longitudinal observational pregnancy study including a subset of 212 pregnancies from which placentas were collected plus other 6 placentas of participants recruited at labour with focus on adverse pregnancy outcomes. Lifestyle, biological and obstetrical data was collected from the first trimester throughout pregnancy by questionnaires and from prenatal check-ups. Fasting blood samples were collected at ≤12, 15, 24-27 and 34 gestational weeks (GW). Blood samples from the mother and cord were also collected at labour. Red blood cell (RBC) and plasma folate, and plasma cobalamin were determined by microbiological assays. Gas chromatography - mass spectrometry was used for plasma total homocysteine (tHcy) concentrations, and liquid chromatography - tandem mass spectrometry for plasma betaine, dimethylglycine (DMG), choline and cotinine. The genotypes of the four polymorphisms were determined in maternal and cord leukocyte DNA by matrix-assisted laser desorption/ionization mass spectrometry. Placenta concentrations of zinc, copper and selenium were determined by inductively coupled plasma - mass spectrometry, and those of iron by inductively coupled plasma - optic emission spectrometry.

The tHcy enhancing effect in *MTHFR* c.665 variant homozygotes was modulated by RBC folate at ≤12 GW and by plasma folate from 15 GW on and in the cord: the *MTHFR* c.665TT genotype was not associated with elevated (tHcy) during pregnancy when folate status was high. We suggest the modulation by RBC or plasma folate at different stages of pregnancy could be due to the pattern of folic acid supplement use. The *BHMT* c.716 variant genotypes were associated with lower DMG concentration and ratio

DMG/betaine in late pregnancy, but in early pregnancy this was found only with high plasma folate status. We speculate that the activity of the variant BHMT might be lower at the extremes of the folate status distribution. Plasma folate was an independent negative predictor of plasma DMG, supporting the idea that the BHMT pathway is upregulated under low folate status conditions. Plasma/RBC folate and tHcy did not vary with *SLC19A1* c.80G>A genotype. Higher plasma tHcy was found in *MTRR* c.66 variant homozygotes only in early pregnancy. After stratification by folate status the effect was lost in the lowest and highest plasma folate tertiles. We suggest the absence of effect in early pregnancy with low folate status and in mid-late pregnancy might be due to upregulation of the BHMT pathway. We also suggest the absence of effect in early pregnancy with high folate status could be due to reduction of this effect by higher S-adenosyl methionine (SAM) and lower S-adenosyl homocysteine (SAH). More IUGR neonates had the *MTHFR* c.665 normal homozygote genotype compared to other genotypes. A speculative explanation for this might be that embryos with variant genotypes are less likely to survive when combined with factors leading to IUGR.

Lower zinc concentrations were found in placentas of male than female neonates. Although the reason is not clear a speculative explanation is that it may be the *NBDY* g.56811695C>T polymorphism in the X chromosome (important for zinc status in RBCs). Users of supplements containing any of the 4 trace elements in the second and third trimesters had lower placenta copper concentration, and given that most of these supplements contain only iron, we suggest non-haem iron intake redistributes copper from the placenta to the intestine to increase ferroxidase activity at that location. Smoking during pregnancy was associated with higher placenta concentrations of copper and selenium. Birth weight was negatively correlated with placenta zinc, copper and selenium, however, after adjustment this was true only for copper. Unlike selenium, copper was associated with higher IUGR risk after adjustment. In addition to the associations with smoking and birth weight, placenta zinc, copper and selenium were positively correlated and not associated with total, food or supplement intake. All this led us to suggest the levels of these 3 trace elements in the placenta depend mostly

on overlapping functions, namely oxidative stress and metal toxicity defence. 1C metabolism was only associated with placenta copper. Plasma cobalamin in early pregnancy was negatively associated with placenta copper and late pregnancy tHcy was positively associated with it. Both associations are unclear but we suggest the mediator of the positive association between tHcy and copper is the placental adenosylhomocysteinase enzyme.

As major conclusions, with the exception of *SLC19A1* c.80G>A, the addressed polymorphisms affect 1C metabolism during pregnancy and their effects are modulated by folate status in different ways in each case. Some of the analysed factors were associated with the 4 trace elements in the placenta, but multivariate models explained very little variability in the observed concentrations. Therefore, other unknown factors, or factors not considered in these models must be involved. The normal allele for *MTHFR* c.665 in the offspring and placenta copper concentration were positively associated with IUGR risk.

INTRODUCTION

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

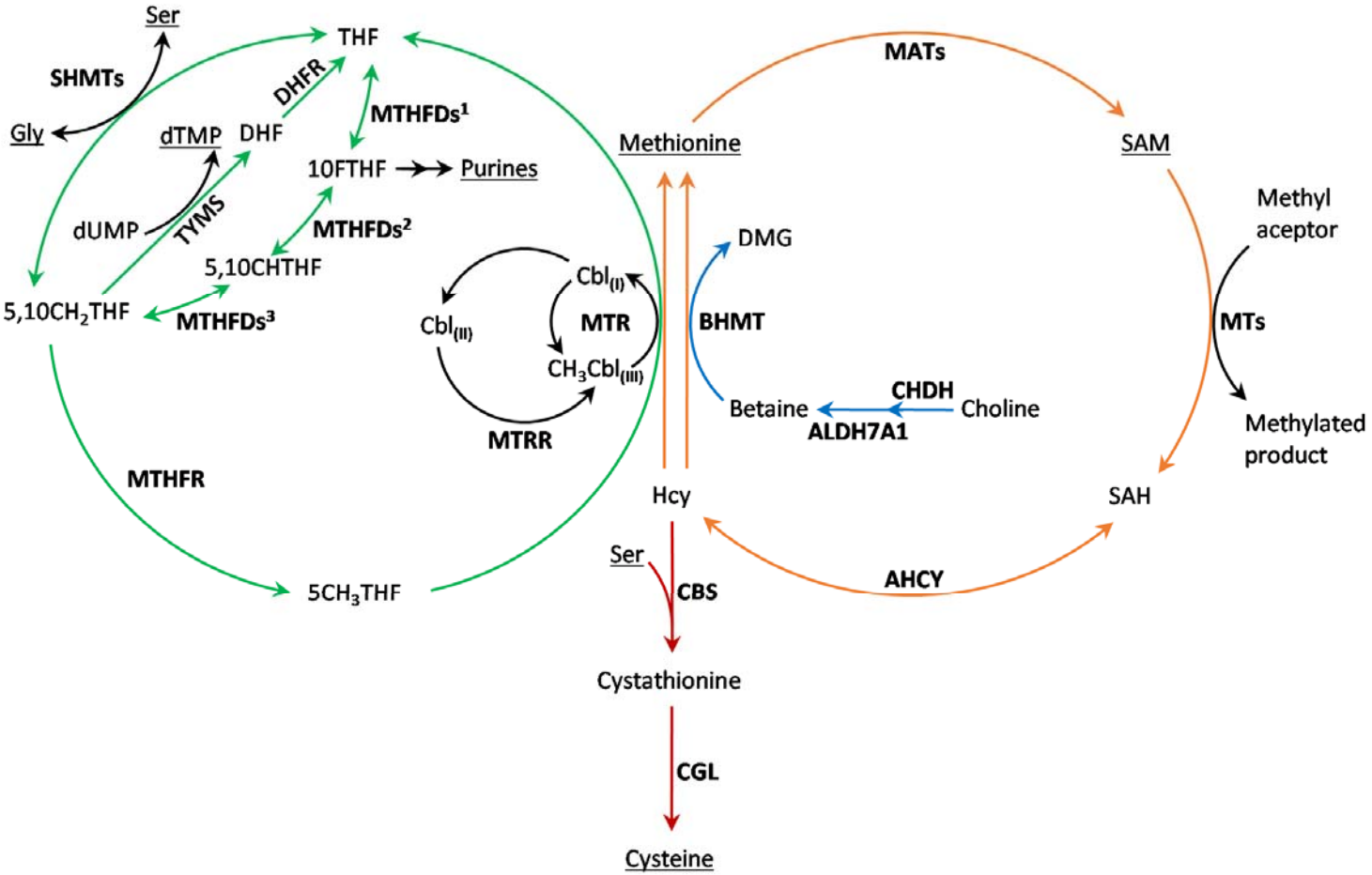
Introduction

1. Overview of one carbon metabolism.

The one carbon (1C) network involves the intracellular transfer of 1C units between molecules (**Figure 1**). These transferable 1C units occur in the forms of formimino (CHNH), formyl (CHO), methylene (CH_2) and especially methyl (CH_3) groups. One carbon donors include amino acids, folates, S-adenosyl methionine (SAM), betaine and dimethylglycine (DMG), among others. Formyl groups can be acquired directly from formate into tetrahydrofolate (THF) in a reaction catalysed by formyltetrahydrofolate synthetases (MTHFDs). 1C metabolism includes the folate cycle, methionine cycle as well as the choline oxidation pathway (terminating with the betaine-homocysteine methyltransferase pathway) and related reactions of 1C transfer. The availability of 1C units is necessary for cell proliferation and metabolism (Molloy 2012), and therefore it is regarded as highly important in pregnancy for embryo/foetal development. Derangements in 1C metabolism have been associated with impaired foetal growth and developmental abnormalities such as neural tube defects. Purines and pyrimidines (from deoxythymidine) derived from 1C metabolism are necessary for DNA and RNA synthesis, and hence for DNA replication, repair and expression. In the 1C metabolic network and transulphuration pathway amino acids are interconverted for protein synthesis. The universal methyl donor SAM can be produced in the methionine cycle subsequently to the homocysteine remethylation to methionine, or from dietary methionine. Homocysteine remethylation can occur by the cobalamin and folate dependent reaction catalysed by the ubiquitous methionine synthase (MTR); or by the betaine dependent reaction catalysed by betaine-homocysteine methyltransferase (BHMT), a minor pathway limited to the liver and kidney (Finkelstein 1990). Three SAMs are necessary for the complete methylation of phosphatidylethanolamine to phosphatidylcholine (a glycerophospholipid with choline in the headgroup) (Vance and Ridgway 1988), which accounts for more than 50% of the phospholipids in mammalian cell membranes (Zeisel 2006). SAM is also involved in the postranslational

modifications of proteins, specifically in protein methylation that can occur in histone and non-histone proteins. The methyl groups for *de novo* and maintenance DNA methylation, as well as for structural and regulatory methylation of RNA, are provided by SAM. Histone and DNA methylation form part of the epigenetic regulation of gene expression, hence determining embryo cell differentiation and specialisation. Epigenetic marks have been shown to be relatively stable and to be heritable and so have been proposed as a foetal programming mechanism , i.e. predisposition to specific phenotypes resulting from exposure to the intrauterine environment (Reynolds et al. 2013). The metabolism and specific roles of homocysteine, folate, cobalamin and BHMT pathway metabolites will be discussed in following sections.

Figure 1. Folate and methionine cycles, BHMT, transulphuration and related pathways.



Scheme of folate cycle (green arrows), methionine cycle (orange arrows), choline oxidation pathway (blue arrows), transulphuration pathway (red arrows) and other related pathways (black arrows). Enzymes are written in bold, substrates and products are not. Substrates and products involved in DNA, RNA and protein synthesis, and methylation reactions are underlined. Enzymes [vitamin cofactor]: ALDH7A1, betaine aldehyde dehydrogenase; AHCY, adenosylhomocysteinase; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine-beta-synthase [B_6]; CGL, cystathionine-gamma-lyase [B_6]; CHDH, choline dehydrogenase [B_2]; DHFR, dihydrofolate reductase; MATs, methionine adenosyltransferases; MTHFDs, formyltetrahydrofolate synthetases[1], methenyltetrahydrofolate cyclohydrolases[2], methylenetetrahydrofolate dehydrogenases[3]; MTHFR, methylenetetrahydrofolate reductase [B_2]; MTR, methionine synthase [B_{12}]; MTRR, methionine synthase reductase [B_2]; MTs, methyltransferases; SHMTs, serine hydroxymethyltransferases [B_6]; TYMS, thymidylate synthetase. Metabolites: $Cbl_{(I/II)}$, cobalamin ($1^+/2^+$ oxidation state); $CH_3Cbl_{(III)}$, methylcobalamin (3^+ oxidation state); DHF, dihydrofolate; DMG, dimethylglycine; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; Gly, glycine; Hcy, homocysteine; SAH, S-adenosyl homocysteine; SAM, S-adenosyl methionine; Ser, serine; THF, tetrahydrofolate; 10FTHF, 10-formylTHF; 5,10CHTHF, 5,10-methenylTHF; $5,10CH_2THF$, 5,10-methyleneTHF; $5CH_3THF$, 5-methylTHF. Information adapted from (Finkelstein 1990; Olteanu and Banerjee 2001; Stover 2010b; Vazquez et al. 2013; Institute of Medicine 2016b).

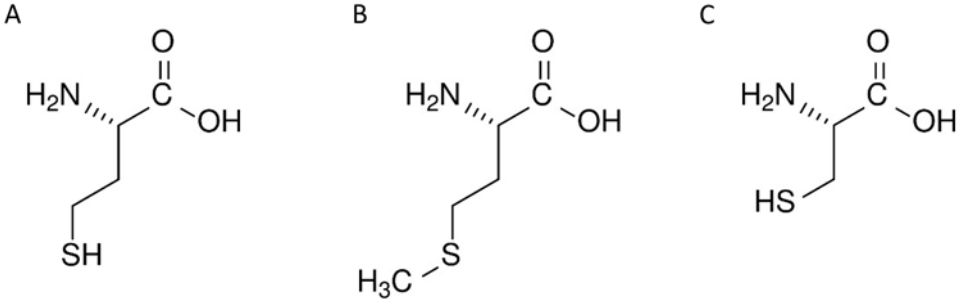
2. One carbon metabolism nutrients, metabolites and cofactors previously shown to affect pregnancy

2.1. Homocysteine

Structure, transport and metabolism

Homocysteine is a sulphydryl-containing amino acid with a molecular structure similar to methionine and cysteine (**Figure 2**).

Figure 2. Sulphur-containing amino acids.



A. Homocysteine. B. Methionine. C. Cysteine. Adapted from (Institute of Medicine 2016a).

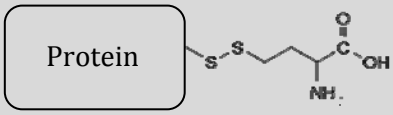
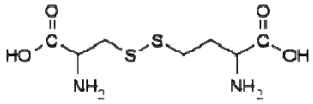
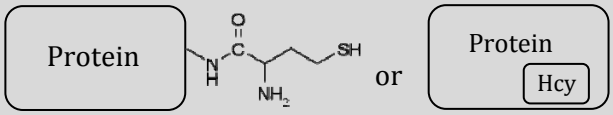
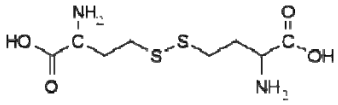
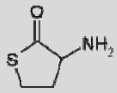
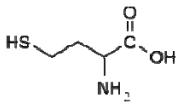
Homocysteine can be remethylated to methionine in the methionine cycle, which intersects with the folate cycle and with the BHMT pathway (*Introduction* section *1*, **Figure 1**). Homocysteine is also metabolised in the transulphuration pathway (**Figure 1**, red arrows), which is pyridoxine dependent and in which the first enzyme of the pathway, cystathionine-beta-synthase (CBS), catalyses a reaction that is irreversible under most *in vivo* conditions (Finkelstein 1990). Homocysteine and serine are converted to cystathionine by cystathionine-beta-synthase, and cystathionine to cysteine, alpha-ketobutyrate and NH_3 in a reversible reaction catalysed by cystathionine-gamma-lyase (CGL) (Matsuo and Greenberg 1959).

Several forms of homocysteine are transported in the blood due to its ability to form disulphides or bind to proteins (**Table 1**). In human plasma, two homocysteine

molecules can be oxidised to form the disulphide homocystine, or one molecule of homocysteine and one cysteine form a mixed disulphide by a mechanism that involves albumin. It is estimated that 20% of these albumin-mediated oxidations are due to albumin-bound copper and 80% through albumin-S-S-cysteine or albumin-S-S-homocysteine by thiol/disulphide exchange (Sengupta et al. 2001). The posttranslational and covalent binding of homocysteine to proteins is called homocysteinylation. In S-homocysteinylation a protein-S-S-homocysteine adduct is formed by the reaction of the thiol group (-SH) of one protein cysteine residue and that of homocysteine. Hydrogen peroxide can be formed in S-homocysteinylation if oxygen is the electron acceptor (Jacobsen 2001). In N-homocysteinylation, the side chain amino group ($-NH_2$) of lysine residues in a protein react with the activated carboxyl of the homocysteine derivative homocysteine thiolactone, resulting in the linkage of homocysteine to a protein via an amide bond (Jakubowski 1997).

Some reported plasma concentration values of different homocysteine forms are shown in **Table 1**. Most of it is transported as protein S-linked homocysteine, especially albumin (Refsum et al. 1985). Less as cysteine-homocysteine mixed disulphide (Gupta and Wilcken 1978), and only trace amounts as the disulphide homocystine (Murphy-Chutorian et al. 1985), protein N-linked homocysteine (Uji et al. 2002; Perna et al. 2006) and reduced homocysteine (Araki and Sako 1987). Homocysteine thiolactone is either absent or found in trace amounts (Chwatko and Jakubowski 2005). According to *in vitro* experiments (Jakubowski 2001), from the total of protein N-linked homocysteine half is incorporated in the backbone of the proteins and the rest as N-homocysteinylation.

Table 1. Homocysteine forms and mean concentrations in human plasma.

Form	Molecule	µmol/L
Protein S-linked homocysteine		6.51♂, 7.29♀[1]
Cys-Hcy disulphide		3.25[2]
Protein N-linked homocysteine		0.35[3]-0.51[4]
Hcy-Hcy disulphide		0.06[5]
Homocysteine thiolactone		< 0.003[6]
Free reduced homocysteine		0.002[7]

Cys, cysteine. Hcy, homocysteine. References: [1](Refsum et al. 1985), [2](Gupta and Wilcken 1978), [3](Perna et al. 2006), [4](Uji et al. 2002), [5](Murphy-Chutorian et al. 1985), [6](Chwatko and Jakubowski 2005), [7](Araki and Sako 1987).

The term total homocysteine (tHcy) refers to the homocysteine that can be determined after a reducing treatment aimed to break disulphide bonds (Refsum et al. 2004); therefore tHcy includes homocysteine molecules that are, prior to the treatment, free homocysteine, homocysteine dishulphides and homocysteine bound to proteins and other molecules by disulphide bonds (but not by amide bonds). Throughout the results of this thesis, with the exception of samples at labour, tHcy will refer to fasting plasma concentrations.

Physiological and clinical relevance

Hyperhomocysteinaemia has been defined as plasma tHcy between 15-30μmol/L (mild), 30-100μmol/L (intermediate) and ≥100μmol/L (severe) (Vollset et al. 2001); and homocystinuria, caused by an inborn error of metabolism is characterised by markedly elevated concentrations of plasma or serum tHcy (Mudd et al. 2000).

In spite of homocysteine being an intermediary in the methionine cycle and a precursor in the synthesis of cysteine by the transulphuration pathway, in excess it is considered to be harmful due to its associations with many pathological conditions and diseases. It has not yet been established whether homocysteine is a cause, consequence or biomarker of the diseases with which it has been associated. Although limited to atherosclerosis and cardiovascular disease, the main mechanisms proposed for a causal role include homocysteinylation, impairment of nitric oxide functions, hypomethylation and oxidative stress.

Homocysteinylation has negative effects on protein on most of the tested proteins which may lead to pathologies, but this has only be assessed in *in vitro* experiments. S-homocysteinylation alters the functions of proteins from plasma (Harpel et al. 1992; Majors et al. 2002; Zinellu et al. 2015), extracellular matrix (Bescond et al. 1999; Hubmacher et al. 2005) and plasmatic membrane (Hajjar et al. 1998). In the cytoplasm, S-homocystenylated metallothioneins have impaired zinc-binding function and inhibited ability to scavenge superoxide anion radicals (Barbato et al. 2007). N-homocysteinylated proteins have altered function (Jakubowski 1999; Zinellu et al. 2015), structure (Paoli et al. 2010), degradation rate (Jakubowski 2004) and antigenic properties (Undas et al. 2004; Jakubowski 2006). It has been proposed that N-homocysteinylation could affect folate receptors (FOLRs), proteins involved in folate uptake, causing the consequent impairment of function due to autoantibodies binding and leading to birth defects that have been associated with low folate status (Taparia et al. 2007; Wallis et al. 2010).

Nitric oxide regulates activity and function of proteins, e.g. activation of DNA methyltransferases (DNMTs) (Hmadcha et al. 1999) and thioredoxin 1 (Haendeler et al. 2002), inactivation of MTR (Danishpajooh et al. 2001) and methionine adenosyltransferases (Ruiz et al. 1998; Pérez-Mato et al. 1999), and release of zinc from metallothioneins (Pearce et al. 2000). Homocysteine could reduce or increase nitric oxide effects through their binding (Gow et al. 2001). Plasma nitric oxide and tHcy are negatively correlated *in vivo* (Selley 2003) and homocysteine reduces nitric oxide levels in human endothelial cells and atherosclerosis model mice (Chen et al. 2014).

Homocysteine can indirectly affect methylation reactions. Adenosylhomocysteinase (AHCY) catalyses the reversible reaction: adenosine + homocysteine ⇄ S-adenosyl homocysteine (SAH) (Prigge and Chiang 2001), and although the synthetic direction is favoured, the efficient removal of homocysteine and adenosine in the organism makes the reaction proceed in the hydrolytic direction (Cantoni 1985). This is especially important because most methyltransferases are inhibited by SAH (Clarke and Banfield 2001). Therefore, impairment in the homocysteine removal mechanisms can lead to an increase in SAH, and this to higher inhibition of methyltransferases. This idea is supported by the associations found in a sample of healthy women between plasma tHcy, plasma SAH but not SAM, and lymphocytes DNA hypomethylation (Yi et al. 2000). The importance of an indirect hypomethylating effect of homocysteine may depend on specific cell types. For example, cell culture media with the addition of homocysteine as 50μM has been associated with higher SAH/SAM ratio and the hypomethylation of specific proteins, in endothelial but not smooth muscle cell lineages (Wang et al. 1997). The authors consider the specific hypomethylation detected in these experiments can occur in other proteins as well (Yang et al. 2005).

If oxygen is the electron acceptor, homocysteine can form superoxide radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) during S-homocystenylation, autooxidation and oxidation with cysteine or other thiols (Jacobsen 2001). The binding of homocysteine to nitric oxide can also produce the reactive oxygen species superoxide (Gow et al. 1997). Some

authors question the role of homocysteine as an oxidant given that: 1) the physiological concentrations of homocysteine are relatively low compared to those of cysteine, which undergoes similar oxidations while being less toxic; and 2) the abundance of antioxidants and antioxidant enzymes might be enough to compensate homocysteine induced oxidative stress (Jacobsen 2000). However, antioxidants can ameliorate the negative effects of homocysteine. Although one-week supplementation with ascorbic acid does not reduce post-methionine load homocysteine, it does reduce the impairment of the arterial flow-mediated dilatation (Chambers et al. 1999). This is mediated by nitric oxide release (Joannides et al. 1995) and its impairment is related to plasma homocysteine (Chambers et al. 1999). In addition, homocysteine seems to dysregulate antioxidant enzymes. The plasma concentration of extracellular superoxide dismutase (SOD3), with copper and possibly zinc cofactors to catalyse the removal of $O_2^{\cdot-}$, is highly positively correlated with tHcy in treated homocysteinuric patients (Wilcken et al. 2000). The activity of extracellular superoxide dismutase in plasma of homocysteine treated rats to mimic a homocysteinuric state is higher than in control animals (Matté et al. 2009). On the other hand, *in vitro* experiments have shown the downregulation of mRNA expression (Upchurch et al. 1997) and activity (Nishio and Watanabe 1997) of the selenoproteins glutathione peroxidases, which catalyse the removal of lipid and hydrogen peroxides with glutathione. Glutathione peroxidase activity is also lower in brain extracts of homocysteinuric rats (Matté et al. 2009). Homocysteine has been shown to downregulate catalase activity, another antioxidant enzyme and, a haemoprotein involved in the removal of H_2O_2, in neuronal (Milton 2008) but not in aortic smooth muscle cell cultures (Nishio and Watanabe 1997). In these rat models of a homocysteinuric state, catalase activity was lower and higher in brain extracts or plasma, respectively, compared with the control group (Matté et al. 2009). Although with many unanswered questions, it seems that as previously proposed for vascular cells (Jacobsen 2000), homocysteine generates oxidative stress due to mechanisms other than thiol oxidation.

Other possible homocysteine toxicity mechanisms include the inhibition of DNA synthesis (as less incorporation of marked thymidine) as a possible growth inhibitory effect in endothelial cell experiments (Wang et al. 1997; Yang et al. 2005). However, it is likely that in some pathological conditions associated with homocysteine, the underlying mechanisms are due to impaired 1C metabolism as will be explained later. In these situations, homocysteine is regarded as a global biomarker of 1C metabolism, although it can be a biomarker of the transulphuration pathway as well. Other factors can affect tHcy levels. Lower glomerular filtration rate (decreased renal function), higher serum creatinine and albumin concentrations are associated with higher plasma tHcy concentrations (Wu et al. 1994; Brattström et al. 1994; Lussier-Cacan et al. 1996; Wollesen et al. 1999). Male sex, increasing age, hypothyroidism, late diabetes, leukaemia and psoriasis are positive determinants, and hyperthyroidism and early diabetes are negative determinants of tHcy (Refsum et al. 2004).

Total homocysteine increase have been associated with various diseases and pathological conditions such as Alzheimer disease (Shen and Ji 2015), schizophrenia (Nishi et al. 2014), multiple sclerosis (Zhu et al. 2011), obstructive sleep apnoea (Niu et al. 2014), type 2 diabetes (Huang et al. 2013), type 1 diabetic retinopathy (Xu et al. 2014), type 2 diabetic nephropathy (Mao et al. 2014), postmenopausal osteoporosis (Zhang et al. 2014), hip fracture (Yang et al. 2012b) and polycystic ovary syndrome (Murri et al. 2013). Homocysteine is considered an independent risk factor of cardiovascular disease (CVD) due to its associations with pathologies encompassed under the CVD threshold (World Health Organization 2011), such as coronary artery disease and stroke (Homocysteine Studies Collaboration 2002; Wald et al. 2002). Furthermore, the variant allele of the polymorphism *methylenetetrahydrofolate reductase* (*MTHFR*) c.665C>T has been associated with some of the these pathologies and higher homocysteine concentrations (see *Introduction* section *3.1*). However, there is some controversy as clinical trials with treatments lowering homocysteine have usually failed in preventing the recurrence of CVD events. In adults with CVD or at risk of CVD, B vitamin (including folic acid) based homocysteine lowering treatments lack

an effect on cardiovascular events (Clarke et al. 2010; Ebbing et al. 2010; McNulty et al. 2012; Martí-Carvajal et al. 2013). On the contrary, a protective effect of these treatments has been reported in stroke when the participants have no history of stroke (primary prevention), as reviewed (McNulty et al. 2012). Folic acid based homocysteine lowering treatment does not reduce myocardial infarction and cardiovascular mortality in patients with kidney disease (Jardine et al. 2012), although for the general category CVD, a protective effect has been reported in these patients, especially evident in the absence of exposure to folic acid fortification (Qin et al. 2013).

Homocysteine in pregnancy

Previously our group described the pattern of changes in tHcy during normal pregnancy in a longitudinal study of women followed from preconception throughout labour (Murphy et al. 2002; Murphy et al. 2004). Total homocysteine decreases between preconception and 8 gestational weeks (GW) and this tendency persists until 20 GW. It then plateaus until 32 GW (Murphy et al. 2002) before gradually increasing until labour when it returns to similar levels to preconception in mothers that do not use folic acid supplements (Murphy et al. 2004). Transversal studies have reported lower tHcy concentrations at all the analysed time points of pregnancy (from 12 to 42 GW) than in non pregnant women (Kang et al. 1986; Holmes et al. 2005; Friesen et al. 2007; Wu et al. 2013). It has been suggested that pregnancy associated endocrine changes may underlie the decrease in tHcy from early to mid pregnancy because it is not completely explained by folic acid supplementation, haemodilution or the decrease in albumin (Murphy et al. 2002). This may also be true for the late pregnancy increase in tHcy, independently of folic acid supplement use and in the face of continued haemodilution and increased renal function (Murphy et al, 2004). A similar tHcy pregnancy pattern has been observed in the Reus-Tarragona Birth Cohort (RTBC) (Fernàndez-Roig et al. 2013) and in other studies (Cikot et al. 2001; Velzing-Aarts et al. 2005; Ubeda et al. 2011). In a Seychelles study where those women taking folic acid

supplements did it probably sporadically, serum tHcy increased between ≈13 and 28 GW and subsequently until delivery (Wallace et al. 2008).

Maternal and cord tHcy concentrations are positively correlated in plasma (Murphy et al. 2004; Molloy et al. 2005) and serum (Guerra-Shinohara et al. 2002; Obeid et al. 2005; Wallace et al. 2008). The plasma/serum concentration of cord tHcy is lower than maternal tHcy concentration at 37-41GW (Friesen et al. 2007) and at labour (Murphy et al. 2004; Molloy et al. 2005; Wallace et al. 2008; Fernàndez-Roig et al. 2013).

Pregnancy tHcy is modulated by folate, choline, betaine and cobalamin as will be described in the *Introduction* sections *2.2*, *2.3*, *2.4* and *2.5*.

In a Spanish study it was reported that neonates born to women with plasma tHcy in the highest tertile at preconception, 8 GW or at labour, weighed less than those born to women in the low-mid tertile (Murphy et al. 2004). The odds of having lower birth weight offspring in high versus low-normal tHcy at these same points of pregnancy was confirmed in the study by multivariate analysis, and maternal plasma tHcy concentration at labour was a negative predictor of birth weight (Murphy et al. 2004).

Congenital heart defects affect the walls or valves of the heart, or the arteries and veins near the heart and can disrupt normal blood flow (National Institutes of Health 2016a). Maternal hyperhomocysteinaemia in non-pregnant women has been associated with a 3.4-fold higher risk of congenital heart defects in the offspring (Verkleij-Hagoort et al. 2007). No association between second trimester serum tHcy and conotruncal heart defects in the offspring was reported in a case control study carried out postfortification with folic acid in the USA (Shaw et al. 2014). Higher concentrations of amniotic fluid tHcy were associated with higher risk of congenital heart defects in a prefortification USA study (Wenstrom et al. 2001). In a rat study, there was higher incidence of congenital heart defects in the offspring of dams receiving three intraperitoneal injections of 8μM homocysteine on gestation days 8, 9 and 10 compared to saline injected dams (He et al. 2009).

Neural tube defects (NTDs) develop in the first month of pregnancy and can affect the foetal brain, spine or spinal cord. NTDs include spina bifida, anencephaly, exencephaly, Chiari malformation and encephalocele (Renuka et al. 2009; National Institutes of Health 2016a; National Institutes of Health 2016b). Higher maternal concentrations of plasma/serum tHcy are associated with NTDs, in cohort and case control studies, as reported in a meta-analysis (Tang et al. 2015). For example, in an Irish case-control study of pregnant women unsupplemented with vitamins, there was higher maternal plasma tHcy at ≈14-15 GW in NTD-affected pregnancies or with previous NTD-affected pregnancies compared to their respective controls (Mills et al. 2014).

Omphalocele is a congenital abdominal wall defect in which the intestine or other abdominal organs are outside of the body due to incomplete closure of the navel area (National Institutes of Health 2016a). In a study from The Netherlands, a country without mandatory folic acid fortification, in a case group of the combined birth defects (54 NTD, 17 congenital heart defect, 7 omphalocele, 4 orofacial clefts cases) there was higher amniotic fluid tHcy than in controls; although the controls specimens had been collected on average 4 weeks earlier than the cases (Brouns et al. 2008).

Hyperhomocysteinaemia is associated with increased risk of recurrent miscarriage (two or more pregnancy losses) (Nelen et al. 2000). There is higher maternal plasma/serum tHcy in pregnancies with hypertension disorders than in uncomplicated pregnancies (Visser et al. 2014).

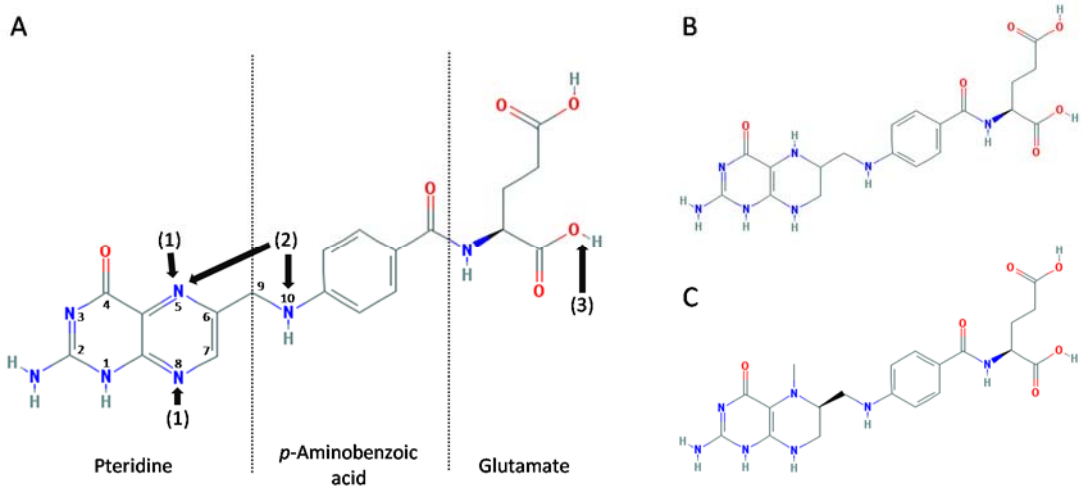
2.2. Folate

Structure, transport and metabolism

The term “folic acid” is used to refer to the most oxidised form of pteroylmonoglutamate (used in supplements and fortified foods), whereas “folates” encompasses a wide range of biological molecules derived from pteroylmonoglutamate. Folic acid is a 2-amino-4-oxo-pteridine moiety bound by a methylene group at the C-6

position to a *p*-aminobenzoyl-glutamate moiety (**Figure 3**). The aforementioned derivatives can have one or more of the modifications stated in **Figure 3** (Shane 2010).

Figure 3. Folates.



A. Folic acid. Atom positions are shown as no-parenthesis numbers. Folic acid modifications: reduction of the N-8 and N-5 of the pyrazine ring of the pterin moiety (1), acquisition and oxidation or reduction of one-carbon units at N-5 and/or N-10 position (2), and elongation of the glutamate chain via gamma-peptide linkage (3). B. Tetrahydrofolate monoglutamate. C. 5-methyltetrahydrofolate monoglutamate. From (Institute of Medicine 2016a).

Natural dietary folates have a polyglutamate chain, and 5-methylTHF is the predominant form. Folate absorption requires the hydrolysis of polyglutamate folate forms to monoglutamate forms that can cross cell membranes. Of the two enzymes that can catalyse the hydrolysis of these glutamates, folate hydrolase 1 (FOLH1, also known as glutamate carboxypeptidase II) and gamma glutamyl hydrolase (GGH), the former is considered to be mainly responsible for removing glutamates from the human intestine. There is low expression of *gamma glutamyl hydrolase* as mRNA in the human small intestine and pancreas, suggesting that it may have a role in intestinal folate absorption, although minor compared to folate hydrolase 1 (Yin et al. 2003). A saturable transport system acts at the physiological concentrations of folate found in

the intestinal lumen (<10μM) with an optimal pH of 6 (proximal small intestine pH) (Rosenberg et al. 1985). These and other characteristics (similar affinity for folic acid as for reduced folate forms, pH-dependence) of this transport system have been identified in the proton coupled folate transporter (SLC46A1), and not in the other candidate for folate intestinal absorption, the reduced folate carrier (SLC19A1) (Qiu et al. 2006). However, the upregulation of the SLC19A1 and folate receptors in the intestine of folate deficient mice suggests they might have a role, although less important than the proton coupled folate transporter, in folate intestinal uptake (Liu et al. 2005). At pharmacological concentrations of folate (>10μM) in the intestinal lumen, a non-saturable transport (diffusion) exists (Rosenberg et al. 1985). The folate in the enterocyte can be exported to the blood stream mainly as monoglutamate, or be transformed into polyglutamate forms that are coenzymatically active and retained in the enterocytes (and all cells) (Shane 2010). The cytosolic and mitochondrial enzyme folypolyglutamate synthetase (FPGS) catalyses the synthesis of folypoly-gamma-glutamates, i.e. the addition of glutamates to the glutamate chain in folates. Mammalian folypolyglutamate synthetase has the following affinity pattern for monoglutamate folate forms: Dihydrofolate (DHF), THF, 10-formylTHF > 5-CH_3THF, 5,10-CH_2THF > Folic acid (Cichowicz and Shane 1987; Chen et al. 1996). It is considered that SLC19A1 is the main importer of folate in systemic tissues (Zhao and Goldman 2013), as will be summarise in *Introduction* section *3.3*.

The most abundant folate forms found in the blood plasma are monoglutamate 5-CH_3THF, in the cytosol polyglutamate 5-CH_3THF, and in the mitochondria polyglutamate 10-formylTHF (Shane 2010). Red blood cell (RBC) folate concentrations are used as a measure of long-term (120 days) folate status because the RBC only accumulates folate during erythropoiesis and cannot release it when the RBCs are mature (Shane 2010).

Folic acid metabolism is of concern because although it is not significantly present in fresh natural foods, it is the form used in most vitamin supplements and that added to

flour in fortification policies. Dihydrofolate reductase (DHFR) catalyses the reduction of DHF to THF (Stover 2010a), being DHF a product of pyrimidines synthesis (see *Introduction* section *1*, **Figure 1**). Human hepatic dihydrofolate reductase has been reported to catalyse the reduction of folic acid to DHF at physiological pH, although this reaction is slower than the reduction of DHF to THF (Bailey and Ayling 2009).

During the folate cycle (**Figure 1**, green arrows), 1C units are transferred from folates to: 1) homocysteine, in homocysteine remethylation; 2) 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), in purine synthesis; and 3) deoxyuridine monophosphate (dUMP), in pyrimidine synthesis (Stover 2010a). Serine is a 1C unit donor that serves to recycle folate by serine hydroxymethyltransferases (SHMTs) in cytosol and mitochondria (Appling 1991). Formate can also be incorporated directly into folates in the cytosol as described in *Introduction* section *1*. Other 1C unit donors for folate recycling are dimethylglycine (DMG), sarcosine and glycine, which are demethylated in the mitochondria (Yoshida and Kikuchi 1970; Wittwer and Wagner 1981).

Physiological and clinical relevance

As explained in *Introduction* section *1*, folate is involved in methylation reactions (of DNA, proteins, and other molecules and compounds), interconversion of amino acids and synthesis of nucleotides and nitrogenous bases. A study in piglets reported that the percentage of methyl groups destined for methyl acceptors in the liver is: 49% for phosphatidylethanolamine (see *Introduction* point *2.3*), 32% for proteins, 18% for guanidinoacetate, 0.1% for DNA, and the rest are destined for other acceptors (McBreairty et al. 2013). The enzyme guanidinoacetate methyltransferase catalyses the transfer of one methyl group from SAM to guanidinoacetate producing creatine (Joncquel-Chevalier Curt et al. 2015). Creatine is an osmolyte and, when phosphorylated, a form of energy storage in muscle and brain (Joncquel-Chevalier Curt et al. 2015). Feeding piglets a diet deficient in folate, betaine and choline lead to an increase in methyl groups destined for phosphatidylethanolamine, a decrease in those

destined for guanidinoacetate but no change in those destined for proteins and DNA methylation (Robinson et al. 2016).

Folate is an essential nutrient and the recommended dietary allowance (RDA) for folate in men and women is 400μg/day (d) of dietary folate equivalents (Institute of Medicine 2000). Dietary folate equivalents take into account the different bioavailabilities of food folates and folic acid, and are defined as the quantity of food folates plus the quantity of folic acid in the diet multiplied by 1.7 (Bailey 1998). In Spain the recommended dietary intake in men and women is 300μg/d of folate (Federación Española de Sociedades de Nutrición Alimentación y Dietética 2010).

The World Health Organization (WHO) has two criteria for assessing folate status in populations (World Health Organization 2012). The first is based on the risk of megaloblastic anaemia, a disease known to be associated with low folate status; and categorises folate status according to the following values for plasma/serum: <6.8nmol/L (deficiency), 6.8-13.4 (possible deficiency), 13.5-45.3 (normal range), >45.3nmol/L (elevated). The second criteria is based on the folate concentration below which homocysteine concentration is not optimal: plasma/serum folate of <10nmol/L (deficiency). These two criteria are also used for the cutoffs of RBC folate. RBC folate concentrations of <226.5nmol/L (megaloblastic anaemia criteria) or <340nmol/L (homocysteine criteria) are considered deficiency. It is important to note that in the megaloblastic anaemia criteria folate determination is the microbiological assay and in the homocysteine criteria is the radioassay (differences between both assays will be described in *Introduction* section *3.1*).

Serum tHcy was higher in patients with folate deficiency compared to normal status (Allen et al. 1993b) and population studies from different geographic regions including the USA (Selhub et al. 2000; Selhub et al. 2008) and China (Hao et al. 2007) have confirmed the negative association between plasma/serum folate and tHcy. Folic acid supplementation was confirmed to have a tHcy lowering effect in a meta analysis of 27 clinical trials (Homocysteine Lowering Trialists' Collaboration. 2005).

In addition to methylation reactions, folate is involved in DNA and RNA synthesis by the transfer of 1C units to form purines and pyrimidines (deoxythymidine) (*Introduction* section *1*, **Figure 1**). Low folate status can limit growth at the two aforementioned biosynthetic pathways (Steegers-Theunissen et al. 2013). Uracil can be incorporated in DNA as the nucleotide deoxyuridine instead of deoxythymidine, then a repair mechanism starts with the removal of the nitrogenous base and breaking of the DNA strand. If the level of uracil in DNA is high DNA breaks accumulate and this leads to genetic instability (Blount et al. 1997; Ciappio and Mason 2010). Healthy splenectomised subjects with <317nmol/L RBC folate had higher blood cell DNA uracil concentration than those with RBC folate >317nmol/L, and DNA uracil decreased after supplementation 5mg/d folic acid for 8wk in both groups (Blount et al. 1997). It has been hypothesized that the resulting genetic instability rather than aberrant methylation, is the main mechanism for tumour occurrence associated with low folate status. Increased intestinal uracil/thymine ratio and tumourigenesis, as well as DNA breaks and damage was observed in mice fed folate deficient diets (Knock et al. 2006; Knock et al. 2008); and this was not corrected after administration of betaine (methyl donor) (Knock et al. 2008). In populations at risk of CVD or with CVD, homocysteine lowering treatments with B vitamins supplementation is not associated with the incidence of any types of cancer (Clarke et al. 2010; Zhou et al. 2011; Martí-Carvajal et al. 2013). Specifically in colorectal cancer, folate is considered to play a dual role. Before the establishment of neoplastic foci, folate suppresses tumour development and progression, and after the establishment folate enhances tumour growth and progression, as reviewed (Smith et al. 2008). Plasma folate concentrations are positively associated with prostate cancer risk in cohort studies, and it has been suggested that this effect is in the rate of progression of localized prostate cancer (Collin et al. 2010).

As for CVD, lower concentrations of plasma/serum folate have been associated with venous thrombosis in a meta analysis (Zhou et al. 2012). Most prospective cohort studies have found an association between low baseline plasma/serum folate

concentrations and risk of stroke but not of coronary artery disease, as reviewed (Kalin and Rimm 2010). Higher intakes of folate were protective against hypertension in a cohort of women in prefortification USA (Forman et al. 2005). Meta analyses of randomised controlled trials have found a protective effect of folic acid supplementation against stroke in populations unexposed to mandatory folic acid fortification but not in fortified populations (Yang et al. 2012a; Zeng et al. 2015).

Severe folate deficiency causes megaloblastic anaemia, and there is more evidence supporting impaired DNA synthesis than impaired methylation, as the main mechanism for the development of this pathology (Stabler 2010). DNA replication is slower than cell growth leading to impaired maturation of the nuclei of erythropoietic precursors, resulting in aberrantly larger RBCs, a typical feature of megaloblastic anaemia.

Folate deficiency has been associated with neurological complications such as cognitive impairment, dementia and depressive disorders (Reynolds 2014). In a meta analysis of six case control studies, low plasma/serum folate concentration was associated with Alzheimer disease (Shen and Ji 2015). Low plasma/serum folate concentration is also associated with cognitive impairment among adults over 65 years old (Michelakos et al. 2013).

Folate in pregnancy

A postfortification Canadian study reported higher plasma folate concentration in pregnant women at 36 GW than in non pregnant controls (mothers of young children) (Wu et al. 2013). In addition to the use of prenatal multivitamin supplements in the pregnant women, the non pregnant controls of this study might not have recover the normal folate pools (Wu et al. 2013), as it has been reported that serum folate pool is not replete at 6 month postpartum (Bruinse et al. 1985). Studies without the use of folic acid supplements in pregnancy have found lower folate concentrations in pregnant than in non pregnant women (Ball and Giles 1964; Ek and Magnus 1981). In the RTBC, plasma folate concentrations decrease when folic acid supplementation is stopped and

RBC folate concentrations decrease in late pregnancy (Fernàndez-Roig et al. 2013). Similar decreases have been found in most studies for plasma/serum (Ek and Magnus 1981; Bruinse et al. 1985; Qvist et al. 1986; Bartels et al. 1989; Cikot et al. 2001; Wallace et al. 2008; Ubeda et al. 2011) and RBC folate (Ek and Magnus 1981; Qvist et al. 1986); but there are also reports of no change (Velzing-Aarts et al. 2005; Wu et al. 2013) or an increase (Cikot et al. 2001; Takimoto et al. 2007). Processes occurring in pregnancy has been proposed as the causes for the usual folate decline in pregnancy, e.g. increased folate demand for the growth of the foetus and uteroplacental structures, haemodilution, increased folate clearance and excretion, and hormonal changes (Tamura and Picciano 2006).

Maternal and cord folate concentrations are positively correlated in plasma (Molloy et al. 2005) and serum (Obeid et al. 2005; Wallace et al. 2008). Plasma/serum cord folate is higher than maternal folate at labour (Molloy et al. 2005; Obeid et al. 2005; Wallace et al. 2008; Fernàndez-Roig et al. 2013).

Although the pregnancy change of tHcy (see *Introduction* section *2.1*) is independent of folate status, folic acid supplementation has been associated with lower tHcy than without supplementation from 20 GW on (Murphy et al. 2002; Murphy et al. 2004). Higher plasma tHcy throughout pregnancy was found in the RTBC with low maternal plasma folate status compared to high (Fernàndez-Roig et al. 2013). Maternal serum folate is a negative predictor of tHcy (Wallace et al. 2008). In the RTBC, maternal plasma folate concentration was a negative predictor of plasma tHcy throughout pregnancy when folate status was low, and only at mid-late pregnancy when folate status was high (Fernàndez-Roig et al. 2013). Maternal plasma/serum folate and tHcy are negatively correlated (Kim et al. 2004; Molloy et al. 2005; Velzing-Aarts et al. 2005; Wallace et al. 2008), and a similar tHcy-folate correlation has been found in cord plasma (Molloy et al. 2005).

According to the Institute of Medicine, the RDA for folate in pregnant women is 600μg/d of dietary folate equivalents (Institute of Medicine 2000). In Spain the

recommended dietary intake is 500µg/d of folate (Federación Española de Sociedades de Nutrición Alimentación y Dietética 2010).

A meta-analysis reported that folic acid supplementation during pregnancy was associated with higher birth weight in humans (Lassi et al. 2013). Deficient or excessive folic acid intake, i.e. 3-fold lower or 20-fold higher than the recommended intake, respectively, in pregnant mice was associated with growth retardation in the foetus (Pickell et al. 2009; Pickell et al. 2011).

In a postfortification USA case control study, low second trimester maternal serum folate was protective against conotruncal heart defects in the offspring (Shaw et al. 2014). Excessive folic acid intake in pregnant mice was associated with less thickness of ventricular walls in the hearts of embryos (Pickell et al. 2011).

Periconceptional use of folic acid supplements has been consistently reported as protective against NTDs (MRC Vitamin Study Research Group 1991; Czeizel and Dudás 1992; Berry et al. 1999), and this led to the mandatory fortification of flour with folic acid in the USA in 1998 (US Food and Drug Administration 1996). Subsequently, other countries adopted the same policy (**Figure 4**). The policy in the USA has led to an increase in total folate intake by a mean of 323µg/d of dietary folate equivalents (Choumenkovitch et al. 2002), as well as increases in serum and RBC folate concentrations (Ganji and Kafai 2006); and a decrease in plasma tHcy (Pfeiffer et al. 2008).

Figure 4. Countries with mandatory folic acid fortification policy[1].

Countries with mandatory folic acid policy are shown in blue, without in white colour. [1] Source: FFI (Food Fortification Initiative 2016).

A meta analysis reported that low maternal concentrations of plasma/serum folate were associated with NTDs affected pregnancies (Tang et al. 2015). Given the association between maternal folate status and NTDs, RBC folate concentrations above 906nmol/L have been recommended for the prevention of NTDs (World Health Organization 2015).

A Swedish case control study found an association between low plasma folate concentrations in pregnant women at 6-12 GW and the risk of miscarriage (George et al. 2002). Deficient folic acid intake (3-fold lower than the recommended intake) in pregnant mice was associated with higher embryonic loss (Pickell et al. 2009).

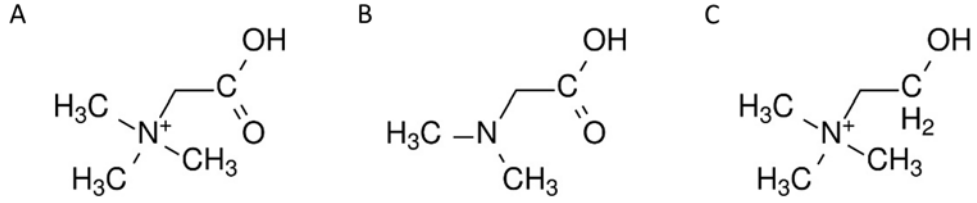
A meta analysis of four clinical trials reported lower incidence of megaloblastic anaemia in pregnant women taking folic acid supplements during pregnancy than in non-supplement user pregnant women (Lassi et al. 2013).

2.3. Betaine, dimethylglycine and choline

Structure, transport and metabolism

The structure of betaine, DMG and choline is shown in **Figure 5** (Institute of Medicine 2016a). Choline is the precursor of betaine, which can form DMG in a reaction catalysed by BHMT.

Figure 5. Main components of the choline oxidation pathway.



A. Betaine. B. Dimethylglycine. C. Choline. Adapted from (Institute of Medicine 2016a).

Choline and its derivatives digestion is a complex process. Several phospholipases from pancreatic secretions remove lipid moieties from phosphatidylcholine (Shapiro 1953; van den Bosch et al. 1965), resulting in liposoluble and hydrosoluble derivatives (Le Kim and Betzing 1976). The liposoluble derivatives are considered to be absorbed by passive diffusion (Jiang et al. 2013), and the hydrosoluble derivatives can be further hydrolysed rendering free choline (Parthasarathy et al. 1974). Three families of choline transporters exist: high affinity choline transporter 1 (SLC5A7), choline transporter-like proteins (SLC44As), and organic cation transporters (SLC22As) (Michel et al. 2006). The choline transporter-like protein family, specifically SLC44A1, is considered the main mediator of free choline intestinal absorption (Horie et al. 2014); and not the other two families (Kamath et al. 2003; Horie et al. 2014).

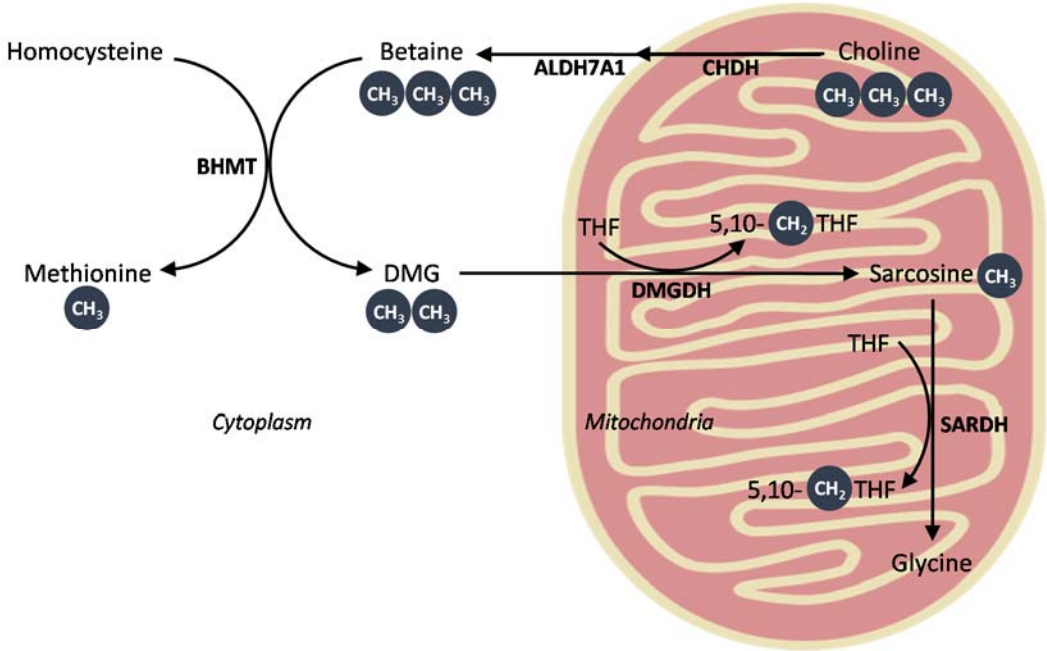
Intestinal absorption of betaine is mediated by the IMINO transporter, which also absorbs proline (Stevens and Wright 1985). This transporter is coded by the *SLC6A20* gene (Kowalczuk et al. 2005; Takanaga et al. 2005). The expression pattern of the IMINO transporter, with high expression in the distal small intestine and low

expression in other organs and regions of the digestive system, supports a role in intestinal absorption (Takanaga et al. 2005). As for systemic betaine uptake, the GABA (gamma-aminobutyric acid) / betaine transporter (SLC6A12) is considered the main mediator (Kempson et al. 2014). This transporter has higher affinity for GABA than for betaine (Borden et al. 1995), and has been detected in many organs including the placenta (Rasola et al. 1995). *In vitro* experiments suggest the amino acid transport system A, in addition to the GABA/betaine transporter, is involved in hypertonic-stress induced betaine uptake in liver (Peters-Regehr et al. 1999) and placenta (Nishimura et al. 2010).

Choline is oxidised in two consecutive reactions to form betaine (**Figure 6**). First choline is converted into betaine aldehyde in a reaction catalysed by choline dehydrogenase (CHDH), a mitochondrial enzyme; then betaine aldehyde is further oxidised to betaine by the betaine aldehyde dehydrogenase (ALDH7A1) (Ueland 2011). Betaine aldehyde dehydrogenase has cytosolic and mitochondrial isoforms (Chern and Pietruszko 1995; Chern and Pietruszko 1999), being both coded by the same gene (Pietruszko and Chern 2001) and the activity higher in the cytosol (Chern and Pietruszko 1999).

In the betaine-dependent homocysteine remethylation, which is less important than the folate-dependent pathway, one methyl group is transferred from betaine to homocysteine to form DMG and methionine in a reaction catalysed by BHMT (**Figure 6**).

Figure 6. Choline oxidation pathway and further demethylation of DMG.



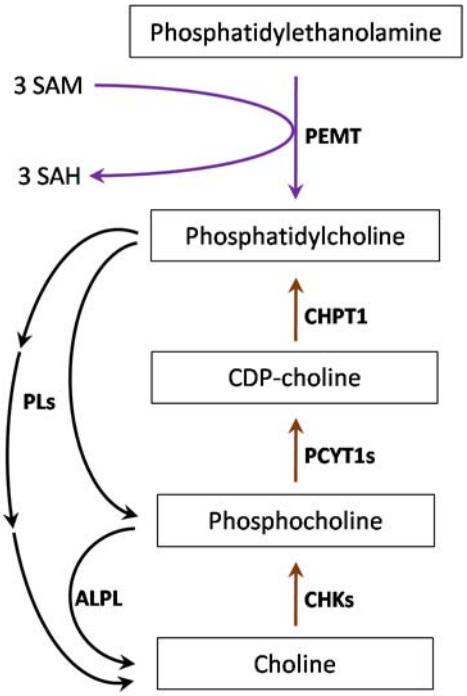
Scheme of the choline oxidation pathway and further demethylation of DMG to glycine. Enzymes are written in bold, substrates and products are not; methyl and methylene groups are illustrated in blue circles. Enzymes [vitamin cofactor]: ALDH7A1, betaine aldehyde dehydrogenase; BHMT, betaine-homocysteine methyltransferase; CHDH, choline dehydrogenase; DMGDH, dimethylglycine dehydrogenase [B_2]; SARDH, sarcosine dehydrogenase [B_2]. Metabolites: DMG, dimethylglycine; THF, tetrahydrofolate; 5,10-CH_2THF, 5,10-methylenetetrahydrofolate. Adapted from (Ueland et al. 2005).

DMG can donate up to three more methyl groups through three mitochondrial reactions which are irreversible *in vivo* (**Figure 6**). First, in a reaction catalysed by the covalent flavoenzyme DMG dehydrogenase (DMGDH), DMG is converted to sarcosine donating one methyl group to THF which is converted to 5,10methyleneTHF. Sarcosine can subsequently be converted to glycine, and THF to 5,10methyleneTHF by another covalent flavoenzyme, sarcosine dehydrogenase (SARDH) (Wittwer and Wagner 1981). Finally, glycine and THF can be transformed to CO_2, NH_3 and 5,10methyleneTHF by the glycine cleavage system (Yoshida and Kikuchi 1970). The glycine N-methyltransferase is a cytosolic enzyme that catalyses the reverse reaction of sarcosine dehydrogenase;

i.e. the transfer of one methyl group from SAM to glycine, to form SAH and sarcosine. The glycine N-methyltransferase reaction is important when methionine and SAM are high due to dietary intake of labile methyl groups exceeding the amount required for obligatory methylation reactions (Mudd et al. 2001; Mudd et al. 2007).

Phosphatidylcholine can be synthesised by two pathways: the phosphatidylethanolamine methyltransferase (PEMT) pathway, and the cytidine diphosphate—choline (CDP-choline) pathway, (**Figure 7**) (Caudill 2010). The PEMT pathway involves three methylation reactions of phosphatidylethanolamine where SAM is the methyl donor (Ridgway and Vance 1987). Choline can be synthesised from phosphatidylcholine by the action of phospholipases, and if phosphatidylcholine was synthesised by PEMT, the process is called "endogenous synthesis of choline".

Figure 7. PEMT pathway, CDP-choline pathway and choline synthesis reactions.



Scheme of phosphatidylcholine and choline biosynthetic pathways: PEMT pathway (violet arrows), CDP-choline pathway (brown arrows), choline synthesis reactions (black arrows). Enzymes: ALPL, alkaline phosphatase; CHKs, choline kinases; CHPT1, choline phosphotransferase; PCYT1s, choline-phosphate

cytidylyltransferases; PEMT, phosphatidylethanolamine methyltransferase; PLs, phospholipases. Metabolites: CDP-choline, cytidine diphosphate—choline; SAH, S-adenosyl homocysteine; SAM, S-adenosyl methionine. Adapted from (Mudd et al. 2001; Caudill 2010).

Physiological and clinical relevance

Betaine, as other methylamines, acts physiologically as an organic osmolyte against osmotic stress (Yancey et al. 1982), especially in medullary kidney cells and chondrocytes (Wehner et al. 2003). In line with this, the expression of the GABA/betaine transporter and the betaine uptake have been reported to increase under hypertonic conditions (Sheikh-Hamad et al. 1994; Peters-Regehr et al. 1999; Nishimura et al. 2010). Betaine is also an stabiliser of macromolecular structure and function (Yancey et al. 1982; Sheikh-Hamad et al. 1994). In canine kidney cell cultures, betaine inhibits the heat-shock protein 70 (HSP70) response to elevated temperature and this is attributed to HSP70 and betaine being both protein stabilisers (Sheikh-Hamad et al. 1994).

Betaine can act as a methyl donor. According to rat experiments, in the liver, betaine-dependent homocysteine remethylation is as important as folate-dependent homocysteine remethylation (Finkelstein and Martin 1984a). However, the latter is thought to prevail globally in mammals based on the fact that BHMT activity has only been detected in some organs whereas MTR is ubiquitous (see *Introduction* section *3.2*). The contribution of each pathway to homocysteine remethylation in the whole organism has not been assessed, but a study of healthy Dutch adults reported that the inverse association between serum folate and fasting plasma tHcy was stronger than that between betaine and tHcy, suggesting that the MTR reaction prevails under normal circumstances (Melse-Boonstra et al. 2005). Complementary treatment with large doses of oral betaine is useful against very high tHcy due to inborn errors of metabolism (Wilcken et al. 1983; Wilcken et al. 1985; Bartholomew et al. 1988). Supplementation with 3-6g/d of betaine also reduces plasma/serum tHcy in healthy (Brouwer et al. 2000; Olthof et al. 2003; Steenge et al. 2003; Alfthan et al. 2004) and

obese individuals (Schwab et al. 2002). Lower doses of betaine seems to require treatments longer than at least one week to have a tHcy-lowering effect (Olthof et al. 2003; Alfthan et al. 2004). Plasma/serum betaine was a negative predictor of post-methionine load plasma homocysteine in Dutch (Holm et al. 2005) and Norwegian populations (Holm et al. 2004) and also of tHcy (Lever et al. 2005; Melse-Boonstra et al. 2005; Imbard et al. 2013).

In spite of the endogenous synthesis of choline, choline is considered an essential nutrient, based on choline deficient individuals showing signs of liver dysfunction (Zeisel et al. 1991). Choline adequate intake is 550mg/d for men and 425mg/d for women according to the Institute of Medicine (Institute of Medicine 2000), and 400mg/d for both sexes according to the European Food Safety Authority (EFSA - NDA Panel 2016).

High plasma DMG concentrations were associated with risk of acute myocardial infarction in a study of a Norwegian angina pectoris patients cohort (Svingen et al. 2013). In another Norwegian cohort, low plasma DMG concentrations were associated with low bone mineral density, and among elder individuals with risk of hip fracture (Oyen et al. 2015).

Betaine, dimethylglycine and choline in pregnancy

Higher plasma choline and lower betaine and DMG concentrations were reported in postfortification Canadian pregnant women at 36 GW (Wu et al. 2013) or at delivery (Friesen et al. 2007) than in non pregnant women. Some authors consider that the higher and lower concentrations of choline and betaine, respectively, in pregnant women are not due to a lower conversion of choline to betaine, but to increased betaine turnover. This is based on the observation that leukocyte *choline dehydrogenase* mRNA is upregulated in pregnancy (Friesen et al. 2007). A postfortification USA clinical trial with diets containing labelled choline suggested a greater use of choline for the

synthesis of phosphatidylcholine (by both the PEMT and the CDP-choline pathways) in third trimester pregnant women than non pregnant women (Yan et al. 2013).

In the RTBC, maternal plasma betaine during pregnancy follows a pattern where the concentration decreases from early to mid pregnancy, and slightly increases afterwards toward labour without returning to early pregnancy concentrations (Fernàndez-Roig et al. 2013). A similar pattern in pregnancy betaine changes was observed in studies from the Netherland Antilles (Velzing-Aarts et al. 2005), Seychelles (Wallace et al. 2008), and in one postfortification Canadian study (Visentin et al. 2015) but not another (Wu et al. 2013). Maternal plasma DMG also decreases toward mid pregnancy and increases afterwards reaching higher concentrations at or close to labour than at early pregnancy (Velzing-Aarts et al. 2005; Fernàndez-Roig et al. 2013; Wu et al. 2013; Visentin et al. 2015). Maternal plasma choline increases from early pregnancy to late pregnancy (Velzing-Aarts et al. 2005; Fernàndez-Roig et al. 2013; Wu et al. 2013; Visentin et al. 2015).

There is a positive correlation between maternal and cord plasma betaine at 34GW in Dutch (Hogeveen et al. 2013), at 37-41GW in Canadian (Friesen et al. 2007) and at labour in Irish women (Molloy et al. 2005). Similar positive correlations have been found for DMG (Molloy et al. 2005; Friesen et al. 2007; Hogeveen et al. 2013); and for choline (Friesen et al. 2007; Hogeveen et al. 2013), except in an Irish study (Molloy et al. 2005). Maternal plasma betaine, DMG and free choline are positive predictors of their respective cord plasma analytes, although borderline significant for free choline (Visentin et al. 2015). Therefore, foetal statuses in betaine, DMG and to a lesser extent choline seem to reflect maternal status.

Cord betaine (Molloy et al. 2005; Friesen et al. 2007; Wallace et al. 2008; Fernàndez-Roig et al. 2013; Hogeveen et al. 2013; Visentin et al. 2015), DMG (Molloy et al. 2005; Fernàndez-Roig et al. 2013; Hogeveen et al. 2013; Visentin et al. 2015), and choline (Molloy et al. 2005; Friesen et al. 2007; Fernàndez-Roig et al. 2013; Hogeveen et al. 2013; Visentin et al. 2015) are higher than maternal concentrations of these

compounds. This suggests a flux of these to the foetus and/or: 1) increased choline oxidation for the synthesis of betaine that can subsequently be used in the BHMT pathway producing DMG in the foetus; 2) increased synthesis of choline from the phosphatidylcholine produced in the PEMT pathway or transported to the foetus. Results from a pregnancy study with labelled choline suggest phosphatidylcholine derived from the PEMT pathway and not from the CDP-choline pathway is preferentially produced/taken up by the foetus (Yan et al. 2013).

Maternal plasma betaine is a negative predictor of tHcy throughout pregnancy (Velzing-Aarts et al. 2005; Wallace et al. 2008) and at labour (Molloy et al. 2005) and there was a tendency for a negative correlation between these in another study (Wu et al. 2013). In the RTBC, the negative association between plasma betaine and tHcy was observed at an earlier stage of pregnancy in mothers with low folate status than in those with high folate status (Fernàndez-Roig et al. 2013). This suggests that the BHMT pathway may be upregulated in response to worsening folate status as pregnancy progresses. Other interactions among 1C metabolism compounds will be explained in *Introduction* point *2.5*.

Some studies suggest the BHMT pathway metabolites are involved in oxidative and osmotic stress protection during pregnancy, and foetal programming. DMG, through its full oxidation to glycine in the mitochondria, is considered to contribute to glutathione synthesis during human pregnancy. This is based on the negative correlation between DMG and 5-oxoproline, a compound formed during glutathione synthesis when glycine is limited, in maternal or cord plasma (Friesen et al. 2007). Betaine added to the media protected against raised osmolarity in *in vitro* mouse embryo experiments (Biggers et al. 1993; Dawson and Baltz 1997). Such experiments also revealed that betaine transport exists in 1-cell stage embryos and is increased with increasing osmolarity (Anas et al. 2007). The transport is by the IMINO transporter and stops at the 2-cell stage (last analysed time point is blastocyst stage) (Anas et al. 2008). In addition, betaine is present in mouse oviduct *in vivo*, and its concentration in dams with embryos

is about twice that of dams with unfertilised eggs (Anas et al. 2007). According to these studies betaine in its roles as methyl donor and as osmolyte, is important during pregnancy. Higher serum methionine concentration was detected in the offspring of betaine supplemented sows suggesting a role for it in foetal programming of methionine (Li et al. 2014a).

Choline adequate intake during pregnancy is 450mg/d (Institute of Medicine 2000), or 480mg/d (EFSA - NDA Panel 2016).

Top versus low quartile cord plasma choline or betaine was associated with a greater risk of low birth weight (LBW, ≤2.5Kg birth weight) and cord choline or betaine and DMG were negative and positive predictors, respectively, of birth weight in a Dutch study (Hogeveen et al. 2013). In a Singapore pregnancy cohort, maternal plasma betaine at 26-28 GW was a negative predictor of birth weight and other neonatal anthropometric values; and higher maternal plasma betaine was associated with a greater risk of an small for gestational age (SGA, $<P_{10}$ birth weight) neonate (van Lee et al. 2016). The causes for these associations are unclear and several hypotheses have been proposed, e.g. increased choline oxidation pathway is able to generate more glycine through the demethylation of DMG, being glycine necessary for foetal growth (Hogeveen et al. 2013).

In a postfortification USA case control study, second trimester maternal serum betaine, DMG and total choline were not associated with conotruncal heart defects in the offspring (Shaw et al. 2014). The incidence of ventricular septal defects was higher in the offspring born to mouse dams fed a choline deficient compared to control diet (Chan et al. 2010).

An Irish pregnancy case-control study reported no differences in plasma betaine and choline at ≈14-15 GW in pregnant women not taking supplements and with NTD-affected pregnancies or with previous NTD-affected pregnancies, and their respective controls (Mills et al. 2014). However in this study there was more proportion of variant

homozygotes for the *PEMT* +5465G>A polymorphism in cases compared to controls suggesting a role for the synthesis of phosphatidylcholine by PEMT in NTDs; although no differences in plasma betaine, choline and tHcy were observed among the alleles (Mills et al. 2014). In an American case control study higher betaine intakes were associated with lower risk of NTD-affected pregnancy in a subsample of Hispanic ethnicity with mixed exposure to folic acid fortification (Chandler et al. 2012). An American prefortification study reported that high intakes of choline were protective against having a NTD-affected pregnancy (Shaw et al. 2004). In a postfortification pregnancy study with maternal blood samples collected at ≈15-18 GW, lower and higher serum choline concentrations increased and decreased, respectively, the risk of a NTD-affected pregnancy (Shaw et al. 2009a). Neurulating mouse embryos exposed to inhibitory analogues of choline or of phosphatidylcholine synthesis derivatives developed NTD (Fisher et al. 2001). Although little is known, the choline oxidation pathway could be necessary for the PEMT pathway and this to form and close the neural tube.

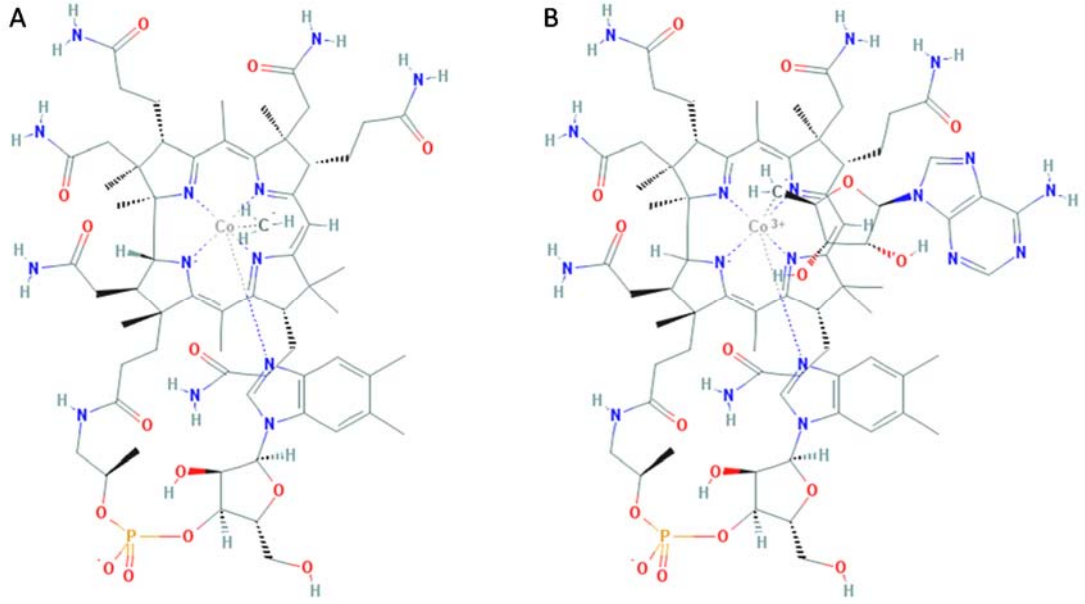
2.4. Cobalamin

Structure, transport and metabolism

Cobalamin and vitamin B_{12} are a water soluble vitamin containing a corrin ring where two of the four pyrrole rings are directly bound to a central cobalt atom (Institute of Medicine 2016a). The cobalt atom is coordinated by four pyrrole nitrogens and a nitrogen of a dimethylbenzimidazole group. A sixth coordinating ligand can be present, such as in methylcobalamin (methyl group carbon atom) and adenosylcobalamin (deoxyribose C5'); or absent such as in cobalamin. The term “cobalamin” refers to cofactor forms, such as methylcobalamin and deoxyadenosylcobalamin (also abbreviated as adenosylcobalamin); and “vitamin B_{12}” refers to non-metabolically active forms, such as aquacobalamin, cyanocobalamin and hydroxycobalamin (IUPAC-IUB 1975). Cyano and hydroxycobalamin are synthetically produced stable compounds

that are added to fortified foods, vitamin supplements and injections; but hydroxycobalamin can also be formed spontaneously from methyl and adenosylcobalamin in the organism (Obeid et al. 2015). The oxidation state of the cobalt atom can be written as roman numerals in parenthesis (IUPAC-IUB 1975). The structure of biologically active cobalamin forms is shown in **Figure 8** (Institute of Medicine 2016a).

Figure 8. Cobalamin cofactors.



A. Methylcob(III)alamin. B. Adenosylcob(III)alamin. From (Institute of Medicine 2016a).

Throughout this thesis, blood and tissue cobalamin concentrations will refer to the combination of metabolically and non-metabolically active cobalamin forms.

The digestion of cobalamin is a complex process. A protein present in saliva, haptocorrin (TCN1), binds free cobalamin forms. In the upper small intestine, haptocorrin is degraded and cobalamin binds to the intrinsic factor (GIF) (Allen et al. 1978). The intrinsic factor-cobalamin complex is absorbed in the distal small intestine (Nexo 1998), mainly by cubilin (Seetharam et al. 1997; Christensen and Birn 2002). Uptaken cobalamin is modified and whether retained in the enterocyte as cofactor or

transported to the basolateral membrane where it is released to the plasma, in complexes with haptocorrin or with transcobalamin 2 (TCN2) (Nexo 1998; Moestrup and Verroust 2001). Haptocorrin and transcobalamin 2, bind mostly the forms methylcobalamin and adenosylcobalamin, respectively (Nexo 1977). Transcobalamin 2—cobalamin complexes are delivered to cells by the ubiquitous transcobalamin 2 receptor (CD320) (Nexo and Hollenberg 1980; Moestrup et al. 1996) and to a lesser extent by megalin in some specific tissues (Moestrup et al. 1996). Plasma cobalamin is found mostly as methylcobalamin, less as adenosylcobalamin, and the lowest concentrations as other forms such as cyanocobalamin and hydroxycobalamin (Linnell et al. 1974; Gimsing et al. 1983). In tissues the major form is adenosylcobalamin (Linnell et al. 1974).

Cobalamin acts as transient methyl group receptacle in the folate-dependent homocysteine remethylation reaction catalysed by the cytosolic enzyme MTR (Weissbach and Taylor 1970). This reaction consists in two steps:

1) $5\text{-}CH_3THF + MTR\text{-}cob(I)alamin \rightarrow THF + MTR\text{-}CH_3cob(III)alamin$

2) $MTR\text{-}CH_3cob(III)alamin + Homocysteine \rightarrow MTR\text{-}cob(I)alamin + Methionine$

Every 200-1000 turnovers the MTR cobalamin cofactor is spontaneously oxidised and a reductive methylation catalysed by the methionine synthase reductase (MTRR) is necessary (see *Introduction* section *3.4*).

Adenosylcobalamin participates in the molecular rearrangement of methylmalonyl-coenzyme A (methylmalonyl-CoA) to form succinyl-coenzyme A; a reaction catalysed by the methylmalonyl-CoA mutase, a mitochondrial enzyme (Banerjee 1998). This reaction links branched-chain amino acid metabolism and degradation of dietary odd chain fatty acids and cholesterol, that produce methylmalonyl-CoA; to the Krebs cycle where succinyl-coenzyme A is targeted (Thoma et al. 1998).

Crosstalk between both cobalamin dependent reactions has been suggested. Experiments with murine leukaemia cells showed intracellular protein-bound cobalamin is associated to MTR and methylmalonyl-CoA mutase, and when 5-methylTHF and homocysteine were added to the media there was an increase in cobalamin associated with MTR (Quadros and Jacobsen 1995).

Physiological and clinical relevance

The known metabolic functions attributed to cobalamin are derived from those of the MTR and the methylmalonyl-CoA mutase reactions, that is, folate dependent homocysteine remethylation and providing metabolites for the Krebs cycle. Several facts, such as an alternative and more efficient pathway for the targeting of branched-chain amino acids and odd chain fatty acids to the Krebs cycle; has led some authors to suggest the methylmalonyl-CoA and its pathway may have unknown non-anaplerotic functions (Allen et al. 1993b).

Cobalamin plays an intimate role with folate in homocysteine remethylation, but it may also affect folate-related DNA synthesis in situations of cobalamin deficiency that can lead to the accumulation of the 5-methylTHF form because the MTHFR reaction that leads to its synthesis is irreversible and the impaired MTR reaction prevents its conversion to THF ("methylfolate trap"). As a result, other forms of folate involved in purine and pyrimidine synthesis decrease, leading to haematological abnormalities (Scott et al. 1981; Scott and Weir 1981). It is considered that this process has evolved from the necessity of providing methyl groups under low supply of methionine; in such circumstances DNA biosynthesis is restricted to reduce cell division and hence the use of methionine for protein synthesis (Scott et al. 1981; Scott and Weir 1981). In rats with cobalamin deficiency that is not severe enough to cause anaemia or illness, there are higher 5-methylTHF and lower THF concentrations, and higher DNA uracil content in colonic mucosa; than in cobalamin replete rats (Choi et al. 2004). In this study there were no differences in plasma folate and colonic mucosa total folate concentrations between the groups (Choi et al. 2004). Therefore, the "methylfolate trap" is considered

the mechanism by which cobalamin deficiency results in secondary intracellular deficiency of all forms of folate except 5-methylTHF, leading to haematological abnormalities indistinguishable from those caused by folate deficiency (Allen 2000). In untreated megaloblastic anaemia, the concentrations of the different forms of cobalamin are equally reduced in cells, but in blood plasma the methylcobalamin concentration is disproportionately reduced (Linnell et al. 1974).

Cobalamin is an essential nutrient, and the RDA for cobalamin in adults is 2.4μg/d (Institute of Medicine 2000). In Spain the recommended dietary intake is 2μg/d of cobalamin (Federación Española de Sociedades de Nutrición Alimentación y Dietética 2010).

The WHO has set the threshold for cobalamin deficiency as a plasma cobalamin below 150pmol/L (de Benoist 2008). Plasma cobalamin >150pmol/L and <221pmol/L has been categorised as marginal cobalamin deficiency (Allen 2009).

In situations of cobalamin deficiency, homocysteine and methylmalonic acid accumulate (Allen et al. 1993b). Methylcobalamin is the cofactor for MTR and so tHcy increases as a result of impairment. Methylmalonic acid is a metabolite upstream the pathway where methylmalonyl-CoA mutase reaction is located, and accumulates for the impairment in this reaction where adenosylcobalamin is the cofactor. Both, tHcy and methylmalonic acid can be considered biomarkers of cobalamin status, although tHcy is not specific. There is a negative association between plasma/serum cobalamin and tHcy in cohort studies (Selhub et al. 2000; Selhub et al. 2008). According to a meta analysis, vitamin B_{12} supplementation in addition to folic acid supplementation, has a higher plasma homocysteine lowering effect than folic acid alone (Homocysteine Lowering Trialists' Collaboration. 2005).

As previously commented for plasma folate, prostate cancer risk is higher per 100pmol/L increase in plasma/serum cobalamin (Collin et al. 2010). It is unknown if in this association cobalamin could be a cause such as the *MTR* c.2756A>G variant allele;

or a consequence due to increased haptocorrin production by prostate tumour cells (Collin et al. 2010). Higher plasma/serum cobalamin concentrations are found in cases of postmenopausal osteoporosis (Zhang et al. 2014), and lower concentrations in cases of venous thrombosis (Zhou et al. 2012) and multiple sclerosis (Zhu et al. 2011), than in controls.

Cobalamin in pregnancy

Plasma cobalamin is higher in pregnant women at 12 (Bartels et al. 1989), 16 and 36 GW (Wu et al. 2013), than in non pregnant women. In the RTBC, plasma cobalamin decreases throughout pregnancy (Fernàndez-Roig et al. 2013) as previously reported in other studies (Bartels et al. 1989; Cikot et al. 2001; Koebnick et al. 2002; Velzing-Aarts et al. 2005; Murphy et al. 2007; Wallace et al. 2008; Ubeda et al. 2011; Wu et al. 2013; McNulty et al. 2013).

Cord and maternal plasma/serum cobalamin are positively correlated (Molloy et al. 2005; Obeid et al. 2005), but higher concentrations are found in the cord than in the maternal compartment (Obeid et al. 2005; Wallace et al. 2008; Fernàndez-Roig et al. 2013).

Plasma cobalamin and tHcy are negatively correlated, in pregnant women (Molloy et al. 2005; Velzing-Aarts et al. 2005) and in the cord (Molloy et al. 2005; Wallace et al. 2008).

The RDA for cobalamin in pregnant women is 2.6μg/d (Institute of Medicine 2000), and in Spain the recommended dietary intake is 2.2μg/d of cobalamin (Federación Española de Sociedades de Nutrición Alimentación y Dietética 2010).

No differences in second trimester maternal serum cobalamin were found in cases of conotruncal heart defects in the offspring and controls in a postfortification USA study (Shaw et al. 2014). In this study no data about cobalamin deficiency is provided being the low serum cobalamin quartile <282pmol/L (Shaw et al. 2014). Lower maternal

concentrations of plasma/serum cobalamin are associated with neural tube defects (NTD), in cohorts and case control studies (Tang et al. 2015).

One link between cobalamin and omphalocele comes from a case control study of a sample of multiple ethnicities from USA and exposed to folic acid fortification. After adjusting for confounders and correcting for multiple comparisons, only the *transcobalamin 2 receptor* c.23A>G polymorphism out of 23 single nucleotide polymorphisms (SNPs) in 13 one-carbon metabolism genes, was associated with a higher risk of omphalocele in the variant homozygote versus the normal homozygote foetuses (Mills et al. 2012).

Serum cobalamin was lower in cases of recurrent miscarriage than pregnant controls at ≈11 GW in a Syrian study whose whole sample cobalamin mean was marginal deficient (180pmol/L) (Hubner et al. 2008).

2.5. Interactions

The necessity to investigate interactions between the different micronutrients is increasingly recognised. Traditionally they have often been studied individually but the importance of nutrient-nutrient and gene-nutrient interactions on metabolic and clinical outcomes is increasingly clear.

The negative association between serum betaine concentration and post-methionine load plasma homocysteine is stronger in individuals with low folate status (Holm et al. 2005). Post-methionine load betaine becomes a weaker predictor of homocysteine after a 3wk treatment with folic acid, vitamin B_{12} and B_6 (Holm et al. 2004). Betaine and choline intakes were negatively correlated with fasting tHcy and post-methionine load plasma homocysteine in a USA cohort, especially with low folate and cobalamin status; and when stratifying according to folic acid fortification the associations of betaine and choline intakes and post-methionine load homocysteine was lost in the postfortification sample (Lee et al. 2010). Higher betaine to DMG conversion (as whether lower betaine, higher DMG or higher DMG/betaine ratio in plasma) has been reported when folate is

low in pregnancy for the RTBC; and betaine becomes a negative predictor of tHcy earlier in pregnancy in women with low folate status than with high (Fernàndez-Roig et al. 2013).

A clinical trial of folic acid supplementation with Northern Irish healthy men and women showed that post-treatment serum cobalamin was a more important predictor of plasma tHcy than folate (Quinlivan et al. 2002). In coronary artery disease patients evaluated 3 month after the complete implementation of folic acid fortification in USA, serum cobalamin was negatively associated with serum tHcy whereas serum folate was not (Liaugaudas et al. 2001). These results suggest the effect of cobalamin on tHcy depends on folate status.

Genetic variations in enzymes and transporters involved in 1C metabolism and related pathways can modulate the relationships between the metabolites addressed in this thesis, and the metabolites themselves can modify the effect of a given polymorphism. A 12wk postfortification USA clinical trial of diets containing 600μg/d of folic acid, 200mg/d of docosahexanoic acid and 480 or 930mg/d of choline during the third trimester of pregnancy, revealed a greater betaine to DMG flux in carriers of the variant allele for the *MTHFR* c.665C>T polymorphism in both diet groups (Ganz et al. 2016). Betaine dependent homocysteine remethylation did not differ between genotypes of the *MTR* c.2756A>G polymorphism in the 480mg/d choline group, but was lower in variant allele carriers compared to normal homozygotes in the 930mg/d choline group (Ganz et al. 2016). Other nutrient-gene interactions affecting 1C metabolism and clinical outcomes are discussed in *Introduction* section *3*.

Advancing the knowledge of the interactions within 1C metabolism may shed light on the discrepancies that have been and are being reported in the field, such as differences between populations exposed and unexposed to mandatory folic acid fortification.

3. Enzymes affecting homocysteine remethylation, the reduced folate carrier and polymorphisms associated with these.

3.1. Methylenetetrahydrofolate reductase and the c.665C>T polymorphism (rs1801133)

Methylenetetrahydrofolate reductase

Methylenetetrahydrofolate reductase (MTHFR; Enzyme Commission, EC 1.5.1.20) catalyses the irreversible reduction of 5,10-methyleneTHF to 5-methylTHF, the methyl donor in folate dependent remethylation (*Introduction* section *1*, **Figure 1**). The *MTHFR* gene is located at chromosome 1 (cytogenetic band 1p36.3), contains 11 exons and 10 introns and is coded in the minus strand (Goyette et al. 1994; Goyette et al. 1998; GeneCards 2016). Additional exons with alternative splicing have been identified (Rozen 2001). Mammalian MTHFR is a flavoenzyme, specifically flavin adenine dinucleotide (FAD) as cofactor; and the electron donor is nicotinamide adenine dinucleotide phosphate (NADPH). MTHFR is strongly inhibited by SAM, but this can be partially reversed by SAH, that can compete with SAM in a non-catalytic site of the enzyme (Kutzbach and Stokstad 1971). Mammalian MTHFR is mainly in the form of homodimers (Matthews et al. 1984).

Phosphorylated and non-phosphorylated forms of MTHFR have been detected in *in vitro* experiments with human recombinant MTHFR (Yamada et al. 2005; Marini et al. 2008), and *in vivo* in mouse liver (Christensen et al. 2015). The degree of phosphorylation state and differences in the designs of the *in vitro* studies could underlie the controversial results: phosphorylation downregulating (Yamada et al. 2005) or upregulating (Marini et al. 2008) enzyme activity. There is evidence *in vivo* (male mice fed a diet with excessive folic acid) supporting the phosphorylation is inhibitory (Christensen et al. 2015).

Like folate dependent remethylation, MTHFR activity is ubiquitous as it has been detected in all the analysed organs in rat, with the highest abundance in kidney

(Finkelstein et al. 1978). As for the cellular location, it has been found mainly in the cytoplasm (Finkelstein et al. 1978).

Diet might modulate the activity and expression of MTHFR. The protein expression of MTHFR was downregulated with excess folic acid intake in a study of male mice wild type or mild deficient for MTHFR (Christensen et al. 2015). But protein content in the diet did not affect MTHFR activity, according to the values of activity per milligram of protein in rat liver experiments (Finkelstein et al. 1978).

MTHFR c.665C>T polymorphism

MHFR is affected by the common polymorphism c.665C>T (also known as 677C>T) in which the substitution of the cytosine at a GCC codon by a thymidine results in a GTC codon and substitution of alanine at position 222 by valine (Frosst et al. 1995; Goyette et al. 1998). The 665 nucleotide position is in the exon 4 (Goyette et al. 1998), whereas the 222 amino acid position is in the N-terminus domain which is catalytic (Shan et al. 1999).

Human lymphocyte (Frosst et al. 1995) and placenta (Daly et al. 1999) studies have shown that MTHFR activity is lower in the heterozygote than in the homozygote normal genotype and lowest in the homozygote variant genotype. This was also true for residual activity after heat inactivation. Thus the variant enzyme is thermolabile and has lower activity (Frosst et al. 1995; Daly et al. 1999). Lower concentrations of 5-methylTHF and higher concentrations of other folate derivatives such as those involved in DNA synthesis would be expected if MTHFR activity is reduced. Supporting this, in TT compared to CC USA Caucasian women (Davis et al. 2005; Huang et al. 2008) and adults (Summers et al. 2010) there were higher RBC THF, 5,10-methyleneTHF, formylTHF, and lower RBC 5-methylTHF concentrations. Also there were lower blood DNA uracil concentrations in TT than CC adults of Puerto Rican origin (DeVos et al. 2008). *In vitro* experiments suggest the lower activity of the variant compared to normal MTHFR is due to a higher dissociation rate of the homodimer with the

subsequent release of the FAD cofactor (Yamada et al. 2001). The affinity for FAD is reduced by SAH, and increased (and to a higher extent in the variant than normal MTHFR) by SAM and 5-methylTHF (Yamada et al. 2001).

Folate may improve the SNP defect in the enzyme. Growth in methionine-absent media was at lower rate in yeast strains with variant compared to normal MTHFR, but this effect was reduced with the addition of 0.21pM 5-formylTHF (Marini et al. 2008). With the variant allele there was similar protein abundance but lower enzyme activity and proportion of phosphorylated MTHFRs than with the normal allele (Marini et al. 2008). Phosphorylatable sites (cysteines) are lost in the variant MTHFR (Yamada et al. 2005; Shahzad et al. 2013). Given the controversy about the phosphorylation effect, it is possible that the phosphorylated states are different in both enzymes, and that the phosphorylation inhibits the normal enzyme but activates or has no effect on the variant enzyme.

A meta analysis of 10 genome wide association studies (GWAS's) of cohorts of European descent people, reported *MTHFR* c.665C>T polymorphism as a genome wide significant positive predictor of tHcy, and the most important predictor out of ≈40000 analysed SNPs (van Meurs et al. 2013). Another meta analysis based on 67 observational studies showed higher homocysteine concentrations with variant allele increase (Holmes et al. 2011). However, when stratifying by folate status world regions in this meta analysis, the increases in homocysteine with the SNP were higher in low folate status regions, intermediate in mid folate status regions and lower in high folate status regions, such as postfortification USA (Holmes et al. 2011). This can be considered as evidence for folate relief of the SNP defect. Another study comparing exposure to folic acid fortification versus non-exposure in two patient cohorts, reported that, despite being a strong positive predictor of tHcy, the effect of the *MTHFR* c.665 variant allele was less important in the fortified cohort (Nagele et al. 2011). Regarding the MTHFR FAD cofactor, the effect of the *MTHFR* c.665C>T polymorphism on homocysteine is increased in the presence of suboptimal riboflavin status in an adult

population unexposed to folic acid fortification (García-Minguillán et al. 2014). An *in vitro* study showed that the variant enzyme was less susceptible to heat-inactivation when the medium contained riboflavin as well as 5-methylTHF (Yamada et al. 2001). In addition to folate and riboflavin status, the effect of *MTHFR* c.665C>T polymorphism on homocysteine seems to be affected by genetic interactions. In a prefortification American study the combination of *MTHFR* c.665TT / *MTRR* c.66GG was associated with lower serum tHcy compared to *MTHFR* c.665TT / *MTRR* c.66AA; while the *MTRR* c.66A>G polymorphism alone had no effect on homocysteine (Yang et al. 2008). This gene-gene interaction was not observed in the Spanish adult population though (Bueno et al. 2016). Unlike the Spanish study, in Yang *et al*. the ethnicity was heterogeneous and folic acid supplement users (≈24% of the sample) were included (Yang et al. 2008). A meta analysis of 4763 adults from two prefortification USA populations reported higher plasma tHcy and lower plasma folate concentrations with increasing number of *MTHFR* c.665 variant alleles, respectively; although the folate result did not reach genome wide significance (Hazra et al. 2009). In this meta analysis no association between *MTHFR* c.665 genotypes and plasma cobalamin was observed (Hazra et al. 2009). These results were confirmed for plasma tHcy, folate and cobalamin in another meta analysis of 3 GWAS's: two from Italy and one from the USA (Tanaka et al. 2009). The association between the SNP and tHcy was lost in the USA study when the analysis was done in each GWAS, and the authors proposed that this was due to folic acid fortification.

The polymorphism has been associated with lower plasma or serum folate concentrations in many studies (Harmon et al. 1996; Molloy et al. 1997; van der Put et al. 1998; Lucock et al. 2001; Castro et al. 2003; Kluijtmans et al. 2003; Vesela et al. 2005; Devlin et al. 2006; Parle-McDermott et al. 2006; Fredriksen et al. 2007; DeVos et al. 2008; Yang et al. 2008; Barbosa et al. 2008a; Laanpere et al. 2011; Ubeda et al. 2011; Zampieri et al. 2012; Qin et al. 2012; Bialecka et al. 2012; de Vogel et al. 2013; García-Minguillán et al. 2014; Barnabé et al. 2015; Plumptre et al. 2015; Li et al. 2015; Bueno et al. 2016). However, others have not confirmed these findings (Molloy et al. 1997;

Chango et al. 2000a; Chango et al. 2000c; Botto et al. 2003; Esfahani et al. 2003; Kim et al. 2004; Terruzzi et al. 2007; Barbosa et al. 2008b; Erdogan et al. 2010; Summers et al. 2010; Biselli et al. 2012; Boas et al. 2015; Misiak et al. 2016), although from the studies conducted in healthy population some have small sample size. In a study with also small sample size, higher concentrations of serum folate was associated with the variant homozygote genotype in "senior" (65-75 years, y) and "elder" (85-102y) individuals (Geisel et al. 2001).

There is some controversy about the effect of *MTHFR* c.665C>T on RBC folate. Many studies have found lower RBC folate concentrations with the variant alleles or genotypes (Molloy et al. 1997; Chango et al. 2000c; Lucock et al. 2001; Kluijtmans et al. 2003; Parle-McDermott et al. 2006; Lucock et al. 2013; Bueno et al. 2016). There are also reported results showing no association (Christensen et al. 1999; Molloy et al. 2002; Esfahani et al. 2003; Yates and Lucock 2003; Barbosa et al. 2008b; Stamp et al. 2010; Summers et al. 2010) or an enhancing effect (van der Put et al. 1998; Castro et al. 2003; Relton et al. 2005; Vesela et al. 2005; Plumptre et al. 2015). Some authors have argued that many of the studies in healthy population finding no effect or higher RBC folate concentrations have one or more of the following features that may confound the results: small sample size, folic acid fortification exposed population, genotypes not in Hardy-Weinberg equilibrium, and radioassay for the RBC folate determination which may overestimate the values in *MTHFR* c.665TT individuals (Molloy et al. 1998); as discussed (Parle-McDermott et al. 2006). A meta-analysis including only women of childbearing age, reported that plasma/serum and RBC folate (measured by microbiological assay) were progressively lower with increasing number of the variant T alleles (Tsang et al. 2015).

A study of Norwegian older adults (50-64y) reported lower plasma betaine and DMG with increasing number of variant T alleles (Fredriksen et al. 2007), whereas in a Brazilian prefortification study no differences were reported in women of childbearing age (Barbosa et al. 2008b). Unlike the Norwegian study, in Barbosa *et al*. the sample

size is small, younger, of only female sex, of heterogeneous ethnicity and blood collection is in fasting conditions. Thus, it is unclear if the *MTHFR* c.665C>T polymorphism can disrupt 1C metabolism to an extent that affect the regulation and activity of the choline oxidation pathway.

Most studies have reported no effect of the SNP on blood cobalamin concentrations (van der Put et al. 1995; Chango et al. 2000a; Botto et al. 2003; Kim et al. 2004; Terruzzi et al. 2007; Fredriksen et al. 2007; Barbosa et al. 2008b; Barbosa et al. 2008a; Erdogan et al. 2010; Summers et al. 2010; Laanpere et al. 2011; Ubeda et al. 2011; Bialecka et al. 2012; de Vogel et al. 2013; García-Minguillán et al. 2014; Barnabé et al. 2015; Misiak et al. 2016). However, some did report that the SNP was associated with lower serum (Harmon et al. 1996; Kluijtmans et al. 2003) or plasma (Chango et al. 2000c) cobalamin. To our knowledge, a link between the MTHFR reaction and cobalamin metabolism has not been proposed to date.

Folate has been reported to modulate the effect of the polymorphism on tHcy. Numerous studies have observed loss of the tHcy-increasing effect of the variant genotype or allele with increasing category of plasma or serum folate (Jacques et al. 1996; Harmon et al. 1996; Geisel et al. 2001; Kluijtmans et al. 2003; Brilakis et al. 2003; Kim et al. 2004; de Lau et al. 2010). Plasma/serum folate has been shown to modulate the effect of *MTHFR* c.665C>T on tHcy in some (Kim et al. 2004; Devlin et al. 2006) but not in other studies (Gaughan et al. 2001; Geisel et al. 2001; van Driel et al. 2009). THcy was higher in the first compared to the fifth serum folate quintile in healthy elderly English adults with the *MTHFR* c.665TT genotype only (Devlin et al. 2006). In Australian gastroenterology patients, with RBC folate below the median, the *MTHFR* c.665C>T polymorphism was no longer a negative predictor of RBC folate (Lucock et al. 2013).

Most pregnancy studies of the effect of the SNP on folate mediated one carbon metabolism are cross-sectional and at varying times of pregnancy ranging from the first trimester (Molloy et al. 1997; Relton et al. 2005; Parle-McDermott et al. 2006), 11-

25GW (Liang et al. 2014), 24-28GW (Kim et al. 2004), labour (Molloy et al. 2002; Lopreato et al. 2008; Barbosa et al. 2008a; Visentin et al. 2015) or at any time of pregnancy (Barnabé et al. 2015). One study does have longitudinal data on serum tHcy, folate and cobalamin (Ubeda et al. 2011). The results of these studies confirm that the *MTHFR* c.665C>T polymorphism is associated with higher tHcy, lower plasma, serum and RBC folate, and not with serum cobalamin. A UK study using a (non radioassay) protein-binding assay reported higher first trimester RBC folate in the *MTHFR* c.665TT compared to CC genotype (Relton et al. 2005). This study is contrary to the lower RBC folate concentration found in another first trimester pregnancy study in Ireland that uses the microbiological assay (Molloy et al. 1997; Parle-McDermott et al. 2006). Relton *et al*. study does not have the aforementioned features that may confound the results in studies finding higher RBC folate in non pregnant variant homozygotes (Parle-McDermott et al. 2006). The protein-binding assays are considered to have low accuracy when mixtures of folates are present, which is the situation in the different genotypes of the polymorphism; and overall protein-binding assays are less recommended than the microbiological assay (Pfeiffer et al. 2010). Overestimation of RBC folate in *MTHFR* c.665TT individuals might also apply in the (non radioassay) protein-binding assays.

To the best of our knowledge the studies assessing cord blood have investigated offspring but not the maternal *MTHFR* c.665C>T genotype, except in an Irish study finding a non significant trend toward higher cord plasma tHcy in TT mothers (Molloy et al. 2002). *MTHFR* c.665 variant genotypes or allele in the offspring is associated with lower serum folate (Lopreato et al. 2008; Plumptre et al. 2015), higher RBC folate (Relton et al. 2005; Plumptre et al. 2015); but not tHcy (Molloy et al. 2002; Lopreato et al. 2008; Plumptre et al. 2015), betaine and DMG (Visentin et al. 2015) or cobalamin (Lopreato et al. 2008) in the cord. In Molloy *et al*. a non-significant trend toward lower RBC folate in the *MTHFR* c.665TT than CC and CT offspring genotypes was observed (Molloy et al. 2002). Again, RBC folate determinations by protein-binding (Relton et al. 2005; Plumptre et al. 2015) or microbiological assays (Molloy et al. 2002) might

explain the differences. Another study reported no differences between offspring genotypes in cord tHcy, serum folate or cobalamin (Lopreato et al. 2008).

Two meta analyses studies have reported that the variant *MTHFR* c.665T allele in mothers, increases the risk of orofacial cleft in the offspring (Luo et al. 2012; Zhao et al. 2014). However, this finding was not confirmed in a separate meta-analysis, based on individual participant data from 4 European studies (Butali et al. 2013). Higher risk of orofacial cleft in the offspring in the presence of the variant allele compared to the CC genotype was reported in Asian (Pan et al. 2012; Zhao et al. 2014) but not other populations (Luo et al. 2012; Pan et al. 2012; Butali et al. 2013).

The maternal *MTHFR* c.665TT genotype was not associated with increased risk of SGA offspring in a meta analysis based on 9 case control studies and 2 cohorts. Most of the studies included were from non fortified countries, while in the rest not all their participants were exposed to mandatory fortification (Facco et al. 2009). Increasing number of variant alleles in the offspring was associated with a lower risk of SGA in a postfortification big Canadian study (Infante-Rivard et al. 2002). But a UK study found non-different allelic and genotype frequencies between cases (SGA) and controls (Glanville et al. 2006). And in a prefortification Mexican cohort of low-to-mid income no association was found between foetal genotypes and birth weight (Kordas et al. 2009). Although from sparse evidence, it seems the offspring *MTHFR* c.665C>T polymorphism has no effect in countries without mandatory folic acid policy, but it might be protective in fortified ones.

The *MTHFR* c.665 variant genotype and allele in the mother or offspring has been associated with risk of congenital heart defects in the offspring, as reported in a meta analysis (Xuan et al. 2014). The risk of NTDs in the offspring is also associated with the variant genotype or allele in the mother (Yadav et al. 2015; Yang et al. 2015) or offspring (Zhang et al. 2013; Yang et al. 2015). To the best of our knowledge no study has assessed maternal *MTHFR* c.665 genotypes and omphalocele risk in the offspring.

MTHFR c.665 variant homozygote or heterozygote genotype in live born infants was not associated with omphalocele risk in a postfortification USA study (Mills et al. 2012).

Recurrent miscarriage is usually defined as ≥2 or ≥3 consecutive pregnancy spontaneous losses, as reviewed (Rai and Regan 2006). There were no differences in the *MTHFR* c.665C>T genotype frequencies between women with recurrent miscarriage and mothers with no previous miscarriage in a Spanish study, even after stratifying by RBC folate status (Creus et al. 2013). But more proportion of variant homozygote was observed in women with recurrent miscarriage compared to women with normal pregnancies in a Chinese study (Zhu 2015). According to meta analyses, the maternal variant genotype and allele is associated with risk of recurrent miscarriage in populations non stratified by ethnicity or of Asians, but not in Caucasians (Cao et al. 2013; Chen et al. 2016b). In relation to sporadic (or unspecified) miscarriage, the effect of the polymorphism, whether in the mother or in the embryo, is not clear. There were no differences in the genotype frequencies between women with miscarriage and control women in a big Irish study (Parle-McDermott et al. 2005). But a smaller study of a postfortification Mexican population found a higher risk in TT mothers compared to CC and CT (Rodríguez-Guillén et al. 2009). The foetal variant genotype or allele has been associated with lower (Isotalo et al. 2000; Bae et al. 2007), higher risk of miscarriage (Callejón et al. 2007) or not associated (Zetterberg et al. 2002).

The *MTHFR* c.665 variant genotype or allele has been associated with risk of male infertility (Gupta et al. 2011), hypertension and pregnancy induced hypertension (Qian et al. 2007), coronary artery disease (Wald et al. 2002; Clarke et al. 2003), peripheral artery disease (Khandanpour et al. 2009) and premature coronary artery disease in Asian populations or when combined with plasma tHcy >15μmol/L (Hou et al. 2015). Thus, the polymorphism is associated with risk of CVD and this is thought to be due to its tHcy-increasing effect (Wald et al. 2002).

3.2. Betaine-homocysteine methyltransferase and the c.716G>A polymorphism (rs3733890)

Betaine-homocysteine methyltransferase

Betaine-homocysteine methyltransferase (BHMT; EC 2.1.1.5) catalyses the transfer of a methyl group from betaine to homocysteine, producing DMG and methionine (*Introduction* section *1*, **Figure 1**). The *BHMT* gene is located at chromosome 5 (cytogenetic band 5q14.1), contains 8 exons and 7 introns and is coded in the plus strand (Park and Garrow 1999; GeneCards 2016; Institute of Medicine 2016b). Despite an initial controversy and based on the high homology with the rat enzyme, it is nowadays accepted that human hepatic BHMT has the general structure of an homotetramer (González et al. 2002). Oligomerisation is required for catalytic function in human recombinant BHMT (Szegedi and Garrow 2004). BHMT is a zinc metalloenzyme whose metal atom is essential for catalytic activity (Millian and Garrow 1998), and each BMHT protein unit contains one atom of zinc (Szegedi et al. 2008).

Human BHMT is contained in liver hepatocytes (no gradient across the organ) and in the kidney cortex (mostly in proximal tubules), in both located in the cytosol (Delgado-Reyes et al. 2001). Mouse experiments have shown that BHMT activity is orders of magnitude lower in the kidney than in the liver (Szegedi et al. 2008), although a previous study in humans implied they might be similar (Gaull et al. 1973). Minor BHMT activity has also been reported in human foetal and adult brain (Gaull et al. 1973) although this has not been confirmed in another study of human brain of unspecified age (McKeever et al. 1991). Moreover, the mRNA transcript has not been detected in human brain (Sunden et al. 1997). BHMT activity was detected in human lens, with highest activity in foetal lens, relatively high in young adults and lowest in older adults (Rao et al. 1998). In Rao *et al*. BHMT protein was not detected in human lens of unspecified age (Rao et al. 1998), but *BHMT* mRNA is reported to be the most abundant transcript in human foetal lens (Wistow 2012).

BHMT has been identified at different stages throughout the development of the embryo and foetus. In a mouse study covering gestational days (GD) 9 and 10, *BHMT* mRNA was observed in the embryo and yolk sac only on gestational day (GD) 10 (Fisher et al. 2002). Therefore, since neural tube closure is considered complete in mice on 10 (Greene and Copp 2009) or 10.5 GD (Zhon and Sarkar 2008), BHMT should not be completely excluded as having a role in neural tube closure in mice. Moreover, *BHMT* mRNA and protein expression do not exactly overlap in the early days of mouse gestation (Lee et al. 2012). Another mouse experiment including several stages of development, e.g. 1-cell (1 GD), 4-cells (≈2 GD), morula (≈3 GD) and blastocyst (≈4 GD) as the final time point; reported *BHMT* mRNA expression from the 4-cell stage, peaking at morula stage and lowest expression by the blastocyst stage (Lee et al. 2012). BHMT protein and activity were detected only at the blastocyst stage. Therefore, *BHMT* expression, at least as mRNA, in early mouse pregnancy, shows a pattern of expression: it is expressed until the blastocyst stage, stops at some time point and then resumes at 10 GD. It is unknown though, if this can be extrapolated to human beings. Regarding its expression at more advanced stages of pregnancy, *BHMT* mRNA has not been detected in human first term and term (Solanky et al. 2010) and unspecified gestational age placentas (Sunden et al. 1997). In human placentas BHMT protein is not detectable by immunohistochemistry (The human protein atlas 2016) and, to our knowledge, there are no published studies investigating the protein in placenta. In human foetus livers ranging in age from approximately 11-32 GW, BHMT protein and activity have been detected. Both are lower than in adults, and the activity is positively correlated with gestational age (Feng et al. 2011). Detection of BHMT human foetal liver activity at lower levels than in adults but higher with increasing gestational age confirms the results of an earlier study and in fact that study also confirmed the same for foetal kidney BHMT activity (Gaull et al. 1973). However, there was no reference to statistical analysis in that study. Foetal BHMT may play a role in development but the lower BHMT activity plus the higher MTR activity in foetal compared to adult liver (Gaull et al.

1973), suggests that betaine dependent remethylation may be less important in foetal than in adult liver.

Early gestation mouse experiments show that inhibition of *BHMT* transcription and activity is associated with impaired embryo development to the blastocyst stage both in terms of reaching it and reduced cell number in the embryoblast (Lee et al. 2012). Inhibition of *BHMT* transcription before transferring the embryos to pseudopregnant dams is associated with a higher rate of embryo resorption (spontaneous abortion) compared to no inhibition despite no differences in implantation rate and posttransfer growth (Lee et al. 2012).

Among other functional roles, truncated forms of BHMT without catalytic function have been found in rat hepatocytes autofagosomal membranes, and this has led some authors to suggest a role in binding proteins for degradation (Overbye et al. 2007). It has been reported in human cell cultures that BHMT interacts and stabilises betaine-homocysteine methyltransferase 2 (BHMT2) (Li et al. 2008), which is a zinc metalloenzyme that can remethylate homocysteine with S-methylmethionine, but not with betaine (Szegedi et al. 2008). BHMT is considered a structural protein (crystallin) in the human lens, especially in the foetus, as reviewed (Wistow 2012).

There was lower expression of a construct of luciferase gene plus *BHMT* promoter when pharmacological doses of SAM (2000μM), but not methionine, are added to the culture media of human hepatoma cells (Castro et al. 2002). A subsequent experiment with the same cell line replicated this, and detected a SAM dose-dependent decrease in endogenous *BHMT* mRNA levels (Ou et al. 2007). Dietary methionine may regulate hepatic BHMT activity in a U-shape fashion. Animals fed methionine-supplemented diets have slightly higher hepatic BHMT activity than those on adequate methionine diets (Finkelstein et al. 1982; Finkelstein and Martin 1986; Emmert et al. 1996; Ohuchi et al. 2009). Hepatic BHMT activity is higher in rats on methionine deficient compared to adequate diets (Finkelstein et al. 1982; Park et al. 1997); although the difference was non significant in another rat report with less severe methionine deficient diets (Park

and Garrow 1999). In cattle, hepatic BHMT activity also responds quadratically to dietary methionine (Lambert et al. 2002). Betaine or choline supplementation enhances the methionine-deficiency mediated increase in hepatic BHMT activity in chickens (Emmert et al. 1996) and rats (Park and Garrow 1999). These hepatic BHMT activity increases due to methionine deficiency (Park et al. 1997; Park and Garrow 1999) or supplementation (Ohuchi et al. 2009) are mirrored by *BHMT* mRNA and protein contents, suggesting upregulated *BHMT* expression. Dietary betaine of preweaning mice, i.e. with dams fed a betaine-supplemented diet, increases liver BHMT activity (Schwahn et al. 2004). Deficient folate intake increases hepatic BHMT specific activity in micropigs (Halsted et al. 2002), but excess folic acid intake does not change hepatic *BHMT* mRNA expression in a study of male wild type mice or with mild MTHFR deficiency (Christensen et al. 2015).

Purified human hepatic BHMT activity was strongly inhibited by DMG and not inhibited by methionine (results not shown) in a study where most of the substrates and inhibitors were at pharmacological concentrations (2500μM betaine and 10^6μM homocysteine; and 500-50000μM DMG or methionine) (Skiba et al. 1982). A subsequent study with purified human hepatic BHMT and substrates and inhibitors at concentrations more close to the physiological ones (200μM betaine and 200μM homocysteine; 20-200μM DMG or methionine or SAM, or 20μM SAH), reported strong inhibition by DMG, weak by methionine and no inhibition by SAM or SAH (Szegedi et al. 2008). Indeed, purified human recombinant BHMT does not seem to bind SAM, as shown in one study by microcalorimetry (Bose et al. 2002). The inhibition by these compounds could vary between species. Methionine had an apparently stronger inhibitory effect in purified mouse hepatic BHMT compared to the human enzyme; but the results for DMG and SAM were similar in both enzymes (Szegedi et al. 2008).

Apart from DMG and methionine, Szegedi *et al*. results were contrary to previous observations from earlier rat liver extract (semi purified BHMT) experiments. The activity was reduced by SAM by 15% in an assay with substrates and inhibitors at near

physiological concentrations (350μM betaine and 20μM homocysteine; 50μM SAM) (Finkelstein et al. 1972); and by 32% with supraphysiological concentrations (2000μM betaine and 7000μM homocysteine; 224μM SAM) (Finkelstein and Martin 1984b). In another study with semi purified BHMTs from rat liver, or human liver or kidney; the activity was reduced by ≈17% by SAM (results not shown) in an assay with physiological concentrations of betaine and supraphysiological concentrations of homocysteine and SAM (200μM betaine and 200μM homocysteine; 1000μM SAM) (Allen et al. 1993a). Thus, according to Finkelstein *et al*. (Finkelstein et al. 1972), SAM inhibition of BHMT may be biologically plausible.

Inhibitory effects of SAH on BHMT activity were reported in rat liver extract experiments containing physiological concentrations of SAH (Finkelstein et al. 1972), but the results are not clear for supraphysiological concentrations of SAH (Finkelstein et al. 1974; Finkelstein and Martin 1984b). The activity was reduced by 16% by 50μM SAH in an assay with substrates and inhibitors at near physiological concentrations (Finkelstein et al. 1972). A study testing a range of SAH doses with substrate concentration ranges at or close to physiological concentrations (120-1030μM betaine and 25-800μM homocysteine; 380-3750μM SAH), shown reduced BHMT activity across the tested SAH range (Finkelstein et al. 1974). But in a subsequent study with supraphysiological concentrations of substrates, 440μM and 880μM SAH led to 15% lower and 16% higher BHMT activity, compared to the control assay with no added SAH, respectively (Finkelstein and Martin 1984b). Possible reasons for the controvert results of the two latter studies have not been proposed. Therefore, it appears that, SAH at similar concentrations to those reported for rat liver, i.e. 10-47μM (assuming rat is equal to human liver density 1.051g/mL, and rounding to 1g/mL) (Overmoyer et al. 1987; Fowler 2001) could inhibit BHMT activity *in vivo*.

Liver extract studies have also found an inhibitory effect of methionine for BHMT activity. Rat liver semi purified BHMT activity was reduced by 93% by 50μM methionine in an assay with substrates and inhibitors at near physiological

concentrations (Finkelstein et al. 1972). In the study with extracts from rat liver, or human liver or kidney; the activity was reduced by ≈8% by 300μM methionine (results not shown) in an assay with physiological concentrations of betaine and supraphysiological concentrations for homocysteine (Allen et al. 1993a). The reported concentrations of methionine in rat liver are 45μM (assuming liver density 1g/ml) (Finkelstein and Martin 1986). Thus, the rat liver extract assay of Finkelstein *et al.* from 1972 has concentrations in the physiological range making the reported results of methionine-mediated inhibition of BHMT the closest to *in vivo* conditions (Finkelstein et al. 1972). Purified/semi purified state of BHMT and different concentrations of substrates may be some of the causes for the different results for methionine inhibition among the commented studies (Finkelstein et al. 1972; Skiba et al. 1982; Allen et al. 1993a; Szegedi et al. 2008).

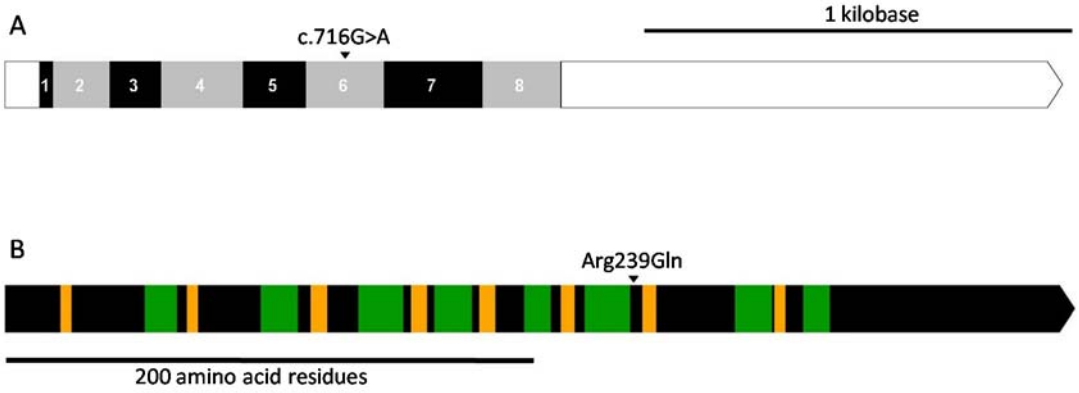
There is evidence in support of *in utero* programming of BHMT. Less *BHMT* mRNA is expressed in the liver of the offspring of rat dams on diets supplemented with folic acid (5mg/Kg diet) and/or protein restricted compared to control diets (folic acid as 2mg/Kg diet and normal protein content) (Chmurzynska and Malinowska 2011). Furthermore, mRNA and protein BHMT expression were higher in the hippocampus of offspring of sows fed a diet supplemented with betaine compared to the unsupplemented group (Li et al. 2014a). Indeed, the *BHMT* promoter contains part of a CpG island known to affect *BHMT* expression according to its degree of methylation (Ganu et al. 2013), suggesting the possibility of epigenetic regulation of *BHMT*.

BHMT c.716G>A polymorphism

BHMT is affected by the common polymorphism c.716G>A (also known as 742G>A) in which the substitution of the guanosine at a CGA codon by an adenosine results in the substitution of arginine at position 239 by glutamine (Park and Garrow 1999). The 716 nucleotide position is in the exon 6 (Park and Garrow 1999), whereas the 239 amino acid position is between α_6 helix and β_7 strand, main secondary structure elements of

the barrel (Evans et al. 2002) (**Figure 9**). In the homotetramer complex, the polymorphic amino acid position is in the protein surface (Li et al. 2008).

Figure 9. *BHMT* c.716G>A (Arg239Gln) polymorphism position.



A. Spliced *BHMT* mRNA diagram with c.716G>A location. Black and grey segments with numbers indicate exons count. White segments indicate non-coding RNA. B. BHMT protein sequence diagram with Arg239Gln location. Yellow and green segments indicate main β strands and α helixes, respectively, of the $(\beta/\alpha)_8$ barrel structure. Black segments indicate protein regions outside of the main secondary structure elements of the barrel. Based on sequences from (Park and Garrow 1999; Evans et al. 2002; Institute of Medicine 2016c).

No differences in betaine affinity have been observed in assays (with unspecified range of betaine and 500μM homocysteine) of crude extract of *E. coli* transfected with the normal or variant human *BHMT* allele (Weisberg et al. 2003). However, the assays of cytosol of monkey kidney cell transfected with human *BHMT* showed that the variant enzyme has a higher affinity for betaine (assay with 2-256μM betaine and 400μM homocysteine) and homocysteine (assay with 64μM betaine and 3.1-400μM homocysteine). However, basal enzyme activities were similar (assay with 64μM betaine and 400μM homocysteine) (Li et al. 2008). A recent study of human liver extract (endogenous BHMT), did not specify kinetic parameters but reported no correlation between *BHMT* c.716G>A genotypes and enzyme activity (assay with 64μM betaine an 400μM homocysteine) (Feng et al. 2011). Rat liver betaine and

homocysteine concentrations are 550μM and 7.6μM, respectively (Svardal et al. 1986; Koc et al. 2002). Thus, none of the aforementioned enzyme assays have used the betaine physiological concentrations; while for homocysteine 100-fold higher concentrations are used (except in the homocysteine affinity assays). Assays using physiological liver concentrations of betaine and homocysteine are needed, although these are unknown, to the best of our knowledge, in human liver. No differences in protein BHMT levels were observed between the normal and variant alleles in monkey kidney cells transfected with human *BHMT* (Li et al. 2008), and no correlation was observed between *BHMT* c.716G>A genotype and protein levels in human liver extracts (Feng et al. 2011). Both enzymes had similar thermostability (Weisberg et al. 2003). Overall it appears from the *in vitro* studies that the only difference between the variant enzyme and the normal enzyme is that the former has a higher affinity for homocysteine, and maybe for betaine.

Some human studies have analysed the possible effects of the polymorphism on the blood concentrations of the metabolites addressed in this thesis (**Table 2** and **Table 3**).

Many studies support the lack of association of the polymorphism with tHcy (Heil et al. 2000; Weisberg et al. 2003; Morin et al. 2003b; Ananth et al. 2007; Terruzzi et al. 2007; Fredriksen et al. 2007; Paré et al. 2009; Liang et al. 2014; Misiak et al. 2016). Only two postfortification studies from Brazil and USA have reported significant associations between variant genotype or allele and homocysteine, and in the opposite sense. Variant homozygote genotype was associated with lower tHcy in a Brazilian Down syndrome cohort (Biselli et al. 2012) whereas increasing number of variant alleles was associated with higher tHcy in healthy adults from a USA cohort (Clifford et al. 2012).

There is no association between the polymorphism and plasma or serum folate (Morin et al. 2003b; Ananth et al. 2007; Terruzzi et al. 2007; Fredriksen et al. 2007; Biselli et al. 2012; Zampieri et al. 2012; Misiak et al. 2016), RBC folate (Morin et al. 2003b), or plasma or serum cobalamin (Morin et al. 2003b; Ananth et al. 2007; Terruzzi et al. 2007; Fredriksen et al. 2007; Misiak et al. 2016). Increasing number of variant alleles

was associated with lower DMG but no difference in betaine in an older adult Norwegian cohort (Fredriksen et al. 2007). The DMG results were replicated in another Norwegian cohort of stable angina pectoris patients, where betaine was not analysed (Svingen et al. 2013).

In pregnancy studies no effect of the polymorphism in the mother on maternal plasma tHcy (Liang et al. 2014), or in the foetus on cord plasma betaine and DMG (Visentin et al. 2015) have been reported.

Table 2. Case control studies investigating the role of the *BHMT* c.716G>A polymorphism on 1C metabolism metabolites.

Reference	Country, year	Mandatory fortification	n Case [outcome][1]	n Controls	Age (y)	Metabolites[2]	Results
(Heil et al. 2000)	The Netherlands, ND[3]	No	190 [CVD]	601	16-68	tHcy	No association (cases, controls, whole sample)
(Morin et al. 2003b)	Canada, ND	ND	54 children [SB] 57 mothers	93 children 86 mothers	1-22 16-55	tHcy, folate (serum and RBC), cobalamin	No association (children and mothers whole sample)
(Weisberg et al. 2003)	USA, 1998-99	Yes	271 [CAD]	233	18-75	tHcy	No association (whole sample)
(Ananth et al. 2007)	USA, 2002-03	Yes	196 [AP]	191	Adult	tHcy, folate, cobalamin	No association (cases, controls)
(Terruzzi et al. 2007)	Italy, ND	No	82 [Obesity]	54	25-53	tHcy, folate, cobalamin	No association (whole sample)
(Zampieri et al. 2012)	Brazil, ND	ND	105 [DS offspring]	185	Adult	Folate	No association (whole sample)
(Misiak et al. 2016)	Poland, ND	No	135 [FES]	146	Adult	tHcy, folate, cobalamin	No association (cases, controls)

[1]Cases outcomes: CVD, Cardio Vascular Disease; SB, Spina Bifida; CAD, Coronary Artery Disease; AP, *Abruptio Placentae*; DS, Down Syndrome; FES, First Episode Schizofrenia. [2]Metabolites in plasma/serum unless otherwise specified. [3]ND, No Data.

Table 3. Retrospective cohort studies investigating the role of the *BHMT* c.716G>A polymorphism on 1C metabolism metabolites.

Reference	Country, year	Mandatory fortification	n [feature][1]	Age (y)	Metabolites[2]	Results
(Fredriksen et al. 2007)	Norway, 1999-01	No	10601	50-64	tHcy, folate, betaine, DMG, cobalamin	Lower DMG with A allele increase
(Paré et al. 2009)[3]	USA, 1992-95	No	13974 [Women]	≥45	Folate adjusted tHcy	No association
(Biselli et al. 2012)	Brazil, 2004-11	Yes	90 [DS]	0-30	tHcy, folate	Lower tHcy in AA genotype
(Clifford et al. 2012)	USA, 2002-04	Yes	373	30-69	Folate adjusted tHcy	Higher tHcy with A allele increase
(Svingen et al. 2013)	Norway, 2000-04	No	2424 [SAP]	44-78	DMG	Lower DMG with A allele increase
(Liang et al. 2014)	China, 2011	No	400 [Pregnant women, 11-25GW]	25-35	Folate adjusted tHcy	No association
(Visentin et al. 2015)	Canada, 2010-12	Yes	≈250 [cord blood samples]	18-45 (mothers)	Betaine, DMG (cord plasma)	No association

[1]Cohort specific features: DS, Down Syndrome; SAP, Stable Angina Pectoris. [2]Metabolites in plasma/serum unless otherwise specified. [3]Genome wide association study.

To the best of our knowledge, no study to date has investigated the effect of the *BHMT* c.716G>A polymorphism, maternal or foetal, on birth weight or LBW, SGA/intrauterine growth restriction (IUGR). It might be expected to affect foetal growth because, as commented above, the BHMT pathway becomes more important during late pregnancy and/or when folate status is low (Fernàndez-Roig et al. 2013).

There is sparse literature regarding the *BHMT* c.716G>A polymorphism and risk of congenital heart defects. In USA studies where whole (Hobbs et al. 2010) or the majority of the sample (Mitchell et al. 2010) was exposed to mandatory folic acid fortification, there was no independent effect of the maternal genotype on congenital heart defect risk in the offspring. There have been no maternal SNP studies before mandatory fortification or in a non-fortified country, to date. Lack of association between offspring genotype and congenital heart defect risk have also been reported in USA studies before (Shaw et al. 2009b) and after folic acid fortification (for the highest percentage of the sample) (Mitchell et al. 2010). All of the aforementioned studies were performed in samples of multiple ethnicities.

Several studies have addressed the *BHMT* c.716G>A polymorphism and risk of NTDs, but only one Canadian study has assessed the SNP in the mother (Morin et al. 2003b). According to the age range (1-22y) and mean age (10y) of the offspring and to the blood draws in the sample being before 1999 (Christensen et al. 1999), most of the pregnancies if not all were before the Canadian folic acid fortification policy in 1998. There was no vitamin supplement use in the sample and maternal serum folate mean ranged 14.3-18.7nmol/L among genotypes. This study reported no difference in risk of NTDs in variant versus normal homozygote mothers or children (Morin et al. 2003b). But other studies have found associations of the offspring genotype and NTDs. A USA family-based study including mothers using vitamin supplements and with most pregnancies exposed to mandatory folic acid fortification (information provided by author), reported the SNP in the offspring was associated with NTDs risk (Boyles et al. 2006). In a postfortification USA study with unknown prenatal folic acid supplement

use, offspring carrying the variant allele compared to normal homozygotes had higher risk of NTDs. However the analysis was not corrected for multiple comparisons despite being a study of 118 SNPs (Shaw et al. 2009b). A Chinese family-based study including mothers using folic acid supplements reported no association between the SNP in the offspring and NTDs risk (results not shown) (Liu et al. 2014). The results of studies assessing the polymorphism in the offspring and NTDs risk according to maternal prenatal vitamin supplement use are controvert. When stratifying by preconception vitamin supplement use in Boyles *et al*. study, higher risk with the offspring SNP is only observed with supplementation (Boyles et al. 2006). However, when stratifying by pre/postconception folic acid supplement use in Liu *et al*. study, higher risk with the offspring SNP is only observed with no supplementation (Liu et al. 2014). Some differences between both studies apart from ethnicity are exposure to mandatory folic acid fortification in most pregnancies in the USA study and the different categories used to define supplement user; e.g. the Chinese study includes postconception use. These inconclusive results highlight the importance of further study of the *BHMT* c.716G>A polymorphism according to folate status.

To the best of our knowledge there are no reports about maternal *BHMT* c.716 genotypes and omphalocele risk in the offspring. *BHMT* c.716 variant homozygote or heterozygote genotype in live born infants was not associated with omphalocele risk in a postfortification USA study (Mills et al. 2012).

To the best of our knowledge there are no studies evaluating the effect of the *BHMT* c.716G>A polymorphism in the mother or embryo, and the risk of miscarriage.

Other studies have investigated other possible clinical effects of the polymorphism. In a meta analysis of 3 studies, the *BHMT* c.716G>A polymorphism was not associated with coronary artery disease (Singh and Lele 2012). The SNP may be a risk factor for placental abruption (Ananth et al. 2007), and a protective factor for uterine cervical carcinoma (Mostowska et al. 2011) and Down syndrome in the offspring (Zampieri et al. 2012; Amorim et al. 2013). A study of USA women reported lower risk of colorectal

adenoma (non-cancerous tumour) in hetero plus variant homozygotes than in normal homozygotes only in the stratum with high dietary methyl status (Hazra et al. 2007). The assessment of dietary methyl status was by a food frequency questionnaire based on folate, methionine and alcohol consumption and the results were the same when exposure to mandatory folic acid fortification was stratified for (Hazra et al. 2007).

3.3. Reduced folate carrier and the c.80G>A polymorphism (rs1051266)

Reduced folate carrier

The reduced folate carrier (SLC19A1, also known as RFC) is along with the proton coupled folate transporter and the folate receptors, a protein involved in the cellular uptake of folate. The *SLC19A1* gene is located at chromosome 21 (cytogenetic band 21q22.3), contains 17 exons (due to the existence of upstream and downstream non-coding exons) and is coded in the minus strand (GeneCards 2016; Institute of Medicine 2016b). Five exons are protein coding. Depending on the earlier discovered upstream non-coding exons number and given names, the first coding exon has been termed "exon 3" (Zhang et al. 1998; Whetstine et al. 2001) or "exon 2" (Tolner et al. 1998; Williams and Flintoff 1998), though the latter term is more used. SLC19A1 consists of 12 transmembrane domains (Ferguson and Flintoff 1999). SLC19A1 has higher affinity for 5-methylTHF (K_t 1.1-2.0μM) and 5-formylTHF (K_t 1.2-4.2μM), i.e. reduced folate forms, than for the oxidised folic acid (K_t from 110-220μM); according to experiments in human cell lines (Sirotnak and Tolner 1999). Experiments of inhibition of methotrexate (chemical drug analogue of folic acid) influx by extracellular organic anions revealed that the mechanism for SLC19A1 mediated folate transport into the cell is the coupling of the downhill efflux of organic phosphates to the uphill influx of folate forms (Henderson and Zevely 1983). Although folate transport by SLC19A1 may be bidirectional, physiologically it is considered to take up folate from the extracellular media due to the high intracellular concentrations of organic anions (Shane 2010)

Since the discovery that proton coupled folate transporter is mainly responsible for folate intestinal absorption (Qiu et al. 2006), it is now considered that SLC19A1 plays a minor role in intestinal absorption. The optimal pH for SLC19A1 mediated transport is 7.5, according to methotrexate influx experiments in mouse lymphocytic leukaemia cells (Sierra et al. 1997). While the optimal pH for folate intestinal absorption is 6 (Selhub and Rosenberg 1981), that *SLC19A1* transcript and protein are up-regulated in the small intestine of folate deficient mice (Liu et al. 2005) suggests a role for it in intestinal absorption. However, due to its optimal pH at the physiological level, SLC19A1 is considered the major route of folate transport into systemic tissues (Zhao and Goldman 2013).

SLC19A1 mRNA is detected ubiquitously in human tissues, with the highest expression values in placenta and liver, and the lowest in skeletal muscle and heart (Whetstine et al. 2002). In human placentas, lower mRNA expression is found at term than in the first trimester (Solanky et al. 2010). Probably due to its role as a transmembrane transporter, SLC19A1 has been found in membranes of several types of cells. These included the epithelia: apical brush-border membrane of the entire intestine (jejunum, ileum, duodenum and colon), basolateral membrane of renal tubular epithelium, plasma membrane of hepatocytes, apical surface of the choroid plexus epithelium (Wang et al. 2001), retinal pigment epithelium (Chancy et al. 2000), apical microvillous plasma membrane and basal plasma membrane of the syncytiotrophoblast (Solanky et al. 2010).

Fifty five CpG sites are close to or within the *SLC19A1* gene, organised in two CpG islands. Several of these CpG sites are reported to differ in methylation state according to tHcy. Most of the sites had lower methylation in leukocytes from individual with high (20-113μmol/L) compared to low (5-10μmol/L) plasma tHcy (Farkas et al. 2013). Methylation states in analysed CpG sites of the *proton coupled folate transporter* and *folate receptor 1* are similar in situations of high or low tHcy. The methylation of many of the CpG sites in *SLC19A1* is negatively associated with its expression. Therefore

SLC19A1 may be a folate transporter which can be epigenetically upregulated in response to high tHcy (Farkas et al. 2013). The inverse association between plasma tHcy and methylation state of a CpG island in the promoter of *SLC19A1*, as well as the inverse association between the latter and *SLC19A1* mRNA expression, has also been reported in lymphocytes from systemic lupus erythematosus cases and controls (Rupasree et al. 2014).

SLC19A1 c.80G>A polymorphism

SLC19A1 is affected by the common polymorphism c.80G>A in which the substitution of the guanosine at a CGC codon by an adenosine results in a CAC codon and substitution of arginine at position 27 by histidine (Tolner et al. 1998; Institute of Medicine 2016c). When the polymorphism was first identified by Chango *et al*. the polymorphic codons were erroneously termed as CGG and CAG (Chango et al. 2000b). The 80 nucleotide position is in "exon 2" (Chango et al. 2000b), which is the first coding exon; whereas the 27 amino acid position is in the most N-terminal part of the first transmembrane domain, close to the cytoplasm (Ferguson and Flintoff 1999).

Experiments in human intestinal cell cultures transfected with truncated constructs of *SLC19A1* show that when the 1-27 amino acid residue segment (which includes the polymorphic amino acid position) is lost, only a small proportion of SLC19A1 proteins reach the cell surface and the rest is contained in the cytoplasm (Marchant et al. 2002). *In vitro* studies investigating the normal and the variant enzyme for the polymorphism report contradictory results (Whetstine et al. 2001; Baslund et al. 2008). In an SLC19A1 deficient erythroleukaemia (myeloid) human cell line transfected with the normal or variant allele from lymphoid leukaemia patients, minor differences in expression and susceptibility to inhibitors are observed (Whetstine et al. 2001). On the one hand, the variant transporter protein is slightly less expressed and the methotrexate uptake is 2-fold less or more inhibited by other substrates (e.g. 5-formylTHF is a stronger inhibitor of the methotrexate transport in the variant protein). On the other hand the variant transporter has similar uptake rates for methotrexate and 5-formylTHF, and similar

kinetic parameters for methotrexate uptake (V_{max} and K_t) than the normal transporter (Whetstine et al. 2001). No statistical analysis of any of the differences were reported in this study. In human unstimulated and antigen-stimulated lymphocytes no significant differences in protein expression of endogenous SLC19A1 were detected among the three *SLC19A1* c.80G>A genotypes, but decreased methotrexate transport efficacy was reported in the variant homozygote stimulated lymphocytes (Baslund et al. 2008). The authors suggest that the different findings between their and Whetstine *et al*.'s might be cell line specific. It is possible that depending on the cell type, the variant transporter is less expressed, and has different affinities and transport efficacy for some of its transportable molecules (e.g. different folate forms).

The polymorphic nucleotide of the SNP is close to position 83, which is one of the five CpG sites in a CpG island within the coding region of the gene; although the methylation states of this island has not been associated with tissue-specific differences or mRNA expression (Farkas et al. 2013). However, the SNP affects the methylation state of other CpG sites related to *SLC19A1* expression: in most of these CpG sites there was lower methylation in *SLC19A1* c.80AA compared to GG leukocytes. Plasma tHcy modified the effect of the SNP on these CpG sites: in *SLC19A1* c.80AA compared to GG leukocytes most of these CpG sites had higher methylation when tHcy was low, and lower methylation when tHcy was high (Farkas et al. 2013). With these finding it can be speculated that in variant compared to normal homozygotes the downregulation with low tHcy and upregulation with high tHcy of the transporter are greater.

Some human studies have investigated the effect of the polymorphism in the metabolites addressed in this thesis (**Table 4** and **Table 5**).

Most of the studied investigating the effect of the *SLC19A1* c.80G>A SNP reported that it had no effect on tHcy (Chango et al. 2000b; Winkelmayer et al. 2003; Födinger et al. 2003; Morin et al. 2003a; Yates and Lucock 2005; Devlin et al. 2006; Fredriksen et al. 2007; Lopreato et al. 2008; Barbosa et al. 2008b; Stanislawska-Sachadyn et al. 2009; de Lau et al. 2010; Summers et al. 2010; Biselli et al. 2012; Rah et al. 2012; Bialecka et al.

2012; Kumudini et al. 2014; Liang et al. 2014; Bueno et al. 2016). A tHcy-increasing effect have been suggested in two studies. In Estonian women undergoing *in vitro* fertilisation higher tHcy was observed in heterozygotes compared to normal homozygotes (Laanpere et al. 2011). Elderly (≥60y) patients of a postfortification Brazilian study had higher tHcy with increasing number of variant alleles (Barnabé et al. 2015). These results do not agree with previous European studies that found no association in individuals aged ≥60y (Devlin et al. 2006; de Lau et al. 2010). In Barnabé *et al*. the ethnicity was heterogeneous and serum folate median was 25.4nmol/L (Barnabé et al. 2015). The sample in Devlin *et al*. were former patients with serum folate means ranging 10.6-12.0nmol/L among genotypes; and in de Lau *et al*. the sample was representative of the Estonian population and plasma folate median was 12.1nmol/L (Devlin et al. 2006; de Lau et al. 2010). Lower tHcy was observed in variant homozygotes compared to hetero and normal homozygotes in a healthy Indian population possibly deficient for folate (plasma folate median 11.1nmol/L) and marginal deficient for cobalamin (plasma cobalamin median 163pmol/L) (Sukla and Raman 2012). But no association between increasing number of variant alleles and tHcy was found in an Indian sample with unknown folate and cobalamin status, and that includes Parkinson's disease patients (Kumudini et al. 2014).

Most studies of the effect of the *SLC19A1* c.80G>A polymorphism on serum or plasma folate have reported no effect (Chango et al. 2000b; Winkelmayer et al. 2003; Födinger et al. 2003; Morin et al. 2003a; Vesela et al. 2005; Devlin et al. 2006; Fredriksen et al. 2007; Lopreato et al. 2008; Barbosa et al. 2008b; Stanislawska-Sachadyn et al. 2009; Summers et al. 2010; Laanpere et al. 2011; Biselli et al. 2012; Rah et al. 2012; Bialecka et al. 2012; Mansoori et al. 2014; Barnabé et al. 2015). In a sample of Brazilian mothers aged ≥35y (including mothers of Down syndrome children), *SLC19A1* c.80 variant allele carriers had less risk of serum folate below P_{25} than normal homozygotes (Zampieri et al. 2012). And in old adults with thromboembolic vascular problems from UK or Australia, variant homozygotes had higher plasma folate than hetero and normal

homozygotes (Yates and Lucock 2005). Both studies determined plasma/serum folate by protein binding assays (Yates and Lucock 2005; Zampieri et al. 2012).

Numerous studies reported no effect of the *SLC19A1* c.80G>A SNP on RBC folate (Chango et al. 2000b; Födinger et al. 2003; Relton et al. 2005; Vesela et al. 2005; Yates and Lucock 2005; Barbosa et al. 2008b; Stamp et al. 2010; Lucock et al. 2013). However, it was associated with higher RBC folate concentrations in healthy young adults from Northern Ireland (Stanislawska-Sachadyn et al. 2009) and with lower RBC folate concentrations in our study of a local Spanish adult population (Bueno et al. 2016). A young sample (20-26y) including multivitamin supplement users was analysed in the former study (Stanislawska-Sachadyn et al. 2009). Bueno *et al*. study excluded supplement users, was conducted at a time voluntary fortification was infrequent in Spain, has a sample with broader age range, and adjusted for other SNPs affecting 1C metabolism as well as cobalamin and betaine status (Bueno et al. 2016).

Increasing number of *SLC19A1* c.80 variant alleles was not associated with betaine or DMG in an older adult Norwegian cohort (Fredriksen et al. 2007). The lack of association with DMG was confirmed in a Brazilian prefortification study of women of childbearing age (results not shown) (Barbosa et al. 2008b).

Most studies of the effect of the *SLC19A1* c.80G>A polymorphism on plasma cobalamin have reported no effect (Morin et al. 2003a; Fredriksen et al. 2007; Lopreato et al. 2008; Barbosa et al. 2008b; Summers et al. 2010; Laanpere et al. 2011; Bialecka et al. 2012; Barnabé et al. 2015). In old adults with thromboembolic event vascular problems there were differences in plasma cobalamin among the *SLC19A1* c.80G>A genotypes, with the lowest concentration in variant homozygotes and the highest concentration in heterozygotes (Yates and Lucock 2005).

Whether folate modulates the possible effect of the *SLC19A1* c.80G>A polymorphism on tHcy was tested in a study of healthy elderly English adults (Devlin et al. 2006). The

distribution of serum tHcy across quintiles of serum folate did not vary among *SLC19A1* c.80G>A genotypes.

Four cross-sectional studies have assessed the effect of the polymorphism on 1C metabolism in pregnancy finding no effect (Relton et al. 2005; Lopreato et al. 2008; Liang et al. 2014; Barnabé et al. 2015).

Table 4. Case control studies investigating the role of the *SLC19A1* c.80G>A polymorphism on 1C metabolism metabolites.

Reference	Country, year	Mandatory fortification	n Case [outcome][1]	Controls	Age (y)	Metabolites[2]	Results
(Morin et al. 2003a)	Canada, ND	ND[3]	57 [SB offspring]	87	16-55	tHcy, folate (serum and RBC), cobalamin	Higher RBC folate in AA genotype (whole sample)
(Laanpere et al. 2011)	Estonia, ≥2000	No	285 [IVF]	225	Child bearing	tHcy, folate, cobalamin	Higher tHcy in GA genotype (cases)
(Bialecka et al. 2012)	Poland, 2008-10	No	248 [PD]	254	Old adult	tHcy, folate, cobalamin	No association (cases, controls)
(Rah et al. 2012)	South Korea, 1999-05	No	100 [IRPL]	125	22-45	tHcy, folate	No association (cases)
(Zampieri et al. 2012)	Brazil, ND	ND	105 [DS offspring]	185	Adult	Folate	Lower risk of P_{25} of folate in AG plus AA genotypes (whole sample ≥35y)
(Kumudini et al. 2014)	India, ND	No	151 [PD]	416	Adult	tHcy	No association (whole sample)
(Mansoori et al. 2014)	India, 2007-10	No	80 [AD] 50 [VD]	120	≥50	folate (serum and RBC)	No association (AD cases, VD cases, controls)

[1]Case outcomes: SB, Spina Bifida; IVF, *In Vitro* Fertilisation; PD, Parkinson's Disease; IRPL, Idiopathic Recurrent Pregnancy Loss; DS, Down Syndrome; AD, Alzheimer's Disease; VD, Vascular Dementia. [2]Metabolites in plasma/serum unless otherwise specified. [3]ND, No Data.

Table 5. Retrospective cohort studies investigating the role of the *SLC19A1* c.80G>A polymorphism on 1C metabolism metabolites.

Reference	Country, year	Mandatory fortification	n [feature][1]	Age (y)	Metabolites[2]	Results
(Chango et al. 2000b)	France, 1994-00	No	169	35-60	tHcy, folate (plasma and RBC)	No association
(Födinger et al. 2003)	Austria, ND[3]	No	120 [ESRD]	Adult	tHcy, folate (plasma and RBC)	No association
(Winkelmayer et al. 2003)	Austria, 1996-98	No	730 [RT]	Adult	tHcy, folate	No association
(Relton et al. 2005)	UK, 1996-03	No	478 [Pregnant women, 1st trimester] 573 [Cord blood]	Child bearing ≥37GW	RBC folate	No association (mothers, neonates)
(Vesela et al. 2005)	Czech Republic, ND	No	591	18-65	folate (plasma and RBC)	No association
(Yates and Lucock 2005)	UK, Australia, ND	No	51 [TE] 105 [NTE]	52-79	tHcy, folate (plasma and RBC), cobalamin	Higher plasma folate, lower cobalamin in AA genotype (TE)
(Devlin et al. 2006)	UK, 1994-96	No	1042	68-102	tHcy, folate	No association

[1]Cohort specific features: ESRD, End-Stage Renal Disease; RT, Renal Transplant; TE, Thromboembolic Event; NTE, Non-Thromboembolic Event vascular problems. [2]Metabolites in plasma/serum unless otherwise specified. [3]ND, No Data.

Table 5. Retrospective cohort studies investigating the role of the *SLC19A1* c.80G>A polymorphism on 1C metabolism metabolites (continued).

Reference	Country, year	Mandatory fortification	n [feature][1]	Age (y)	Metabolites[2]	Results
(Fredriksen et al. 2007)	Norway, 1999-01	No	10601	50-64	tHcy, folate, betaine, DMG, cobalamin	No association
(Barbosa et al. 2008b)	Brazil, <2004	No	102 [Women]	Child bearing	tHcy, folate (serum and RBC), DMG, cobalamin	No association
(Lopreato et al. 2008)	Brazil, 2001-03	No	275 [Pregnant women, labour] 275 [Placenta[3] blood]	Child bearing	tHcy, folate, cobalamin	No association (mothers, neonate)
(Stanislawska-Sachadyn et al. 2009)	Northern Ireland, 1997-99	No	489	20-26	tHcy, folate (serum and RBC)	Higher RBC folate with A allele increase (whole sample, women)
(de Lau et al. 2010)	The Netherlands, ND[4]	No	1011	60-90	tHcy	No association
(Stamp et al. 2010)	New Zealand, 2005-08	No	191 [MTXR]	18-84	RBC folate	No association
(Summers et al. 2010)	USA, 2007	Yes	49 [Women] (26 Caucasian, 23 African American)	Child bearing	tHcy, folate and derivatives (serum and RBC), cobalamin	Higher RBC THF with A allele increase (African American)
(Biselli et al. 2012)	Brazil, 2004-11	Yes	90 [DS]	0-30	tHcy, folate	No association

[1]Cohort specific features: MTXR, Methotrexate treated Rheumatoid arthritis; DS, Down Syndrome. [2]Metabolites in plasma/serum unless otherwise specified. [3]From placenta veins (neonate blood). [4]ND, No Data.

Table 5. Retrospective cohort studies investigating the role of the *SLC19A1* c.80G>A polymorphism on 1C metabolism metabolites (continued).

Reference	Country, year	Mandatory fortification	n [feature][1]	Age (y)	Metabolites[2]	Results
(Sukla and Raman 2012)	India, ND	No	1426	35 median	tHcy	Lower tHcy in AA genotype
(Lucock et al. 2013)	Australia, ND	No	212 [GEP]	18-89	RBC folate	No association
(Liang et al. 2014)	China, 2011	No	400 [Pregnant women, 11-25GW]	25-35	Folate adjusted tHcy	No association
(Barnabé et al. 2015)	Brazil, 2006-07	Yes	106 [Children]	≤6	tHcy, folate, cobalamin	Higher tHcy with A allele increase (elderly)
			291 [Pregnant women, variable[4]]	Child bearing		
			60 [Lactating women]			
			262 [Elderly]	≥60		
(Bueno et al. 2016)	Spain, 1998-02	No	789	18-75	tHcy, folate (plasma and RBC)	Lower RBC folate, lower plasma folate (borderline significance) in AA genotype

[1]Cohort specific features: GEP, Gastroenterology Patients.. [2]Metabolites in plasma/serum unless otherwise specified. [3]ND, No Data. [4]The pregnancy trimester where the blood sample is drawn varies in the sample: 25%, 50% and 25% of the participants in first, second and third trimester, respectively.

As far as we know only one study has assessed the effect of the *SLC19A1* c.80G>A polymorphism on birth weight. In a UK cohort, there was no effect of the maternal or foetal SNP on birth weight, even when comparing the most extreme categories of maternal RBC folate status at first trimester (Relton et al. 2005).

There is conflicting evidence in the literature regarding the association between the *SLC19A1* c.80G>A polymorphism and congenital heart defect risk. Two separate studies reported no effect of the maternal *SLC19A1* c.80G>A polymorphism on congenital heart defect risk in the offspring (Pei et al. 2006; Christensen et al. 2013). A prefortification USA study reported a higher risk in foetal heterozygote compared to variant homozygote genotypes (but not to normal homozygotes). However, the association was lost in the offspring of maternal folic acid supplement users, suggesting a modest interaction between infant *SLC19A1* c.80 genotype and periconceptional intake of vitamins (Shaw et al. 2003). A Chinese study reported no association between offspring genotype and congenital heart defect risk but further analysis of the subsample born to mothers that had not used folic acid supplements revealed a higher risk in heterozygotes or normal homozygotes versus variant homozygotes (Pei et al. 2006). Another Chinese study reported a higher risk for foetal heterozygote, but not variant homozygotes, compared to the normal homozygote genotype (Gong et al. 2012). Another Chinese study found no association between the foetal *SLC19A1* c.80G>A polymorphism alone and congenital heart defects. It only conferred greater risk when combined with 2 other SNPs affecting 1C metabolism (*MTHFD1* c.1958G>A and *MTR* c.2756A>G) (Wang et al. 2013).

Meta analyses have concluded that neither the polymorphism in the mother (Wang et al. 2012) nor the offspring (Wang et al., 2012; Zhang et al., 2013) affects NTD risk. Studies that have considered folate status suggest that the SNP may be protective against NTDs. The normal homozygote genotype in mothers with low RBC folate (Morin et al. 2003a) or not taking periconceptional folic acid (Pei et al. 2009) was associated with higher risk of NTDs in the offspring. Similarly, the normal homozygote genotype in

the offspring had higher NTD risk only in the absence of periconceptional folic acid supplement use (Pei et al. 2009).

The *SLC19A1* c.80G>A genotype in live born infants was not associated with omphalocele risk in a postfortification USA study (Mills et al. 2012).

To the best of our knowledge only three case control studies, from South Korea, China and Iran, have assessed the effect of the *SLC19A1* c.80G>A polymorphism and miscarriage; and reported no effect of the maternal SNP on recurrent miscarriage risk. (Rah et al. 2012; Luo et al. 2015; Mohtaram et al. 2016).

3.4. Methionine synthase reductase and the c.66A>G polymorphism (rs1801394)

Methionine synthase reductase

Methionine synthase reductase (MTRR, EC 1.16.1.8) is the protein that catalyses the reductive methylation of cob(II)alamin to methylcob(III)alamin, which can be used by MTR for the folate-dependent homocysteine remethylation (Olteanu and Banerjee 2001). The *MTRR* gene is located at chromosome 5 (cytogenetic band 5p15.31), contains 15 exons and 14 introns and is coded in the plus strand (Leclerc et al. 1998; Leclerc et al. 1999; GeneCards 2016).

It is estimated that the cobalamin cofactor of mammal MTR is spontaneously oxidised to the inactive cob(II)alamin every 200-1000 turnovers (Molloy 2010). This is based on experiments with purified recombinant cobalamin dependent MTR from *E. coli* in the absence of SAM, where a loss of enzymatic activity is detected towards ≈2000 turnovers (Drummond et al. 1993). MTRR has binding domains for FAD, flavin mononucleotide (FMN) and NADPH (Leclerc et al. 1998). In the reaction catalysed by MTRR, NADPH is the electron donor (Gulati et al. 1997) whereas SAM is the methyl donor (Foster et al. 1964; Ludwig and Matthews 1997). MTRR is a monomeric enzyme with one molecule of FAD and one of FMN (Olteanu and Banerjee 2001). The similarities of mammal MTRR to *E. coli* proteins with FAD and FMN that are responsible

for the reductive activation of cob(II)alamin (Fujii et al. 1977), has led to the acceptance that the function of FAD and FMN is to sequentially transfer the electron from NADPH to the cob(II)alamin in the MTRR reaction (Olteanu et al. 2002).

Another function of MTRR is the stimulation of the formation of the holoenzyme MTR when combined with NADPH. The proposed mechanisms by which MTRR achieves this stimulation are two: reduction of free or MTR-bound aquacob(III)alamin to cob(II)alamin, and the conversion of free cob(II)alamin to MTR-bound methylcob(III)alamin. MTRR, independently of NADPH, also protects the apoenzyme MTR from denaturalization at 37ºC. This stabilisation effect adds to the promotion of holoenzyme MTR formation (Yamada et al. 2006).

MTRR mRNA has been detected in all the human organs analysed, including the placenta and with the highest expression in skeletal muscle (Leclerc et al. 1998). In mice, the protein and activity has been detected in the liver, heart, kidney and brain (Elmore et al. 2007). Due to alternative transcription start sites and splicing, two isoforms of MTRR are proposed. One shorter MTRR starts the protein coding at exon 2 and is cytoplasmic, while the larger starts at exon 1 and has a mitochondria targeting sequence (Leclerc et al. 1999). Later it has been proven that both mRNAs are transcribed but the protein is only detected in the cytoplasm (Froese et al. 2008).

MTRR might be regulated by 1C metabolism. In human hepatoma cell line, *MTRR* mRNA is downregulated when cell are transferred from folate-repleted medium to folic acid-depleted medium (Chango et al. 2009). Broilers injected once *in ovo* with 100 or 150µg folic acid have higher liver *MTRR* mRNA expression at 21 days after hatching than with 0 or 50µg folic acid. This suggests that folic acid supplementation during embryogenesis could modulate the postnatal expression of *MTRR* (Li et al. 2016). On the contrary, there are no differences in hepatic *MTRR* mRNA and protein expression between mice fed a folate-adequate diet or a folate-deficient diet (Christensen et al. 2010). This is also true for hepatic mRNA from lactating cows receiving weekly

injections of 160mg of folic acid with or without 10mg vitamin B_{12} (for 19wk) or no supplement injections (Preynat et al. 2010).

The natural genetic variations in a DNA locus including the *MTRR* gene have been shown in a GWAS of mice strains to determine the methylation state of the biggest number of CpG sites both *in cis* and *in trans*. The methylation state of 27% of the CpG sites affected by this locus has been reported to be due to the *MTRR* gene in a severely deficient *MTRR* mouse model (gene trapped) (Orozco et al. 2015). Previous studies with the same mouse model reported higher tHcy (Elmore et al. 2007) and betaine, and lower DNA global methylation in the hippocampus than in the wild type (Jadavji et al. 2014). Offspring that were homozygote for this mutation, or born from homozygote dams, had lower birth and placenta weights, and higher risk of congenital heart defects compared to heterozygote or wild type genotypes. In addition, the *MTRR* mutant homozygote dams had a higher proportion of embryo resorptions compared to heterozygote or wild type genotypes (Deng et al. 2008). Higher plasma tHcy and transgenerational defects comprising restricted growth in the second generation, and congenital malformations up to the fifth generation were reported in a study of a similar mouse model. The transgenerational effects suggest a high impact of MTRR on the phenotype by epigenetic programming mechanisms (Padmanabhan et al. 2013). To sum up, MTRR can affect epigenetic regulation, 1C metabolism, and foetal growth and development according to studies in *MTRR* deficient mice.

MTRR c.66A>G polymorphism

MTRR is affected by the common polymorphism c.66A>G in which the substitution of the adenosine at an ATA codon by a guanosine results in a ATG codon, and substitution of isoleucine at position 22 by methionine (Wilson et al. 1999; Institute of Medicine 2016b). The 66 nucleotide position is in the exon 2 (Leclerc et al. 1999), whereas the 22 amino acid position is in the FMN binding domain (Olteanu et al. 2002).

In vitro experiments with the purified human recombinant variant and normal MTRR and in which the shorter isoform was evaluated, revealed no differences in affinity for the flavonoid cofactors (FAD and FMN) and electron donor (NADPH) or in the maximal velocity of the reaction of activation of MTR between the variant and normal MTRR (Olteanu et al. 2002). The polymorphism has no relevant effects on the flavin redox potentials or electron transfer (Olteanu et al. 2004). However, the higher ratio of MTRR/MTR required to reach the maximal velocity in the variant MTRR suggests defects in the formation of the MTRR-MTR complex and/or its stability (Olteanu et al. 2002). In experiments with the isolated domains of MTRR and MTR that interact, the dissociation constants of the MTRR-MTR complex did not differ between the variant and normal MTRR (Wolthers and Scrutton 2007). Therefore, it appears that the reduced efficiency of the variant enzyme is due to impaired formation of the MTRR-MTR complex, i.e. variant MTRR with less affinity for MTR.

Human studies assessing the possible metabolic effect of the *MTRR* c.66A>G polymorphism have focused on serum or plasma tHcy, folate and cobalamin concentrations (**Table 6** and **Table 7**).

Most of these studies have found no association in patients or healthy individuals between *MTRR* c.66A>G polymorphism and plasma/serum tHcy, folate (including RBC folate) or cobalamin (Brown et al. 2000; Gaughan et al. 2001; Geisel et al. 2001; Lucock et al. 2001; Botto et al. 2003; Kluijtmans et al. 2003; Yates and Lucock 2003; Brilakis et al. 2003; Feix et al. 2004; Martínez-Frías et al. 2006; van der Linden et al. 2006; Ananth et al. 2007; Terruzzi et al. 2007; Fredriksen et al. 2007; Barbosa et al. 2008b; Yang et al. 2008; Barbosa et al. 2008a; van Driel et al. 2009; de Lau et al. 2010; Summers et al. 2010; Montjean et al. 2011; Nagele et al. 2011; Biselli et al. 2012; Clifford et al. 2012; Zampieri et al. 2012; Lucock et al. 2013; Coppedè et al. 2014; Liang et al. 2014; García-Minguillán et al. 2014; Li et al. 2015; Bueno et al. 2016).

Two studies from Morocco and India that include cases of coronary artery disease found higher tHcy in variant homozygotes compare to hetero and normal homozygotes

(Laraqui et al. 2007) or with increasing variant allele number (Vinukonda et al. 2009). An Indian study including Parkinson's disease cases found also higher tHcy with increasing variant allele number (Kumudini et al. 2014). However, a study of Northern Irish men reported lower tHcy in variant homozygotes or heterozygotes versus normal homozygotes (Gaughan et al. 2001). The results of this study contrasted with those of a previous one in Northern Ireland that reported no differences in tHcy among *MTRR* c.66A>G genotypes, although the studied population was younger and included women (Kluijtmans et al. 2003).

A UK study reported lower serum folate with increasing variant allele number in mothers without previous NTD affected pregnancies (Lucock et al. 2001). However, an Italian Down syndrome offspring mothers case-control study reported higher serum folate in: variant versus normal homozygotes (whole sample), variant homozygotes versus heterozygotes (cases), hetero versus normal homozygotes (controls) (Coppedè et al. 2014). In both studies determination of serum folate was by protein-binding assays. These assays were radioassay (Lucock et al. 2001) or non radioassay (Coppedè et al. 2014). As previously commented protein-binding assays are considered less reliable for folate determination than the microbiological assay (*Introduction* section *3.1*).

In Lucock *et al*. lower RBC folate was also found in mothers without previous NTD affected pregnancies with increasing variant allele number (Lucock et al. 2001). In a methotrexate treated rheumatoid arthritis patients cohort there was lower RBC folate in variant homozygotes than in hetero or normal homozygotes (Stamp et al. 2010). But differences in RBC folate among *MTRR* c.66A>G genotypes, with higher concentrations in variant homozygotes and heterozygotes; were observed in patients with thromboembolic events (Yates and Lucock 2003). While in the Spanish population no independent effect of the *MTRR* c.66A>G polymorphism on RBC folate was observed, it was reported to reduce the RBC folate lowering effect of the *MTHFR* c.665TT genotype (Bueno et al. 2016).

Two studies assessing betaine and/or DMG have found no association between these and *MTRR* c.66A>G genotypes in Norwegian cohort of older adults (Fredriksen et al. 2007) and in Brazilian women of childbearing age (Barbosa et al. 2008b).

Higher plasma cobalamin in participants with the *MTRR* c.66AG genotype compared to the AA genotype were reported in one European study (Botto et al. 2003) whereas lower plasma cobalamin in AG compared to AA or GG genotypes was reported in another (García-Minguillán et al. 2014). Among the differences between the studies, the former was conducted in an Italian cohort of patients that underwent coronary angiography (Botto et al. 2003), while the latter was in a Spanish population of healthy adults with a broader age range (García-Minguillán et al. 2014). The cobalamin determination methods were also different, with a (non radioassay) protein-binding assay in the Italian study (Botto et al. 2003), and the microbiological assay in the Spanish study (García-Minguillán et al. 2014).

Whether folate status modulates the effect of the *MTRR* c.66A>G polymorphism on tHcy has been investigated in pregnant women at labour (Barbosa et al. 2008a) and in old adults (60-90y) (de Lau et al. 2010). Both studies reported no association between *MTRR* c.66A>G genotypes and tHcy, between individuals above or below the serum/plasma folate median.

The pregnancy studies to date that have investigated the association between the *MTRR* c.66A>G polymorphism and tHcy, folate, cobalamin, betaine or DMG were based on maternal blood determinations at delivery (Barbosa et al. 2008a) or postpartum (Ananth et al. 2007), or on foetal genotype and amniotic fluid concentrations at amniocentesis (Brouns et al. 2008), or in cord plasma (Visentin et al. 2015). The former studies reported no association between the maternal *MTRR* c.66A>G genotypes and plasma/serum tHcy, folate and cobalamin (Ananth et al. 2007; Barbosa et al. 2008a). This was also true for foetal genotype and amniotic fluid tHcy (Brouns et al. 2008) and for cord plasma betaine and DMG (Visentin et al. 2015). Clearly a common factor in all

of these studies is the inevitable variation in gestational age at the time of blood or amniotic fluid collection.

Table 6. Case control studies investigating the role of the *MTRR* c.66A>G polymorphism on 1C metabolism metabolites.

Reference	Country, year	Mandatory fortification	n Case [outcome][1]	Controls	Age (y)	Metabolites[2]	Results
(Wilson et al. 1999)	Canada, ND[3]	ND	56 children [SB] 578 mothers	97 children 88 mothers	1-19 22-55	tHcy	No association (children and mothers whole sample)
(Lucock et al. 2001)	UK, ND	No	38 [NTD offspring]	56	Adult	tHcy, folate (serum and RBC) and derivatives	Lower serum and RBC folate, RBC 5formylTHF; higher RBC $5CH_3THF$ with G allele increase (whole sample)
(Jacques et al. 2003)	USA, 1994-95	No	ND [HRCHD families]	ND	>25	tHcy (fasting and PML[4]), folate, cobalamin	No association (whole sample of 562 individuals)
(Martínez-Frías et al. 2006)	Spain, 2001-04	No	91 [DS offspring]	90	16-44	tHcy	No association (whole sample)
(van der Linden et al. 2006)	The Netherlands, ND	No	99 children [SB] 116 mothers	213 children 264 mothers	Child Adult	tHcy	No association (children and mothers whole sample)
(Ananth et al. 2007)	USA, 2002-03	Yes	196 [AP]	191	Adult	tHcy, folate, cobalamin	No association (cases, controls)
(Laraqui et al. 2007)	Morocco, ND	ND	151 [CAD]	79	20-76	tHcy	Higher tHcy with G allele (whole sample)

[1]Case outcomes: SB, Spina Bifida; NTD, Neural Tube Defect; HRCHD, High Risk of Coronary Heart Disease; DS, Down Syndrome; AP, *Abruptio Placentae*; CAD, Coronary Artery Disease. [2]Metabolites in plasma/serum unless otherwise specified. [3]ND, No Data. [4]PML, Post-methionine load test.

Table 6. Case control studies investigating the role of the *MTRR* c.66A>G polymorphism on 1C metabolism metabolites (continued).

Reference	Country, year	Mandatory fortification	n Case [outcome][1]	Controls	Age (y)	Metabolites[2]	Results
(Terruzzi et al. 2007)	Italy, ND[3]	No	82 [Obesity]	54	25-53	tHcy, folate, cobalamin	No association (whole sample)
(Brouns et al. 2008)	The Netherlands, ND	No	58 [CBD]	68	12-37 GW	Amniotic fluid tHcy	No association (cases, controls)
(Vinukonda et al. 2009)	India, ND	No	108 [CAD]	108	36-79	tHcy	Positive correlation tHcy - G allele (whole sample)
(Montjean et al. 2011)	France, ND	No	50 [OZP]	53	Adult	tHcy	No association (whole sample)
(Zampieri et al. 2012)	Brazil, ND	ND	105 [DS offspring]	185	Adult	Folate	No association (whole sample)
(Coppedè et al. 2014)	Italy, ND	No	172 [DS offspring]	187	Adult	tHcy, folate, cobalamin	Higher folate with G allele (cases, controls, whole sample)
(Kumudini et al. 2014)	India, ND	No	151 [PD]	416	Adult	tHcy	Positive correlation tHcy - G allele (whole sample)

[1]Case outcomes: CBD, Complex Birth Defect (combination of NTD, orofacial cleft, congenital heart defect, omphalocele); CAD, Coronary Artery Disease; OZP, Oligozoospermia; PD, Parkinson's Disease. [2]Metabolites in plasma/serum unless otherwise specified. [3]ND, No Data.

Table 7. Retrospective cohort studies investigating the role of the *MTRR* c.66A>G polymorphism on 1C metabolism metabolites.

Reference	Country, year	Mandatory fortification	n [feature][1]	Age (y)	Metabolites[2]	Results
(Brown et al. 2000)	USA, ND[3]	ND	180 [CC]	<58	tHcy, folate, cobalamin	No association
(Gaughan et al. 2001)	Northern Ireland, ND	No	601 [Men]	30-49	tHcy, folate, cobalamin	Lower tHcy with G allele
(Geisel et al. 2001)	Germany, ND	No	280	20-102	tHcy, folate	No association
(Botto et al. 2003)	Italy, ND	No	68 [CA]	Old adult	tHcy, folate, cobalamin	Higher cobalamin in AG genotype
(Brilakis et al. 2003)	USA, 1998-99	Yes	504 [CAD]	27-77	tHcy	No association
(Kluijtmans et al. 2003)	Northern Ireland, 1997-99	No	452	20-25	tHcy, folate (serum and RBC), cobalamin	No association
(Yates and Lucock 2003)	UK, Australia, ND	No	51 [TE] 101 [NTE]	52-79	tHcy, RBC folate	Higher RBC folate with G allele (TE)
(Feix et al. 2004)	Austria, 1996-98	No	733 [RT]	Adult	tHcy, folate, cobalamin	No association
(Vaughn et al. 2004)	USA, 2000-01	Yes	362 [Women]	20-30	tHcy, folate, cobalamin	No association
(Fredriksen et al. 2007)	Norway, 1999-01	No	10601	50-64	tHcy, folate, betaine, DMG, cobalamin	No association
(Barbosa et al. 2008b)	Brazil, <2004	No	102 [Women]	Child bearing	tHcy, folate (serum and RBC), DMG, cobalamin	No association

[1]Cohort specific features: CC, Cardiac Catheterization; CA, Coronary Angiography; CAD, Coronary Artery Disease; TE, Thromboembolic Event; NTE, Non-Thromboembolic Event vascular problems; RT, Renal Transplant. [2]Metabolites in plasma/serum unless otherwise specified. [3]ND, No Data.

Table 7. Retrospective cohort studies investigating the role of the *MTRR* c.66A>G polymorphism on 1C metabolism metabolites (continued).

Reference	Country, year	Mandatory fortification	n [feature][1]	Age (y)	Metabolites[2]	Results
(Barbosa et al. 2008a)	Brazil, 2001-03	No	275 [Pregnant women, labour]	Child bearing	tHcy, folate, cobalamin	No association
(Yang et al. 2008)	USA, 1991-94	No	6793	≥12	tHcy, folate	No association
(van Driel et al. 2009)	The Netherlands, 2003-07	No	336 [Mothers]	20-48	tHcy	No association
(de Lau et al. 2010)	The Netherlands, ND[3]	No	1011	60-90	tHcy	No association
(Stamp et al. 2010)	New Zealand, 2005-08	No	191 [MTXR]	18-84	RBC folate	Lower RBC folate in GG genotype
(Summers et al. 2010)	USA, 2007	Yes	49	Child bearing	tHcy, folate and derivatives (serum and RBC), cobalamin	No association
(Nagele et al. 2011)	Austria, 2005-07	No	139 [NOP]	18-92	tHcy	No association (separated and combined cohorts)
	USA, 2008-12	Yes	281			
(Clifford et al. 2012)	USA, 2002-04	Yes	373	30-69	Folate adjusted tHcy	No association
(Biselli et al. 2012)	Brazil, 2004-11	Yes	90 [DS]	0-30	tHcy, folate	No association

[1]Cohort specific features: MTXR, Methotrexate treated Rheumatoid arthritis; NOP, Nitrous Oxide-exposed Patients; DS, Down Syndrome. [2]Metabolites in plasma/serum unless otherwise specified. [3]ND, No Data.

Table 7. Retrospective cohort studies investigating the role of the *MTRR* c.66A>G polymorphism on 1C metabolism metabolites (continued).

Reference	Country, year	Mandatory fortification	n [feature][1]	Age (y)	Metabolites[2]	Results
(Lucock et al. 2013)	Australia, ND[3]	No	212 [GEP]	18-89	RBC folate	No association
(Liang et al. 2014)	China, 2011	No	400 [Pregnant women, 11-25GW]	25-35	Folate adjusted tHcy	No association
(García-Minguillán et al. 2014)[4]	Spain, 1998-02	No	771	18-75	tHcy, folate, cobalamin	Lower cobalamin in AG genotype
(Li et al. 2015)	China, 2005	No	480 [HTA]	28-75	Folate	No association
(Visentin et al. 2015)	Canada, 2010-12	Yes	≈250 [cord blood samples]	18-45 (mothers)	Betaine, DMG (cord plasma)	No association
(Bueno et al. 2016)[4]	Spain, 1998-02	No	789	18-75	tHcy, folate (plasma and RBC)	No association

[1]Cohort specific features: GEP, Gastroenterology Patients; HTA, Hypertension. [2]Metabolites in plasma/serum unless otherwise specified. [3]ND, No Data. [4]References from the same study sample.

Maternal and offspring *MTRR* c.66A>G genotype frequencies did not differ between SGA cases and controls in a prefortification Australian study (Furness et al. 2008). To the best of our knowledge, no other study has investigated the effect of this SNP on birth weight.

The maternal SNP was reported not to be associated with congenital heart defect risk in two Dutch studies (van Beynum et al. 2006; Verkleij-Hagoort et al. 2008). Subsequently, a prefortification Canadian study also reported no effect of the maternal polymorphism on congenital heart defect risk (Christensen et al. 2013). However, the offspring SNP could be associated with congenital heart defects. Two different meta analyses did report that the foetal *MTRR* c.66 variant allele compared to normal allele was associated with increased risk of congenital heart defects (Cai et al. 2014; Yu et al. 2014). This was also true in Cai *et al*. when stratifying analyses by ethnicity (Chinese Han or non-Chinese Han) (Cai et al. 2014). After stratification (Asian or Caucasian) in Yu *et al*. higher risk in variant versus normal allele was observed in Asians but not in Caucasians (Yu et al. 2014). This was also true for the comparison of variant versus normal homozygotes (Yu et al. 2014). The analysed studies were not the same in both meta analyses; e.g. Cai *et al*. included transmission disequilibrium test studies in addition to case control studies and also a USA Down Syndrome study (Locke et al. 2010). These meta analyses did not include two previous case control studies that found no association between foetal *MTRR* c.66A>G genotypes and congenital heart defect risk in Canada (Christensen et al. 2013) and China (Wang et al. 2013). Stratification by cobalamin status did not change the absence of association between the maternal or foetal *MTRR* c.66A>G genotype and risk of congenital heart defect in a Dutch study (Verkleij-Hagoort et al. 2008). However, an interaction of cobalamin status on the effect of the maternal or foetal genotype cannot be ruled out as: 1) to the best of our knowledge no study from India, where low cobalamin status is prevalent, has assessed this; and 2) in Verkleij-Hagoort *et al*. cobalamin status was normal (241-284pmol/L serum cobalamin means among genotypes) and the low cobalamin group (≤175pmol/L serum cobalamin) was not deficient but marginally deficient. To sum up,

the maternal genotype seems not to affect congenital heart defect risk in the offspring, although this is based on only three studies. The variant allele in the offspring may increase the risk in Asians but not Caucasians.

Several studies have investigated the association between the *MTRR* c.66A>G polymorphism and NTD risk. In a big Irish case control study there were not differences in the *MTRR* c.66A>G genotype frequencies of mothers of children with NTDs or children with NTDs, and controls (O'Leary et al. 2005). A meta analysis assessing 11 studies of the polymorphism in the mother reported that there was higher NTD risk only in the comparison of hetero and variant homozygotes versus normal homozygotes (Yadav et al. 2015). Another meta analysis of 12 studies reported higher NTD risk in the following foetal *MTRR* c.66A>G genotype comparisons: G versus A allele, GG versus AA, and GG and GA versus AA (Wang et al. 2015b).

Omphalocele risk did not differ between foetal *MTRR* c.66A>G genotypes, in a case control study of a sample of multiple ethnicities from USA and exposed to folic acid fortification (Mills et al. 2012).

Few studies have investigated the effect of the *MTRR* c.66A>G polymorphism on risk of miscarriage. In regard to the SNP in mothers, a South Korean study of sporadic miscarriage reported no differences in allelic and genotypic frequencies between cases and controls (Kim et al. 2011). A Chinese study of the risk of recurrent miscarriage, including stillbirths, reported similar findings (Luo et al. 2015). However, another Chinese study of recurrent miscarriages (of unspecified gestational age) reported higher proportion of variant homozygotes and variant allele in the case compared to the control group (Zhu 2015). The sample sizes of these three studies were: 188, 260 and 292. Insufficient sample size for the detection of an effect in the two former studies cannot be ruled out. Thus, the evidence for an association between the maternal SNP and miscarriage occurrence is scarce to date. To the best of our knowledge, there have been no epidemiological reports, so far, on the effect of the polymorphism in the embryo.

Two meta analysis reported that the *MTRR* c.66A>G polymorphism is a risk factor for Down syndrome affected pregnancies (Yang et al. 2013; Coppedè et al. 2014). Also two meta analyses reported no association with coronary artery (Singh and Lele 2012) or heart (Chen et al. 2012) diseases.

4. Placenta

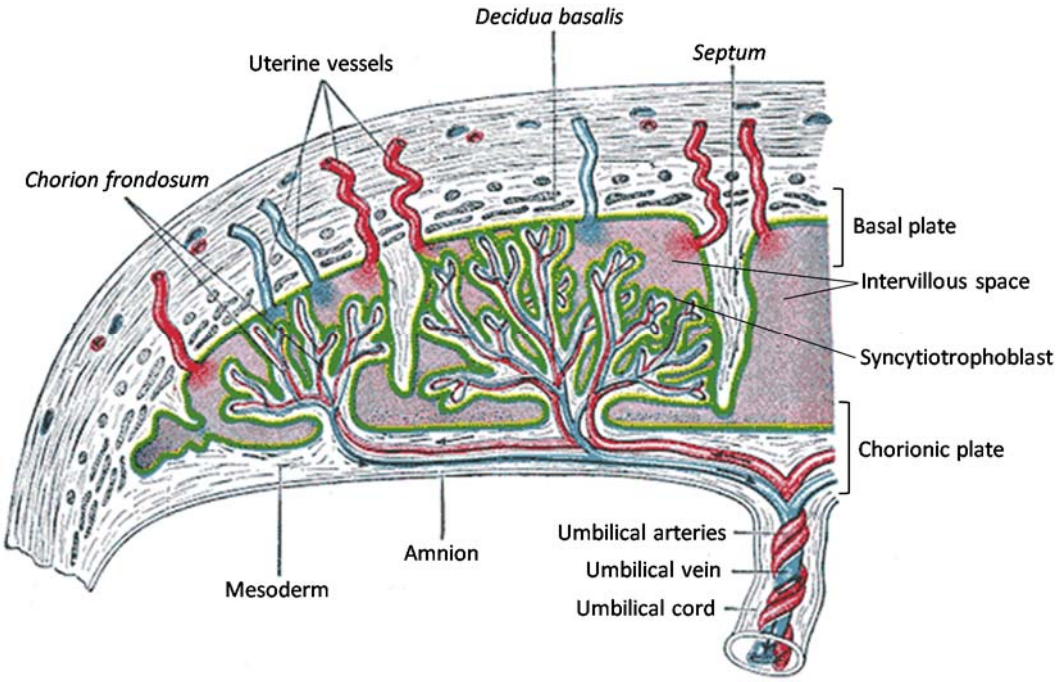
4.1. Brief overview of anatomy

The placenta is a transient organ formed by cells of foetal origin. The human placenta is haemochorial, that is, the maternal blood is separated from the foetal blood by only the chorion, which is part of the placenta (Enders 1965). Human placenta is also deciduate, that is, part of the decidua (a specialised layer of the endometrium) is expelled together with the placenta (Huxley 1869). The placenta anatomy is complex and only main features will be explained briefly (Kraus et al. 2004). A scheme of the placenta circulation and anatomy is shown in **Figure 10**. A high proportion of the placenta volume consist of a space where the uterine arteries discharge maternal blood. This space is called the intervillous space and the *chorium frondosum* forms tree-like structures (chorionic villi) in this space. These structures protrude from the mesoderm of the chorionic plate (the part of the placenta closest to the foetus) and branch into villi of decreasing diameter, to enhance the surface in contact with the maternal blood (**Figure 11**). Inside the chorionic villi, vessels transport foetal blood. Some chorionic villi grow up to the *decidua basalis*, forming specialised structures to anchor the placenta to the endometrium. Nutrients, gases and other molecules are transported from the maternal to the foetal blood and vice versa penetrating through several cell layers in the chorionic villi. One of these layers is the syncytiotrophoblast, a continuous space with multiple nuclei which is in direct contact with the maternal blood. Under syncytiotrophoblast is the cytotrophoblast, a layer of unfused cells. The surface of the chorionic plate is covered with an epithelium called amnion and the cord protrudes

from this plate. The part of the placenta closest to the uterus is called the basal plate, and includes the decidua located under the placenta: *decidua basalis*.

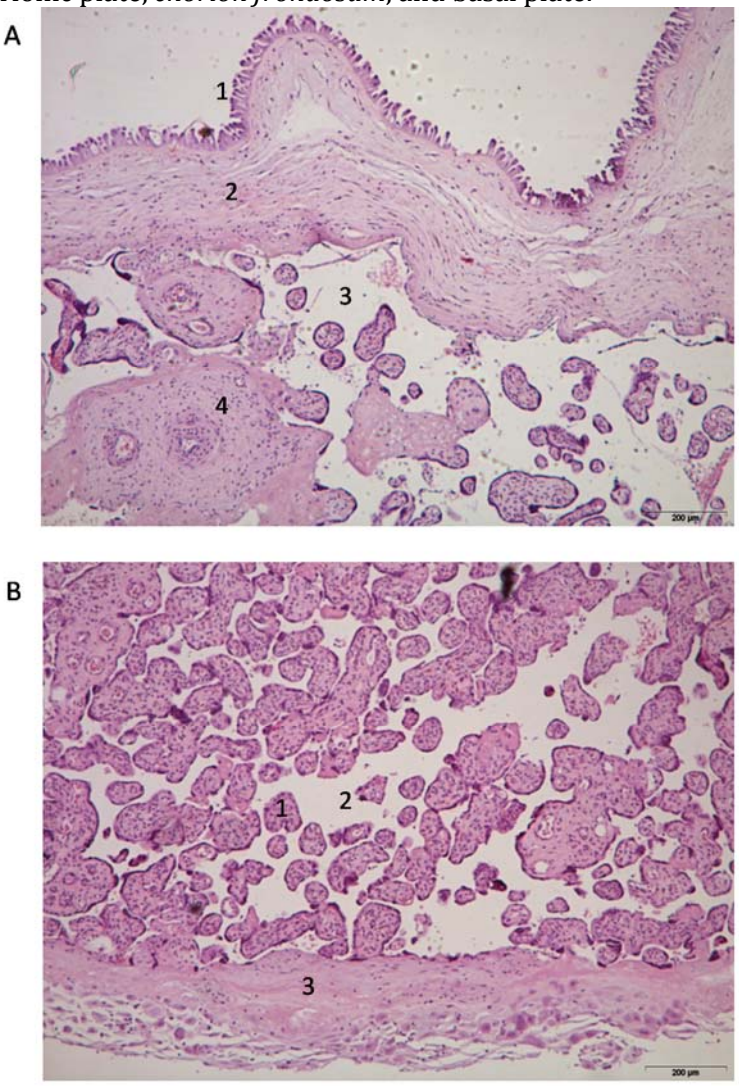
Radial growth of the placenta and continuous sealing of the placental edges with the decidua (which also anchors the placenta to the uterus), leads to the formation of septa that protrude into the intervillous space marking compartment like sections within this space. These compartment like sections are cotyledons and once the placenta is expelled in the delivery they can be identified macroscopically as lobes on the maternal surface of the placenta.

Figure 10. Placenta blood circulation and anatomy.



Side view scheme of the placenta blood circulation and anatomy. Arrows indicate the direction of the blood flow. Adapted from (Gray 1918).

Figure 11. Chorionic plate, *chorion frondosum*, and basal plate.



Optic microscope photographs of formalin fixed placentas from participants (standard haematoxylin and eosin stain). A. Chorionic plate and *chorion frondosum*. 1, amnion; 2, mesoderm; 3, intervillous space; 4 major chorionic stem villi (greater diameter). B. *Chorion frondosum* and basal plate. 1, terminal chorionic villi (lesser diameter); 2, intervillous space; 3, *decidua basalis*.

4.2. Function and one carbon metabolism

The placenta controls the transfer of nutrients, wastes and gases between the maternal and foetal blood and is also an important endocrine organ. It is estimated that the placental metabolic rate is as high as that in brain, liver and many tumour cells; and this high oxygen consumption is considered to be necessary for its transport and synthesis activities (Hay 1991). Among the hormones produced in the placenta there are steroid hormones such as progesterone and oestrogens; and peptide hormones such as insulin-like growth factors, relaxin, prolactin, placental lactogen, urocortins, leptin, ghrelin, and growth hormone, thyrotropin, gonadotropin and their respective releasing hormones (Imperatore et al. 2006; Tal et al. 2015).

During human placental development, the foetal blood begins to flow in the placenta vessels at ≈3GW (Enders and Blankenship 1999), while maternal blood flow to the intervillous space is not achieved until the end of the first trimester (Wang and Zhao 2010). Initially, during the first trimester foetal nutrition is histiotrophic, i.e. from endometrium and uterine glandular secretions (Burton et al. 2002). From the end of the first trimester on, is mainly haemotrophic, i.e. from the nutrients contained in the maternal blood. The advantages of histiotrophic nutrition in early gestation are considered to be the hypoxia (the foetus at early stages of development is highly susceptible to reactive oxygen species), and the more efficient uptake of maternal cytokines (compared to haemotrophic nutrition) (Burton et al. 2001). It can be considered that the placenta is fully developed once the final transport way (haemotrophic nutrition) is achieved, that is, at the end of the first trimester.

The placenta can act as a barrier to several toxins, in some cases accumulating them. The foeto toxic heavy metal cadmium is an example (Goyer and Cherian 1992). Placental tissue cadmium concentration (median 68.6ng/g dw, dry weight) is much higher than in umbilical cord tissue (median 1.16ng/g dw) (Sakamoto et al. 2013). This effect is thought to be mediated by metallothioneins. Human placentas perfused with pharmacological cadmium concentrations (20μM) up-regulate *metallothionein 2A*

mRNA (Breen et al. 1994). Placenta cadmium and metallothionein protein concentrations are positively correlated (Serafim et al. 2012). However, the placenta is not an effective barrier for other toxins such arsenic (Hall et al. 2007).

BHMT mRNA has not been detected in human placenta (Sunden et al. 1997; Solanky et al. 2010), and *cystathionine-beta-synthase* mRNA levels are very low. On the other hand, *MTHFR* and *MTR* mRNAs are highly expressed so it has been suggested that human placenta (in the first trimester and at term) homocysteine is mainly remethylated to methionine and by the cobalamin dependent reaction (Solanky et al. 2010). By experiments of competitive inhibition of the transport of substrates or homocysteine in syncytiotrophoblast apical membrane vesicles, three amino acid transport systems (L, y^+L and A) have been shown to be involved homocysteine transport (Tsitsiou et al. 2011). Several amino acids are transported by each system, and methionine is the common amino acid to all of these (Tsitsiou et al. 2011). The system L is the major mediator of homocysteine transport from the intervillous space to the syncytiotrophoblast cytoplasm (Tsitsiou et al. 2009). It is unknown whether homocysteine transport *in vivo* occurs towards the foetus, towards the mother, or both because the system L mediated transport is bidirectional (Verrey 2003). That maternal and cord serum or plasma tHcy are positively correlated (Guerra-Shinohara et al. 2002; Murphy et al. 2004; Molloy et al. 2005; Obeid et al. 2005; Wallace et al. 2008) supports a homocysteine foetal-maternal transport mechanism; although other factors such as the relationship between maternal and foetal methyl donor status cannot be excluded as the main reason. Apart from possible toxic effects, homocysteine may induce apoptosis and lower chorionic gonadotropin production in the placenta; as shown in trophoblast cultures with physiologically plausible levels of homocysteine in the media (20μM) (Di Simone et al. 2003). Also, potential importing of homocysteine to the placenta would result in a lower flux of amino acid to the foetus, and the supply of amino acids such as leucine and phenylalanine (transported by system L) is reported to not be limiting just by a small margin (Chien et al. 1993).

Serum folate in the maternal vein, placenta intervillous space and umbilical cord (neonate blood) is positively correlated in humans (Giugliani et al. 1985). *In vitro* experiments show the membrane-bound form of folate receptor alpha, the proton coupled folate transporter and the reduced folate carrier (SLC19A1) are involved in human placental folate transport (Yasuda et al. 2008). The mRNA levels of these proteins increase throughout pregnancy in rat placenta suggesting an increased need of folate for the foetus and placenta as pregnancy progresses (Yasuda et al. 2008). Experiments with isolated human placental cotyledons perfused with labelled 5-methylTHF suggest that folate receptor binding and the release of folate on the surface of the syncytiotrophoblast in contact with the maternal blood, is the first step in the maternal-to-foetal folate transport (Henderson et al. 1995). The different localisations of the membrane-bound form of folate receptor alpha, the proton coupled folate transporter and SLC19A1 in the human syncytiotrophoblast (Yasuda et al. 2008; Solanky et al. 2010), has led to the proposal of the current model for placenta folate transport (Solanky et al. 2010). Folate receptors alpha, proton coupled folate transporter and SLC19A1 are present in the syncytiotrophoblast membrane exposed to the maternal blood. Folate receptors alpha bind 5-methylTHF and are internalised in an endosomal vesicle together with proton coupled folate transporters. The lumen of the vesicle becomes acidic facilitating the export of folates to the cytoplasm by the proton coupled folate transporter, and afterwards folate receptors and transporters in the vesicle are recycled back to the maternal blood exposed syncytiotrophoblast membrane. SLC19A1 in this membrane are an alternative pathway for the import of folates from maternal blood. The export of folate from the syncytiotrophoblast to the foetal blood (vessels in the chorionic villi) is achieved by SLC19A1 and possibly other exporters, but not folate receptor alpha or the proton coupled folate transporter (Solanky et al. 2010). In cultured human cytotrophoblasts, folate receptor alpha, the proton coupled folate transporter and SLC19A1 are involved in folate uptake (Keating et al. 2009).

A study of 8 women undergoing hysterotomy for termination of pregnancy shows folic acid is not found in the placenta or foetal liver at different times after labelled folic acid intravenous injections (Landon et al. 1975). It is found as folate derivatives though, indicating rapid and extensive metabolism by the mother and/or placenta (Landon et al. 1975). Supporting a possible role of the placenta in the metabolism of folic acid is the finding of dihydrofolate reductase activity in human placenta (Jarabak and Bachur 1971). Human placental folate mainly occurs in the polyglutamated forms (Landon 1975) and gamma glutamyl hydrolase activity has been found in human placental extracts (Landon 1972). The optimal pH for this enzymatic activity is acid (Landon 1972). Two enzymes can remove glutamates from the glutamates chain of folates: soluble gamma glutamyl hydrolase and membrane folate hydrolase 1; but only the former has acid pH as optimal (Galivan et al. 2000). In Landon *et al*'s experiment, the placenta extract is the supernatant from homogenising and centrifuging placental tissue in water. Therefore, it is improbable that the placental enzyme is folate hydrolase 1, a transmembrane enzyme (Landon 1972). Another study detected abundant *gamma glutamyl hydrolase* mRNA in human placenta (Yin et al. 2003). Moreover, immunohistochemically gamma glutamyl hydrolase is detectable in human placenta whereas folate hydrolase is not (The human protein atlas 2016). Therefore, the enzyme involved in placenta folate mobilisation is gamma glutamyl hydrolase.

In human placentas at term from a sample containing preeclampsia cases (40%), placental folate concentration measured by microbiological assay ranged 2-32μg/g wet weight (ww) (Mislanova et al. 2011). Human placenta density have been reported to be 0.995 or 1.020g/ml (Younoszai and Haworth 1969; Del Nero et al. 2002), therefore rounding to 1g/ml the concentration in Mislanova *et al*. is 4.5-72.5μmol/L. In this study the highest folate concentrations occurred in preeclampsia cases with *MTHFR* c.665TT maternal genotype, and the lowest in cases with CT genotype. Without genotype stratification cases and uncomplicated pregnancies did not differ in placental folate concentration (Mislanova et al. 2011). A previous study had reported about 1μg/g ww (2.3μmol/L) in human placentas of ≈16 GW (Landon 1975).

In vitro experiments with human trophoblasts suggest that out of the three previously commented choline transporter families, the choline transporter-like protein family is the main responsible of placenta choline uptake (Yara et al. 2015). Specifically, high and low affinity transports are mediated by SLC44A1 (CTL1) and SLC44A2 (CTL2), respectively (Yara et al. 2015). These two transporter mRNAs are highly expressed *in vivo* in human placentas, whereas other members of the three families are not expressed or at much lower levels (Yara et al. 2015). Choline oxidation is not possible in the placenta because, as commented previously, BHMT is not expressed in this organ. The human placenta produces high amounts of acetylcholine, and the placenta choline acetyltransferase enzyme is indistinguishable from the enzyme in brain (Roskoski et al. 1975). Experiments with fragments of human placentas at term shown choline is mainly metabolised to acetylcholine, with a small proportion to phosphocholine (a precursor of phosphatidylcholine) and none to betaine (Welsch 1976).

The GABA/betaine transporter acts as the main mediator of betaine uptake into the cells (Kempson et al. 2014), and two mRNA transcripts for it have been found in human placenta. Even though this is at weaker levels than in other organs such as kidney and brain (Rasola et al. 1995); this transporter can be regarded as responsible for placental betaine uptake. A part from the GABA/betaine transporter, other transporters may be involved in the placental betaine transport. A study with rat syncytiotrophoblast cell line revealed higher betaine uptake and up-regulation of GABA/betaine transporter and one of the amino acid transport system A members in hypertonic conditions compared to normal conditions (Nishimura et al. 2010). The addition of a ligand of the amino acid transport system A, but not of a ligand of the GABA/betaine transporter; inhibits hypertonic-stress induced betaine uptake (Nishimura et al. 2010). Thus, it is possible that in addition to the GABA/betaine transporter, system A is also involved in placenta betaine import and with greater importance under hyperosmotic stress. This experiment also suggests betaine's main function as an osmolyte against hyperosmotic stress (Yancey et al. 1982) may be relevant in the placenta. The IMINO transport system, responsible for the betaine intestinal absorption, can be excluded from

placenta betaine uptake, as no mRNA as been detected in rat placentas (Takanaga et al. 2005).

As in folate, serum cobalamin concentrations in the mother, placenta and neonate are positively correlated in humans (Giugliani et al. 1985). The cobalamin bound to transcobalamin 2 (holotranscobalamin) but not to haptocorrin or intrinsic factor, is the form which is delivered to the foetus, according to a rabbit study (Fernandes-Costa and Metz 1979). Experiments with human placenta cell membrane preparations reported higher binding of holotranscobalamin than of transcobalamin 2 or cobalamin alone, and that this binding is mediated by a membrane protein that needs divalent cations (Friedman et al. 1977). It has also been reported that the intrinsic factor - cobalamin complex does not bind to this preparation (Friedman et al. 1977), adding more evidence for the exclusion of the intestinal cobalamin uptake pathway in the placental transport. The membrane protein has been subsequently purified and is the transcobalamin 2 receptor, although additionally two different proteins or isoforms of transcobalamin 2 receptor that bind holotranscobalamin have also been detected (Seligman and Allen 1978). In line with the possibility of other holotranscobalamin receptors is the fact that transcobalamin 2 receptor knockout mouse is not embryonic lethal (Lai et al. 2013). As the receptor megalin can bind and internalise holotranscobalamin in the rat renal proximal tubule and is expressed in placenta, it has been proposed as an alternative pathway for transcobalamin 2 receptor (Moestrup et al. 1996). Although haptocorrin and intrinsic factor do not seem to participate in cobalamin placental transport, a study with perfused human placenta suggests 19% of the cobalamin internalised in the placenta is bound to haptocorrin and/or intrinsic factor (Perez-D'Gregorio and Miller 1998). The transport of cobalamin toward the foetus is thought to involve a concentrating step as proposed for folate, based on the higher concentration of serum cobalamin found in the maternal blood of the intervillous space than in the maternal veins (Giugliani et al. 1985). It is considered that holotranscobalamin in the placenta follows the same pathway discovered in cultured human skin fibroblast. Holotranscobalamin binds to its receptor and is internalised by

endocytosis, then cobalamin is released by the lysosomal degradation of transcobalamin 2 (Youngdahl-Turner et al. 1979). In human placentas, methylmalonyl-CoA mutase has been isolated, with cobalamin bound to it (Kolhouse et al. 1980) and the same has been reported for MTR (Utley et al. 1985). In cultured cells of human chorionic villi collected at 9-10 GW, labelled cobalamin in the media is transformed to the active cofactors methylcobalamin and adenosylcobalamin, and these bound to MTR and methylmalonyl-CoA mutase, respectively (Begley et al. 1993). In these cultures the reactions catalysed by both enzymes are functional (Begley et al. 1993). Therefore the two pathways in which cobalamin is a cofactor are present in human placenta.

5. Trace elements

5.1. Zinc

Metabolism and pregnancy

Zinc is present in many proteins: 10% of the proteome is estimated to be bound to zinc *in vivo* (Andreini et al. 2006). Zinc has catalytic, structural and regulatory functions (King 2011). It is essential for the catalytic activity of BHMT (Millian and Garrow 1998), folate hydrolase 1 (Halsted et al. 1998), DNA methyltransferases (Chuang et al. 1996), alkaline phosphatases (Kim and Wyckoff 1991) and carbonic anhydrases (Håkansson et al. 1992) among other enzymes (Coleman 1998). Zinc allows the structure of the zinc finger domains involved in DNA recognition (Miller et al. 1985), and of superoxide dismutase 1 (SOD1) (Banci et al. 2009). Transcription of genes containing metal responsive elements can be regulated by zinc levels; e.g. via the metal-binding transcription factor 1 that acts as an intracellular zinc sensor (Giedroc et al. 2001; Zhang et al. 2003). Among these genes are the genes coding for metallothioneins, metal binding proteins involved in heavy metal detoxification, redox and zinc homeostasis (Lazo et al. 1995; King 2011; Kimura and Kambe 2016), whose synthesis is also induced by copper and cadmium (Hamer 1986).

The zinc transporters ZIPs (SLC39As) are responsible for the flow of zinc toward the cytosol and the zinc transporters ZNTs (SLC30As) for flow out of the cytosol (Cousins 2010). Intestinal zinc uptake occurs throughout the small intestine, with higher rate in the jejunum, lower in the duodenum and the lowest in the ileum (Lee et al. 1989). Zinc intestinal absorption is an active process facilitated by the zinc transporter ZIP4 (SLC39A4) in the enterocyte apical membrane (Cousins 2010).

An accepted biomarker of zinc status has not yet been established. Plasma zinc concentrations are subject to high physiological variability (King 2011), and other potential biomarkers such as RBC metallothioneins are being investigated (Caulfield et al. 2008). Zinc is transported in blood plasma as 70% bound to albumin, 18% to α_2-macroglobulin and the rest to other proteins and amino acids (Gibson et al. 2008). Within the cells most zinc is bound to proteins and free zinc concentration is tightly controlled (Outten and O'Halloran 2001).

Zinc transporter ZNTs have been identified in human placentas (Helston et al. 2007) and the zinc transport is by an active mechanism (Nandakumaran et al. 2003), but it is not yet fully understood (Donangelo and King 2012). Cord serum zinc concentrations are higher than in maternal serum (Hyvonen-Dabek et al. 1984; Krachler et al. 1999), but the contrary occurs in RBC (Kuhnert et al. 1987b). The lower zinc concentration in foetal RBC may reflect a worse zinc cellular uptake in the foetus than the mother.

The RDA for zinc in non-pregnant women is based on quantities of absorbed zinc to exceed endogenous zinc losses. Foetus zinc content in late pregnancy is taking into account for the RDA in pregnancy. The zinc RDA in pregnancy is 12mg/d or 11mg/d for women of 18y or under, or over 19y, respectively. The zinc tolerable upper intake level (UL) is based on the appearance of adverse effects (low copper status as low RBC superoxide dismutase 1 activity). The zinc UL in pregnant women is considered the same as in non-pregnant women, i.e. 34mg/day or 40mg/day for women of 18y or under, or over 19y, respectively (Institute of Medicine - Panel on Micronutrients 2001).

Variables that may affect or be affected by the concentrations of the trace elements addressed in this thesis will be commented throughout this and the following sections: smoking, birth weight, gestational age, maternal age and BMI, socioeconomic level, neonate sex, trace elements interrelationships and food and supplement intake.

No differences in serum, plasma and RBC zinc concentrations were observed between pregnant smokers and non-smokers (Vir et al. 1981; Kuhnert et al. 1987b; Kantola et al. 2000). There is controversy about whether maternal smoking reduces the zinc concentration in RBC of the foetus (Kuhnert et al. 1987b; Kuhnert et al. 1988a) or not (Aydogan et al. 2013).

In the placenta, higher zinc concentrations are found in pregnant smokers from Chile (Ronco et al. 2005) and Croatia (Stasenko et al. 2010); but not Turkey (Sorkun et al. 2007), Ukraine (Zadorozhnaja et al. 2000), and Finland, Estonia and Russia (Kantola et al. 2000). In USA, the association between smoking and higher placenta zinc concentration is found in some studies (Kuhnert et al. 1987b), but not others (Punshon et al. 2016). As smoking is an important source of cadmium exposure (EFSA - CONTAM Panel 2009), the controversial finding of higher zinc concentration in placentas of smokers in some studies has been attributed to a protective mechanism where cadmium induces higher metallothionein levels in placenta, with a subsequent side effect of zinc retention in the organ (Ronco et al. 2005; Ronco et al. 2006). The ratio of placenta zinc/cadmium concentration is lower in pregnant smokers from USA (Kuhnert et al. 1988b) and Chile (Ronco et al. 2005) than in non smokers. The proportion of placenta zinc bound to metallothioneins is ≈6% in non-smokers and ≈11% in smokers (Ronco et al. 2006).

Second trimester plasma zinc is negatively correlated with birth weight (Mukherjee et al. 1984), with some studies reporting similar results at delivery only in smokers (Kuhnert et al. 1988a). Other studies reported no association between maternal plasma, serum or whole blood at delivery and birth weight (Díaz et al. 2002; Al-Saleh et al. 2004; Ozdemir et al. 2007) or found a positive association with maternal plasma

collected in the first half of pregnancy (Crosby et al. 1977). Cord plasma, serum and whole blood zinc have not been associated with birth weight either, in most studies (Osman et al. 2000; Díaz et al. 2002; Al-Saleh et al. 2004; Ozdemir et al. 2007). However, cord RBC zinc is positively associated with birth weight (Kuhnert et al. 1988a; Kippler et al. 2010). In a USA study, cord plasma zinc is negatively correlated with birth weight only in smokers (Kuhnert et al. 1988a). In a big Chinese observational study, maternal zinc deficiency (as serum zinc <56μg/dL) in the first trimester is associated with SGA, and in the second and third trimester with SGA and LBW (Wang et al. 2015a). The serum zinc deficiency cutoff of Wang *et al*. is based on the $P_{2.5}$ of first trimester pregnant women in the National Health and Nutrition Examination Survey (NHANES) II study (Hess et al. 2007), and in another big study from USA using a higher cutoff (P_{25}) no differences in SGA risk are found (Tamura et al. 2000). In general the association, if any, between maternal plasma/serum and LBW and SGA is controversial in the literature (Wilson et al. 2016). There are no differences in cord serum zinc concentrations between SGA and appropriate for gestational age (AGA) neonates from Japan (Osada et al. 2002).

There is also controversy regarding the relationship between zinc status in the placenta and birth weight or categories of it. Placenta zinc concentrations have been reported to be positively (Ward et al. 1987), or not associated (Kantola et al. 2000; Díaz et al. 2002; Odland et al. 2004; Ozdemir et al. 2009; Grant et al. 2010) with birth weight. There is a similar discrepancy in smokers (Kuhnert et al. 1987a; Kantola et al. 2000; Ronco et al. 2005). Higher placenta zinc concentration was found in pregnancies with LBW in a USA well drinkers cohort (Punshon et al. 2016), but not in other studies (Dawson et al. 1969; Llanos and Ronco 2009). Also no differences were observed between placentas of SGA and control neonates (Malhotra et al. 1990; Osada et al. 2002; Zadrozna et al. 2009). It is currently unknown whether higher placenta zinc concentrations reflect more zinc available for foetal development or retention of zinc to compensate toxics such as cadmium. The ratio of placenta zinc/cadmium concentration is positively correlated with birth weight in pregnant smokers from USA (Kuhnert et al. 1987a), but

this correlation is not found in pregnant smokers and non-smokers from Chile (Ronco et al. 2005).

Lower zinc concentrations are found in pregnant than non pregnant women in serum, plasma, whole blood and RBC (Qvist et al. 1986; Atamer et al. 2005; Ghneim et al. 2016). In most cross-sectional studies, maternal plasma/serum zinc concentrations are lower in more advanced pregnancies (Hyvonen-Dabek et al. 1984; Tamura et al. 2000; Kantola et al. 2000), but some studies have reported no differences (Reddy et al. 2014). In longitudinal studies, the maternal concentrations of zinc decrease in plasma and increase in whole blood and RBC (Vir et al. 1981; Qvist et al. 1986; Caulfield et al. 2008); although the RBC increase in a clinical trial of zinc supplementation is only found in the zinc supplemented group (Caulfield et al. 2008). At delivery, cord whole blood zinc is positively associated with gestational age (Kippler et al. 2010), but cord serum is not associated (Reddy et al. 2014). There is controversy about the possible association between maternal plasma/serum zinc and preterm risk. Results range from higher preterm delivery risk in lower compared to higher categories of serum zinc in China, no association in USA and Denmark, and in Indian studies higher plasma/serum zinc concentrations in preterm compared to term deliveries or no association (Wilson et al. 2016).

Cross-sectionally, lower placenta zinc concentrations are found in term pregnancies than during the first trimester (Kantola et al. 2000), but this has not been observed in other studies assessing more advanced pregnancies (Ward et al. 1987; Custódio et al. 2003; Reddy et al. 2014). Placenta zinc concentrations from preterm deliveries are not different than those of term deliveries (Zadrozna et al. 2009), but zinc-uptake capacity is higher in placentas from preterm than term neonates (Vargas Zapata et al. 2000). A greater need for zinc transport in placentas of preterm neonates is a possibility.

Maternal age is not associated with maternal serum zinc (Wang et al. 2015a), but it is positively associated with cord serum zinc (Osman et al. 2000).

Studies assessing maternal age and placenta zinc concentration have led to inconsistent results. Maternal age is negatively correlated with placenta zinc concentration in USA women (Kuhnert et al. 1988b), positively correlated in Turkey (Ozdemir et al. 2009) and not associated in England (Ward et al. 1987) and Jamaica (Grant et al. 2010) .There are no differences in placenta zinc concentration with maternal age above or below 30y in urban Ukrainian women (Zadorozhnaja et al. 2000) and USA well drinkers (Punshon et al. 2016). But Brazilian adult pregnant women have higher zinc concentrations on the foetal side of the placenta than in the 15-19y group (De Moraes et al. 2011).

Higher maternal serum zinc was reported in Chinese women with 18.5-24.9 compared to below 18.5 pre-pregnancy body mass index (BMI) (Wang et al. 2015a). Differences in placenta zinc according to 24 GW BMI were found in USA well drinkers with the highest concentrations in normal BMI (<25) and the lowest in obese (BMI ≥30) (Punshon et al. 2016).

Income is not associated with maternal serum zinc in pregnant women from China (Wang et al. 2015a), and to the best of our knowledge no study has assessed this on placenta concentrations.

There is higher placenta zinc in placentas of male newborns in the USA well drinkers cohort (Punshon et al. 2016), but other studies has found no association (Osman et al. 2000; Phuapradit et al. 2000).

Placenta zinc is positively correlated with placenta copper in pregnant women from England (Ward et al. 1987), Ukraine (Zadorozhnaja et al. 2000), and Northern Norway and Russia (Odland et al. 2001). However, this is not the case in Turkey (Ozdemir et al. 2009), Finland (Kantola et al. 2000), Sweden (Osman et al. 2000), Portugal (Serafim et al. 2012) and Japan (Tsuchiya et al. 1984). Placenta zinc is positively correlated with placenta concentrations of selenium in several studies (Osman et al. 2000), including a USA well drinkers study (Punshon et al. 2016); but not in other studies (Odland et al. 2001). Placenta zinc and iron concentrations are negatively correlated in Northern

Norway and Russia (Odland et al. 2001), positively correlated in Japan (Tsuchiya et al. 1984), and not associated in Turkey (Ozdemir et al. 2009). Supporting an absence of interaction between zinc and iron, no differences in placenta zinc concentration are found between iron supplement users and non-users from England (Ward et al. 1987).

Lower placenta zinc compared to control diets is observed in animals fed a zinc deficient diet (McKenzie et al. 1975; Fosmire et al. 1977; Masters et al. 1983a). However, no differences are observed in diets low in zinc (Kalinowski and Chavez 1991) or with twice the zinc requirements (Marques et al. 2016). There is controversy regarding whether the food restriction and weight loss that accompany zinc deficiency are greater determinants than dietary zinc intake itself of the placenta concentrations (Masters et al. 1983b), or not (McKenzie et al. 1975). Zinc intake seems not to affect blood zinc in human studies. In a clinical trial, maternal plasma and RBC zinc are not different in the group supplemented throughout pregnancy with zinc in addition to iron and folic acid than in a group without zinc (Caulfield et al. 2008).

Potential effects of 1C metabolism on zinc status

Effect of folate on zinc uptake and status

Six human studies tested the effect of concomitant folic acid and zinc intake on zinc absorption and four of these reported no effect (Keating et al. 1987; Krebs et al. 1989; Arnaud et al. 1992; Hansen et al. 2001). These studies observed no difference in zinc absorption following a zinc tolerance test (ZTT) combined with 10-200mg of folic acid or 5-formylTHF compared to the ZTT without folic acid in healthy volunteers (Keating et al. 1987; Krebs et al. 1989; Arnaud et al. 1992; Hansen et al. 2001) or no effect on Zn^{65} absorption from bread made with flour fortified with folic acid (Hansen et al. 2001). The former was also true in ZTT with 200mg folic acid compared to 5-formylTHF (Arnaud et al. 1992). Two other studies reported that zinc absorption was lower in the presence of folic acid (Milne 1989; O'Brien et al. 2000). Lower Zn^{65} absorption from meals with 800µg of folic acid compared to without folic acid was

found in 5/13 healthy adults that had a tendency to low zinc intake and plasma zinc (Milne 1989). This was also true for zinc isotopes absorption from intravenous injections and oral doses combined with 250µg folic acid, 60mg iron and 15mg zinc compared to without supplementation in pregnant Peruvian women (O'Brien et al. 2000). Along these lines, another study reported folate to be a negative predictor of the amount of bioavailable zinc from vegetarian meals (Chiplonkar and Agte 2006).

The results of animal studies of the effect of simultaneous folic acid on zinc absorption are also controversial, reporting lower absorption with folic acid (McMaster et al. 1985; Ghishan et al. 1986; Southon et al. 1989) or no effect (Keating et al. 1987). Lower zinc absorption was observed in rats fed 500µg/d compared to 5µg/d folic acid (McMaster et al. 1985), or with perfusates with 460µM folic acid compared to without folic acid (Ghishan et al. 1986), or fed 6mg folic acid, 308mg iron and 11.6g calcium compared to 2.8mg folic acid, 37.7mg iron and 5.8g calcium (/Kg diet) (Southon et al. 1989). The latter finding was also true for pregnant rats (Southon et al. 1989). But no differences in zinc absorption were observed among groups of rats administered 1ml of aqueous solution or milk formula, containing 13µg zinc plus Zn^{65} and 4.4µg, 176µg or no folic acid intragastrically (Keating et al. 1987). Folic acid and zinc were shown to bind and to form complexes *in vitro*, but this mechanism for the possible effect of folic acid on zinc absorption seems unlikely because the complexes only occurred at pH 2 and were dissolved at pH 6 (Ghishan et al. 1986). The pH is changed to ≈6 towards the jejunum in individuals without gastrointestinal pathologies (Evans et al. 1988). Endocytosis of folate bound to membrane folate receptors alpha plus proton coupled folate transporters has been proposed as a folate uptake a mechanism in the placenta, together with a necessary acidification of the endocytic vesicle lumen for the release and transport of folate to the cytoplasm (Solanky et al. 2010). This mechanism has been verified in cervical cancer cells and proposed as the mechanism by which the proton coupled folate transporter takes up folate when the extracellular milieu is at neutral pH (Zhao et al. 2009). The pH of these vesicles is as low as 6-6.5, but this is in reclycling vesicles for 5-methylTHF uptake, and a non recycling pathway with lower pH for folic

acid is possible (Yang et al. 2007). If this process occurs in the intestine, a sufficiently acidic pH in the vesicles might promote the formation of complexes between zinc and folate. In *in vitro* studies, zinc uptake did not differ in pig purified brush border membrane vesicles for a folic acid concentration range (0.05-50μM) (Turnbull et al. 1990), or in human intestinal cells after the addition of beverages digestates with 1.75μg/g folic acid or without folic acid (Pullakhandam et al. 2011). But zinc uptake in rat liver slices (Tupe et al. 2007) and zinc transport through a human intestinal cell layer (Tupe and Agte 2010) were lower with increasing folic acid concentrations (0.02-0.57μM).

Four human studies tested the chronic effect of folic acid on zinc absorption and three of these reported a decreasing effect (Meadows et al. 1983; Simmer et al. 1987; Milne 1989). There was lower zinc absorption in healthy volunteers following a ZTT or the intake of a meal with Zn^{65} after 2wk treatments with folic acid as 550μg/d (plus 100mg/d iron) (Meadows et al. 1983), or 350μg/d (Simmer et al. 1987), or 800μg/d (Milne 1989), compared to before the interventions. This was also true for a 2wk treatment with 350μg/d folic acid plus 100mg iron in pregnant women (Simmer et al. 1987). The finding in Milne study only occurred in the 5/14 healthy adults with a tendency to low zinc intake and plasma zinc (Milne 1989). But there were no differences in Zn^{65} absorption from meals after a 3wk 400μg/d or 800μg/d folic acid treatment, compared to before the interventions in healthy volunteers (Milne et al. 1990). Higher zinc absorption was reported in the pups of rat dams fed an ethanol diet plus folic acid, compared to the ethanol/no folic acid group, from 8 weeks before pregnancy throughout 21 days of lactation. Rats in the folic acid group had about 80-100μg/d more folic acid than the no folic acid group (Tavares et al. 2000).

Most clinical trials of folic acid supplementation assessing zinc status did not specify if folic acid was simultaneous to zinc intake, and have found no differences in plasma, serum, RBC and urinary zinc concentrations in treated (400-10000μg/d of folic acid for 24d up to 4 months) compared to untreated patients (Butterworth et al. 1988; Reid et

al. 1992) or untreated healthy volunteers (Milne et al. 1990; Hambidge et al. 1993; Kauwell et al. 1995; Green et al. 2003). However, lower maternal but not cord plasma zinc was found in pregnant women taking 250μg folic acid plus 60mg iron compared to the unsupplemented group (O'Brien et al. 2000). Animal studies have assessed the effect of folic acid on zinc status in tissues. No differences in liver or femur zinc concentrations were observed in rat fed diets supplemented with folic acid as 100mg/Kg diet compared to unsupplemented diets (Fuller et al. 1988), or with folic acid as 6mg/Kg diet compared to 2.8mg/Kg diet (Southon et al. 1989). However, in Fuller *et al*. study higher kidney zinc concentration was found in pregnant or lactating rat dams fed the folic acid supplemented diet compared to the unsupplemented diet (Fuller et al. 1988).

In observational studies folate and zinc concentrations are positively correlated in amniotic fluid (Weekes et al. 1992) and negatively correlated in plasma/serum of preterm neonates (Fuller et al. 1992) but not of pregnant women (Mukherjee et al. 1984; Tamura et al. 1992). Folic acid supplements use was not associated with amniotic or plasma zinc in pregnant women (Weekes et al. 1992; Tamura et al. 1994)

Effect of choline on zinc uptake and status

Rats fed a choline deficient diet have lower hepatic zinc concentrations than fed a control diet (Keefer et al. 1973). The author of this experiment did not test zinc intestinal uptake in perfused rat intestine but proposed impaired zinc absorption (Keefer et al. 1973). *In vitro* experiments with human neuroblastoma cell line in medium with 1μM zinc, show higher intracellular zinc levels when M1 muscarinic acetylcholine receptor or its downstream effector are stimulated (Zuchner et al. 2006). Because this receptor is localised in the intervillous space membrane of the syncytiotrophoblast according to pharmacological experiments (Pavía et al. 1997) and there is high acetylcholine synthesis in the placenta (Welsch 1976), a link between placenta zinc status and choline might exist.

5.2. Copper

Metabolism and pregnancy

Copper is necessary for the catalytic function of several proteins, such as the diamine oxidase (Wolvekamp and De Bruin 1994) and three ferroxidase enzymes: hephaestin (Chen et al. 2004), ceruloplasmin (Zaitseva et al. 1996), and zyklopen (Danzeisen et al. 2000; Chen et al. 2010). Although ceruloplasmin contains >95% of the plasma copper, experiments with mutant aceruloplasminemic mice show ceruloplasmin has little effect on copper homeostasis (Meyer et al. 2001). However, liver copper and iron concentration are higher in aceruloplasminemic mice and this has been attributed to loss of ferroxidase activity (Meyer et al. 2001).

The copper transporter 1 (SLC31A1) is considered the main mediator of copper intestinal uptake (Nose et al. 2006). In tissues, the superoxide dismutase 1 is the major copper containing protein (Rakhit and Chakrabartty 2006), but metallothioneins have been reported to be involved in intracellular storage of copper (Tapia et al. 2004).

In the placenta, copper uptake from the intervillous space is through passive carrier-mediated transport (Tong and McArdle 1995; Nandakumaran et al. 2003). In addition two copper transporting ATPases have been identified in human placenta (Hardman et al. 2004), and according to human placenta cells and mouse experiments, the ATP7A pump delivers copper to the foetus, and the ATP7B pump returns excess copper to maternal blood (Hardman et al. 2007; Wadwa et al. 2014). Copper concentrations in cord plasma, serum or whole blood are lower than in the mother (Goel and Misra 1982; Hyvonen-Dabek et al. 1984; Tsuchiya et al. 1984; Schramel et al. 1988; Ong et al. 1993; Krachler et al. 1999; Al-Saleh et al. 2004). This has been attributed to lower ceruloplasmin levels in foetal than maternal blood, but an endocytic pathway against the copper gradient may also be involved (Hardman et al. 2006).

The calculation of RDA for copper in pregnancy is based on the reported content of copper in term foetus, placenta, amniotic fluid and maternal tissues developed in

pregnancy. The copper RDA in pregnancy is 1mg/d. The pregnancy copper UL is based on liver damage as an adverse effect in non-pregnant women. Copper UL in pregnancy is 8mg/d or 10mg/d for women ≤18 or ≥19 years, respectively (Institute of Medicine - Panel on Micronutrients 2001).

Maternal smoking seems not to affect the copper concentrations in maternal serum at different stages of pregnancy (Mochizuki et al. 1984; Kantola et al. 2000) or in cord RBC (Aydogan et al. 2013). There are no differences between placenta copper concentrations of pregnant smoker and non-smokers from Chile (Ronco et al. 2005), Croatia (Stasenko et al. 2010), Ukraine (Zadorozhnaja et al. 2000) and Finland, Estonia and Russia (Kantola et al. 2000).

Maternal plasma copper concentrations at delivery are negatively associated with birth weight (Ozdemir et al. 2007), but not in whole blood (Al-Saleh et al. 2004) or during the first half of pregnancy (Crosby et al. 1977). Cord plasma or whole blood copper is negatively associated with birth weight (Al-Saleh et al. 2004; Ozdemir et al. 2007). The results from studies comparing SGA and AGA pregnancies report either lower copper concentrations in cord plasma of SGA neonates (Goel and Misra 1982) or no differences in cord or maternal serum (Osada et al. 2002).

Placenta copper concentrations have been reported to be negatively associated with birth weight (Kantola et al. 2000), but other studies reported a positive association (Ozdemir et al. 2009) or none (Ward et al. 1987; Odland et al. 2004; Ronco et al. 2005). There are no differences in placenta copper between LBW neonates and controls (Dawson et al. 1969; Llanos and Ronco 2009). Lower placenta copper concentrations are found in SGA cases from Poland (Zadrozna et al. 2009) but not in Japan (Osada et al. 2002).

Lower serum copper was reported in non pregnant than pregnant women towards the end of pregnancy (Atamer et al. 2005), but the opposite was reported in a recent study for plasma and whole blood at delivery (Ghneim et al. 2016). In two cross-sectional

studies, maternal serum copper concentrations are higher in more advanced pregnancies (Hyvonen-Dabek et al. 1984; Kantola et al. 2000), with one study finding no differences at deliveries before, at, or after 37 GW (Reddy et al. 2014). A longitudinal study reported that maternal serum copper increases between 28 to 37 GW, maintaining a plateau thereafter (Mochizuki et al. 1984). Cord serum copper though recently reported not to be associated with gestational age (Reddy et al. 2014) had previously been reported to be lower in preterm than term neonates (Goel and Misra 1982).

Higher placenta copper concentrations were reported in term pregnancies than during the first trimester (Kantola et al. 2000), but this was not confirmed in other studies assessing more advanced pregnancies (Ward et al. 1987; Custódio et al. 2003; Reddy et al. 2014). Regarding preterm delivery, no differences were reported in placenta copper concentrations between preterm and control deliveries (Zadrozna et al. 2009).

Maternal age was positively correlated with placenta copper concentrations in Finnish pregnant smokers but not in non smokers (Kantola et al. 2000), nor in a study from Turkey with unknown smoking status of the participants (Ozdemir et al. 2009). In Ukrainian urban pregnant women placenta copper did not differ with maternal age (Zadorozhnaja et al. 2000). However, foetal side placenta copper concentrations were reported to be higher in adult pregnant women than the 15-19y group (De Moraes et al. 2011). This was not observed on the maternal side of the placenta.

To the best of our knowledge no studies to date have assessed the effects of maternal BMI or socioeconomic status on placenta copper status.

Lower placenta copper concentrations were reported in placentas of male compared to female neonates in some studies (Ward et al. 1987), but not others (Phuapradit et al. 2000).

Placenta copper and selenium are positively correlated (Osman et al. 2000; Odland et al. 2001). Placenta copper and iron concentrations were negatively correlated in

studies from Northern Norway and Russia (Odland et al. 2001), but not associated in Turkey (Ozdemir et al. 2009) and Japan (Tsuchiya et al. 1984). Placenta copper and iron are negatively correlated in a rat study of low iron or deficiency, and this is not due to changes in the transcription of copper transport proteins in liver (including ceruloplasmin) or placenta (Gambling et al. 2004). In addition to the possible postranslational regulation of these proteins, the placental copper containing ferroxidase (zyklopen), which is downregulated and upregulated by iron and copper status, respectively (Danzeisen et al. 2002) may be involved in the placenta iron-copper relationship.

There were higher placenta copper concentrations in cattle fed a diet with organic (but not inorganic) supplementation with trace elements including copper, than fed a control diet (Marques et al. 2016). The opposite was reported in rat dams fed copper deficient diets compared to copper adequate (Masters et al. 1983a) or low copper diets (Ebesh et al. 1999; Andersen et al. 2007).

Potential effects of 1C metabolism on copper status

Effect of folate on copper uptake and status

A clinical trial of zinc depletion induction in 8 healthy USA men found no differences in copper absorptions (as faecal copper loss as percentage of dietary copper intake) and serum copper concentrations in the supplemented group (400μg folic acid every other day for 5-6 months), and in the unsupplemented group (Milne et al. 1984). In a pregnant rat study where methotrexate was added in intraperitoneal injections chronically and also concomitantly to copper intake, no differences in serum copper concentration were detected between the methotrexate treated and the untreated group (Al-Saleh et al. 2009).

Higher serum copper concentrations was reported in mice two hours after receiving a single oral dose of folic acid as 100mg/Kg body weight than in animals not receiving

the dose (Hamed et al. 2009). The authors proposed that this acute folic acid oral dose without copper mobilises tissue copper stores towards the blood.

In observational studies, no differences in copper concentrations at ≈17 GW were observed between users and non-users of prenatal supplements (800-1000μg folic acid daily) in USA pregnant women, in amniotic fluid (Weekes et al. 1992; Tamura et al. 1994) or maternal plasma (Tamura et al. 1994). However, in a study with 57 Scottish pregnant women, iron-folate supplement users had lower serum copper concentrations that non users (Burns and Paterson 1993).

In rats a foetal programming effect of folic acid on copper status was reported. The adult offspring of dams fed folic acid supplemented diet (5mg/Kg diet) during pregnancy had lower copper concentration in kidney but not in liver, than those born of dams fed the control folic acid diet (2mg/Kg diet) (Król et al. 2011).

Effect of choline on copper uptake and status

In a rats fed diets low in protein and with 0.1% copper sulphate (non-toxic levels), there were larger quantities of copper in the liver when the diet is supplemented with choline than when the diet is low in choline (Hegsted et al. 1948).

5.3. Selenium

Metabolism and pregnancy

Selenium is contained in several human proteins (selenoproteins), mostly as the amino acid selenocysteine. Enzymes such as glutathione peroxidases, which catalyse the conversion of lipid or hydrogen peroxide and reduced glutathiones to water and oxidised glutathione, have selenocysteine in their sequence (Diamond 2015). Other selenocysteine-containing enzymes are the thioredoxin reductases, which reduce the thioredoxin proteins that can then reduce other proteins to avoid oxidative stress (Lu and Holmgren 2014). Non-selenocysteine selenoproteins includes selenium binding

protein 1 (SELENBP1), whose specific function is still unknown but has been suggested to have anticarcinogenic properties (Diamond 2015).

Selenium absorption and assimilation are not clearly understood (Fairweather-Tait et al. 2011). Organic selenium, such as selenomethionine and selenocysteine, is absorbed in the intestine by two amino acid transport systems (B^0 and b^{0+} rBAT) (Nickel et al. 2009). Inorganic selenium as selenate (SeO_4^{2-}) is possibly absorbed in the intestine by the anion exchangers SLC26s (Fairweather-Tait et al. 2011). Selenate uptake in the placenta is possibly mediated by SLC26s (Shennan 1988) and SLC13A4, which also transport chromium (Miyauchi et al. 2006). Cord blood selenium concentrations are lower than in the mother (Hyvonen-Dabek et al. 1984; Wasowicz et al. 1993; Al-Saleh et al. 2004). However, a Polish study reported similar concentrations in both compartments (Dobrzynski et al. 1998).

The calculation of the RDA for selenium is based on the selenium needed to reach the plateau concentration of plasma glutathione peroxidase, the concentration assumed to correspond with tissue selenoenzyme activity level. The RDA in pregnancy takes into account the selenium content of a 4kg foetus. Selenium RDA in pregnancy is 60µg/d. The pregnancy selenium UL is based on hair and nail brittleness and loss as adverse effects in non-pregnant women. In addition, uncertainty due to more sensitive individuals is taken into account. Selenium UL in pregnancy is 400µg/d (Institute of Medicine - Panel on Dietary Antioxidant and Related Compounds 2000).

Smoking has been reported not to affect maternal serum or whole blood selenium (Kantola et al. 2004) although it had been negatively associated with blood selenium in a previous study (Kosanovic et al. 2002). A similar controversy occurs in cord whole blood selenium concentrations (Kosanovic et al. 2002; Kantola et al. 2004). Passive smoking was recently reported not to be associated with cord serum selenium (Al-Saleh et al. 2015).

Smoking has been reported not to be associated with placenta selenium (Osman et al. 2000) but a later study in rural Finland women reported that it was higher in smokers than non smoker (Kantola et al. 2004). These findings were not confirmed in Estonian women (Kantola et al. 2004). Passive smoking was not associated with placenta selenium (Al-Saleh et al. 2015).

Maternal serum selenium is negatively associated with birth weight only in non smokers (Kantola et al. 2004), but no association is found in maternal whole blood selenium (Kantola et al. 2004). Cord whole blood selenium is positively associated with birth weight only in non smokers (Kantola et al. 2004), and is not associated in cord serum (Osman et al. 2000; Al-Saleh et al. 2015). Maternal or cord serum selenium do not differ between SGA and AGA pregnancies (Osada et al. 2002; Al-Saleh et al. 2015).

Placenta selenium and birth weight were positively associated in AGA pregnancies of a study of Croatian non-smokers (Klapec et al. 2008), but these findings were not confirmed in other studies (Ward et al. 1987; Kantola et al. 2004; Odland et al. 2004; Grant et al. 2010; Al-Saleh et al. 2015). Placenta selenium did not differ between LBW neonates and controls in a Chilean study of non-smokers (Llanos and Ronco 2009) and in a USA well drinkers cohort (Punshon et al. 2016). There have been varying reports on placenta selenium of SGA neonates, being reported to be lower (Klapec et al. 2008) or higher (Osada et al. 2002; Zadrozna et al. 2009) than AGA neonates. In a big Saudi Arabia study no association between placenta selenium and SGA risk was observed (Al-Saleh et al. 2015).

Lower serum selenium was observed in pregnant than non pregnant women (Atamer et al. 2005). Maternal serum and whole blood selenium were lower at delivery or in more advanced pregnancies (Hyvonen-Dabek et al. 1984; Kantola et al. 2004), but such a difference was not observed in samples of smokers only (Kantola et al. 2004). Several studies reported no association between placenta selenium and gestational age (Ward et al. 1987; Osman et al. 2000; Odland et al. 2001) but lower selenium was reported at delivery than in first trimester placentas in Finnish and Estonian studies (Kantola et al.

2004). No differences in placenta selenium were observed in rats at different gestational ages (Bou-Resli et al. 2001).

Maternal blood selenium in term and preterm deliveries were reported to be similar (Wilson et al. 1991; Mask and Lane 1993; Wasowicz et al. 1993; Lorenzo Alonso et al. 2005), except in a study in which plasma selenium was lower in preterm pregnancies (Dobrzynski et al. 1998). Reports on cord blood selenium and preterm delivery also have inconsistent findings with some studies showing lower concentrations in plasma of preterm neonates (Mask and Lane 1993; Dobrzynski et al. 1998) and others no differences in plasma (Wilson et al. 1991; Wasowicz et al. 1993) or whole blood (Lorenzo Alonso et al. 2005) compared to control neonates. Higher placenta selenium in preterm deliveries was reported in one study (Zadrozna et al. 2009) but not in others (Dobrzynski et al. 1998; Lorenzo Alonso et al. 2005).

No association was observed between maternal age and maternal serum or whole blood selenium (Kantola et al. 2004; Lorenzo Alonso et al. 2005). There is controversy regarding the association between maternal age and cord blood selenium, with studies reporting in cord whole blood negative (Kantola et al. 2004), positive associations (Lorenzo Alonso et al. 2005), or no association with cord serum (Al-Saleh et al. 2015). Maternal age is not associated with placenta selenium (Osman et al. 2000; Kantola et al. 2004; Lorenzo Alonso et al. 2005; Grant et al. 2010; Al-Saleh et al. 2015).

Maternal BMI was not associated with cord serum or placenta selenium (Al-Saleh et al. 2015; Punshon et al. 2016). Socioeconomic level was not associated with placenta selenium (Al-Saleh et al. 2015), and that is true also for neonate sex (Osman et al. 2000; Al-Saleh et al. 2015; Punshon et al. 2016).

Placenta selenium and iron are negatively associated (Odland et al. 2001). As for selenium intake, higher serum selenium concentration was found in cattle fed diets supplemented with 1mg/d or 2mg/d inorganic selenium, than fed a control diet (de Toledo and Perry 1985). In a clinical trial of organic selenium supplementation, plasma

selenium concentrations increased in old adults taking 100µg/d or 300µg/d selenium during 6 months, while remained unchanged in the placebo group (Bekaert et al. 2008).

Potential effects of 1C metabolism on selenium status

Selenium is usually considered a nutrient, but as a trace element is toxic at high concentrations. One carbon metabolism can have an effect on selenium status because, as in arsenic, methylation of selenium is required for its detoxification. The methylated intermediaries, i.e. mainly the pulmonary-excreted dimethylselenide and the urine-excreted trimethylselenonium, are less toxic than selenite ion (SeO_3^{2-}) (Olson 1986). SAM is required for its methylation (Tandon et al. 1986; Hoffman and McConnell 1987).

Effect of folate on selenium uptake and status

Folic acid can chronically impair organic selenium absorption. Lower seleno-methionine absorption in the duodenum was reported in the rat pups born to dams fed 8mg folic acid compared to 2mg folic acid (/Kg diet), from 8 weeks before pregnancy throughout 21 days of lactation (Nogales et al. 2011).

Folic acid simultaneous to selenium intake has been reported to affect selenium status in several tissues. Serum and urine selenium were lower in rat pups born to dams fed a diet supplemented with inorganic selenium plus folic acid compared to selenium alone (Ojeda et al. 2010; Nogales et al. 2011). This was also true for selenium concentration in the heart, kidney, spleen and testes but not the lungs and brain, where it was higher (Ojeda et al. 2010). Although folate receptors seem to play a minor role in folate intestinal uptake, the authors suggested competition in the intestine between selenium (as selenite) and soluble folate receptors for megalin, an endocytic receptor confirmed to be involved in cellular uptake of soluble folate receptors in kidney and yolk sac (Birn et al. 2005) and of the selenoprotein P (SELENOP) in kidney (Olson et al. 2008).

In a pregnant rat study where methotrexate was added in intraperitoneal injections chronically and also concomitantly to selenium, no differences in serum selenium

concentration were detected between the methotrexate treated and the untreated group (Al-Saleh et al. 2009).

Effect of choline on selenium uptake and status

Methyl groups derived from choline oxidation appear not to be involved in selenium detoxification by methylation. Rats fed a choline deficient diet had similar urinary selenium excretion to the control diet following an acute dose of selenite with ^{75}Se (Zeisel et al. 1987). A previous study reported similar dimethylselenide formation after injection of selenium (1.4mg/kg body weight) in choline-deficient rats and the control group (Hirooka and Galambos 1966).

Effect of cobalamin on selenium uptake and status

Higher kidney selenium (but not liver) was reported in cobalt deficient sheep compared with the cobalt supplemented group (Andrews et al. 1963). This was true both in the case of low natural selenium supply and for selenium supplementation (Andrews et al. 1963). More susceptibility to selenium toxicity induced by oral supplements was suggested in cobalt deficient sheep or those with low protein intake in an Australian study, although no statistical test was used (Gardiner 1966). In a study with rats injected intraperitoneally with 1.5mg selenium and ^{75}Se, the cobalamin depleted group had reduced formation of dimethylselenide, reduced urine trimethylselenonium, higher retention of ^{75}Se in liver, kidney and muscle, and lower retention of ^{75}Se in blood (Chen and Whanger 1993). In this study, liver extracts or hepatocytes from cobalamin deficient rats transformed less selenium to dimethylselenide, and the hepatocytes were less resistant to selenium toxicity (Chen and Whanger 1993). A subsequent study with perfused rat intestines and labelled selenium discarded a chronic effect of dietary cobalamin on selenium intestinal absorption (Chen et al. 1993). Liver and kidney selenium were higher and blood selenium lower in rats after 6 weeks on a cobalamin deficient compared to supplemented diet, each including different sub-toxic concentrations of selenium (Chen

et al. 1993). In other tissues (heart, spleen), lower selenium concentrations were reported with cobalamin deficiency after eight weeks (Chen et al. 1993). Therefore, cobalamin seems to affect selenium status but not absorption.

5.4. Iron

Metabolism and pregnancy

Physiologically, iron exists in two stable oxidation states: ferric (Fe^{3+}) and ferrous (Fe^{2+}). Body iron content (about 4.0g in men and 3.5g in women) is distributed between RBC haemoglobin (60-70%), muscle myoglobin (10%), and storage pools (20-30%) as ferritin and haemosiderin in the liver and in the monocytes and macrophages of the reticular connective tissue (Geissler and Singh 2011). The ferritin complex contains iron as Fe^{3+} (up to 4500 atoms) which is easily released and reduced to Fe^{2+} when ferritin is degraded in the cytosol (Koorts and Viljoen 2007). If ferritin is internalised in lysosomes for degradation, haemosiderin can be formed and in this case the stored iron is less mobilisable, thus protecting against iron toxicity (Koorts and Viljoen 2007). About 1% of total body iron is contained in enzymes: haem enzymes such as cytochromes, catalase and haem-dependent peroxidases; and non-haem iron enzymes. Less than 0.2% of total body iron is transported in plasma bound to soluble transferrin proteins (Geissler and Singh 2011). Each transferrin binds one Fe^{3+} atom and this holotransferrin can interact with its membrane receptor, undergoing endocytosis, and releasing the iron to the acidic lumen of the vesicle (Luck and Mason 2012). The transferrin-mediated cellular iron delivery process ends with the export of Fe^{3+} to the cytosol and the recycling of transferrin and its receptor back to the blood plasma and cell membrane, respectively (Luck and Mason 2012). Extracellular iron not bound to transferrin, and in the Fe^{2+} state can be transported into cells by ZIP14 (SLC39A14) (Liuzzi et al. 2006), which is one of the cellular importers of Zn^{2+} (King 2011). The export of Fe^{2+} from the cells is carried out only by the ferroportin protein (SLC40A1) (McKie et al. 2000; Gulec et al. 2014).

Non-haem iron intestinal uptake occurs mainly in the duodenum, by the divalent cation transporter 1 (SLC11A2) (Gunshin et al. 1997). The divalent state of iron in the intestinal lumen is achieved by ferrireductase activities of enzymes (such as cytochrome b reductase 1) and certain molecules (such as ascorbic acid) (Luck and Mason 2012). The non-haem iron absorbed by the enterocytes (in Fe^{2+} state) is exported by the ferroportin transporter (McKie et al. 2000). The exported iron needs to be oxidised to Fe^{3+} (by proteins with ferroxidase activity, which in the enterocyte basolateral membrane is hephaestin, a copper-containing enzyme) to be transported by transferrin in blood (Chen et al. 2004). Ceruloplasmin, another copper-containing ferroxidase enzyme can contribute to the oxidation of Fe^{2+} to Fe^{3+} after intestinal absorption (Osaki et al. 1966; Zaitseva et al. 1996), although it is currently considered as involved mostly in the oxidation of iron for the correct export from the liver (Meyer et al. 2001).

There is controversy regarding the pathways involved in haem iron intestinal uptake (Gulec et al. 2014). The later termed proton coupled folate transporter (Qiu et al. 2006), was firstly identified as a low affinity intestinal haem transporter (Shayeghi et al. 2005). An *in vitro* experiment with colorectal adenocarcinoma human cells showed that when the transcription of this transporter is inhibited, both haem iron and reduced folates uptakes are decreased (Le Blanc et al. 2012). This suggests that the proton coupled folate transporter is important for haem iron intestinal uptake. More about this controversy, and a potential competition between folate and haem iron is explained in the following section. Other experiments with the same cell line report absorbed haem iron is mostly converted into free iron, although a fraction remains unmetabolised and is exported from the basolateral membrane (Mendiburo et al. 2011).

Iron intake is used during pregnancy to expand maternal RBC mass, to meet foetal iron needs, for placental development, and to provide for blood loss at delivery (Cetin et al. 2011). Based on experiments in pregnant rats fed control or iron deficient diets, some authors have proposed that during pregnancy, iron absorbed in the maternal gut is

preferentially destined in the following order: foetal RBC > foetal iron stores > maternal RBC > maternal iron stores (with iron stores as hepatic iron concentrations) (Gambling et al. 2009). From the approximately 1g of iron in the neonate at birth, 200-400mg are estimated to come from the maternal stores and the rest from maternal dietary intake and cessation of menses (Bothwell 2000).

In the placentas from rat dams on iron deficient diets, there is an up-regulation of proteins involved in iron uptake from the maternal blood: transferrin receptor (responsible for transferrin-bound iron uptake) and divalent metal transporter 1 (importer of free Fe^{2+}), suggesting that these are two pathways for iron uptake in the placenta. Although ferroportin (Fe^{2+} exporter) expression is not changed, these placentas may have a higher efflux of iron towards foetal blood, given that the activity of placental ferroxidase (zyklopen) is higher (Gambling et al. 2001). In the same study, human placenta cell growth in iron deficient medium have higher expression of transferrin receptors and there is a higher iron efflux from the basolateral side, corresponding with the foetal circulation (Gambling et al. 2001). These results add to the idea that in maternal iron deficiency conditions, the flux toward the foetus is prioritised. Cord serum or whole blood iron concentrations are higher than in the mother (Singla et al. 1979; Hyvonen-Dabek et al. 1984; Tsuchiya et al. 1984; Al-Saleh et al. 2004).

The RDA for iron in pregnant women of 27mg/d was set by modelling the components of iron requirements (basal losses, iron deposited in foetus and related tissues, and iron utilized in haemoglobin mass expansion), estimating the requirement for absorbed iron at the $P_{97.5}$, and taking into account iron absorption efficiency during pregnancy (25%). The UL in pregnancy is based on the appearance of gastrointestinal distress in non-pregnant women, and it is 45mg/day of iron (Institute of Medicine - Panel on Micronutrients 2001).

Lower placenta iron concentration is found in pregnant smokers from Chile (Ronco et al. 2005), but not Croatia (Stasenko et al. 2010). Exposure to nicotine by smoking

during pregnancy has been discarded as an inhibitor of iron transport due to the very high concentrations needed to exert such effect in placenta cells (McArdle and Tysoe 1988).

Neither maternal plasma or whole blood iron (Crosby et al. 1977; Al-Saleh et al. 2004) or cord whole blood iron are correlated with birth weight (Al-Saleh et al. 2004). There are no differences in maternal or cord serum iron between SGA and AGA pregnancies (Osada et al. 2002).

Placenta iron was positively correlated with birth weight in pregnancies from Turkey (Ozdemir et al. 2009), but not from England (Ward et al. 1987), Northern Norway and Russia (Odland et al. 2004), Chile (Ronco et al. 2005) and Jamaica (Grant et al. 2010). LBW neonate placentas had lower iron concentrations than controls in a Chilean study of non-smokers (Llanos and Ronco 2009), but this was not found in another study with unknown smoking status in the participants (Dawson et al. 1969). Also, no differences were observed between SGA and AGA pregnancies from Japan (Osada et al. 2002). The mRNA and protein levels of the transferrin receptor are lower in placentas of SGA than AGA neonates, suggesting impaired iron transport (Mando et al. 2011). The available evidence suggests that, mobilisable iron in placenta may be associated with foetal growth, but not placenta total iron.

In cross-sectional studies, maternal (Hyvonen-Dabek et al. 1984; Reddy et al. 2014) and cord (Reddy et al. 2014) serum iron did not vary with gestational age. Placenta iron concentration was not associated with gestational age in pregnant women from England (Ward et al. 1987) and India (Reddy et al. 2014).

Placenta iron was not correlated with maternal age in studies from Turkey (Ozdemir et al. 2009) and Jamaica (Grant et al. 2010). However, Brazilian adult pregnant women had higher placenta foetal side iron concentrations than the 15-19y group, although this was not observed in the maternal side specimens (De Moraes et al. 2011).

No studies assessing maternal BMI, socioeconomic status and neonate sex with placenta iron concentrations has been identified. Regarding iron intake, no differences in placenta iron concentration were found between iron supplement users and non-users from England (Ward et al. 1987).

Potential effects of 1C metabolism on iron status

Effect of folate on iron uptake and status

The proton coupled folate transporter is considered a high affinity folate importer, and according to some studies a low affinity haem iron importer. Proton coupled folate transporter can mediate haem iron uptake according to studies using labelled haem iron (Shayeghi et al. 2005; Latunde-Dada et al. 2006) but not to studies measuring electrogenic proton flux (Qiu et al. 2006; Umapathy et al. 2007). This could mean haem transport does not depend on proton flux. Studies using a fluorescent haem analogue (zinc protoporphyrin) report effective transport in transfected Chinese hamster ovary cells (Shayeghi et al. 2005) but not in human embryonic kidney cell (Nakai et al. 2007; Salojin et al. 2011). In human cases of hereditary folate malabsorption, caused by mutations in the *proton coupled folate transporter* gene, large doses of 5-formylTHF are sufficient to apparently correct the associated haematological disorder (Geller et al. 2002; Qiu et al. 2006). Mutant mice deficient for the proton coupled folate transporter have survival rates improved with oral doses or injections of various folate forms, but not with oral non-haem iron or injections of haem iron (Salojin et al. 2011). Oral 5-methylTHF completely rescues the anaemia in these mutant mice (Salojin et al. 2011). Serum and hepatic iron are increased in these mutant mice, which the authors propose to be due to diminished iron use when haemoglobin synthesis is lower (Salojin et al. 2011). In the mouse duodenum, the mRNA transcripts of the *proton coupled folate transporter* are not modulated by iron (deficiency or loading conditions), but the cellular location is, with it ranging from predominantly apical membrane under iron deficient diets to cytoplasmic location under iron-loaded conditions (Shayeghi et al. 2005). In a human intestinal cell line higher haem iron concentrations in the media

down-regulate *proton coupled folate transporter* mRNA (Le Blanc et al. 2012), and inhibition of the translation of this transporter results in lower haem iron and folate uptakes (Laftah et al. 2009; Le Blanc et al. 2012). In the retina and retinal pigment epithelium cells there is higher expression of *proton coupled folate transporter* transcripts in mutant mouse models of haemochromatosis, than in the wild type strain (Gnana-Prakasam et al. 2011). Due to the high bioavailability of haem iron, in Western societies haem iron intake makes up two-thirds of the total iron stores in spite of constituting only one-third of the ingested iron (West and Oates 2008). Although the role of the proton coupled folate transporter in intestinal haem iron uptake is not well defined, a competitive inhibition might exist between haem iron and folate at the intestinal absorption site.

Iron uptake in human thyroid slices incubated in sera with ^{59}Fe was not correlated with serum folate (Buchanan 1971). Non-haem iron and folic acid seem not to interact *in vivo*. In an *in vitro* experiment, the cations Fe^{2+} and Fe^{3+} cannot form complexes with folic acid in conditions resembling physiological ones (pH 6 and experimental medium consisting in aqueous buffer in addition to the used dimethyl sulfoxide) (Campbell et al. 1994). No effect of inorganic iron on folic acid absorption was observed in rat jejunum perfusion experiments using the equivalent dose of folic acid to 1000μg and iron (ferrous sulphate) equivalent to 6.4, 64 or 300mg in humans (Campbell et al. 1994). Iron uptake was higher in human intestinal cell cultures incubated with media (containing iron, zinc and vitamin A) with added folic acid and ascorbic acid compared to the basic media alone. This effect was specific to an experiment using a high-phytate food matrix (Pullakhandam et al. 2011). However, the interpretation of this study is more likely to be due to the inorganic iron uptake enhancing effect of ascorbic acid than to folic acid (Lynch and Cook 1980). To the best of our knowledge, there are no studies regarding the potential effect of folic acid simultaneous to iron where the iron form is haem.

Folate status may affect non-haem iron absorption but there is controversy regarding iron status. Higher iron (ferrous citrate) absorption and plasma iron concentrations were observed in rabbits fed a folate deficient compared to normal diet for 10 weeks and both parameters returned to normal following intramuscular injection of 15mg folic acid (Celada et al. 1979). In the previously mentioned clinical trial of zinc depletion in 8 healthy USA men, there were no differences in total iron absorption (haem and non-haem), serum iron, total iron binding capacity, serum transferrin or ferritin between the group supplemented with 400μg folic acid every other day for 5-6 months and the unsupplemented group (Milne et al. 1984). Higher serum iron was reported two hours after receiving a single oral dose (100mg/Kg body weight) of folic acid compared to no folic acid in mice (Hamed et al. 2009). The authors proposed folic acid mobilises tissue iron stores towards the blood.

In a USA observational study, no differences in total iron concentrations in amniotic fluid or maternal plasma at ≈17 GW were detected between users and non-users of prenatal supplements (800-1000μg folic acid daily) (Weekes et al. 1992; Tamura et al. 1994).

The adult offspring of rat dams fed folic acid supplemented diets during pregnancy had similar liver and kidney iron concentrations to the offspring born to unsupplemented dams suggesting a lack of foetal programming effect by folic acid on postnatal iron status (Król et al. 2011).

Effect of choline on iron uptake and status

Substituting sodium chloride by choline chloride in the medium in which mouse duodenal fragments were incubated led to no changes in intestinal iron absorption suggesting that choline probably does not interfere with the intestinal reduction of non-haem iron in the ferric state and its subsequent absorption (Raja et al. 1989). There are *in vitro* reports based on spectrophotometry showing that choline and non-

haem ferrous iron can form insoluble complexes. However this was reported at a non physiological pH (11.7) (Geisser 1990).

Intestinal uptake of non-haem iron (Fe^{2+}) was un affected after 14 weeks of a choline deficient diet in female rats (Batey and Johnston 1993).

Uptake of non-transferrin bound iron (Fe^{2+}) decreased in rat liver when sodium in the perfusate had been substituted with choline (Wright et al. 1988). The choline concentration used in the perfusate in this experiment was much greater than the physiological (≈105μM) (Zeisel et al. 1989). In the study of female rats fed a choline deficient diet to induce liver steatosis of Batey and Johnson, lower hepatic transferrin-bound iron (Fe^{3+}) uptake was detected as ^{59}Fe signal in the liver 6 hours after an injection in the tail vein of 400μg/mL transferrin (50% saturation) (Batey and Johnston 1993). This is a physiological dose of transferrin (≈475μg/mL in rat serum) (Gentry-Nielsen et al. 2001). Therefore, the study with the best replication of *in vivo* conditions shows that choline enhances liver iron uptake (transferrin-bound, which is the major form).

In RBC of 14 GD chicken embryos, Fe^{3+} (from ovotransferrin) uptake was lower when the cells were incubated with 50μg phosphatidylcholine than without it (D'Andrea et al. 1995). It was proposed that phosphatidylcholine lowers RBC membrane cholesterol and that this affects expression and/or mobility of the ovotransferrin receptors (D'Andrea et al. 1995).

6. Summary of current knowledge and contributions of this thesis

Genetic study

What is known	What is unknown	What this thesis adds
In vitro the *MTHFR* c.665 variant allele is associated with thermolability, lower enzymatic activity and lower affinity of the enzyme for its cofactor. 5-methylTHF alleviates the effect of the variant enzyme. *In vivo* the variant allele is associated with lower plasma/serum and RBC folate, and higher plasma/serum tHcy concentrations. The effect on tHcy is reduced/lost when folate status is high.	Whether folate status modulates the effect of the maternal *MTHFR* c.665C>T SNP on tHcy during pregnancy.	The effect of the SNP on tHcy is not observed during pregnancy when folate status is high.
In vitro the *BHMT* c.716 variant allele is associated with higher affinity of the enzyme for Hcy. There is controversy about whether higher or similar affinity of the variant compared to normal enzyme for betaine. *In vivo* the variant allele is associated with lower plasma DMG, but not betaine or tHcy.	How the maternal *BHMT* c.716G>A SNP affects 1C analytes during pregnancy. How the maternal *BHMT* c.716G>A SNP affects cord blood 1C analytes. Whether folate status modulates the effect of the maternal *BHMT* c.716G>A SNP on DMG and the DMG/betaine ratio during pregnancy.	The *BHMT* c.716G>A polymorphism does not affect pregnancy changes in betaine and tHcy . In the AA genotype the increase in DMG starts later compared to the other genotypes. Cord DMG is lower in AA compared to GG, but without differences in betaine or tHcy. The GA genotype combined with high folate status is associated with lower plasma DMG and the AA genotype combined with low folate status is associated with a higher DMG/betaine ratio in early pregnancy.

What is known	What is unknown	What this thesis adds
In vitro the *SLC19A1* c.80 variant allele is associated with higher affinity for formylTHF, and possibly wrong cellular location of the transporter. There is controversy about whether lower or similar methotrexate transport and expression of the variant compared to normal transporter. *In vivo* no differences in plasma/serum and RBC folate or plasma/serum tHcy concentration have been reported between alleles or genotypes. The variant homozygote genotype was associated with lower RBC folate in healthy Spanish adults.	Whether the maternal *SLC19A1* c.80G>A SNP affects 1C analytes during pregnancy. Whether the maternal *SLC19A1* c.80G>A SNP affects cord blood 1C analytes. Whether the SNP affects tHcy during pregnancy depending on folate status.	The changes in plasma and RBC folate throughout pregnancy are similar among *SLC19A1* c.80G>A genotypes. THcy has a U shape pattern where the increase starts later in GA and AA. There are no differences in cord plasma folate and tHcy among maternal genotypes. There are no differences in tHcy among genotypes and plasma folate does not interact with this.
In vitro the *MTRR* c.66 variant allele is associated with lower formation of the MTRR-MTR complex. *In vivo* no differences in plasma/serum and RBC folate, plasma/serum tHcy or cobalamin concentrations between the alleles or genotypes have been reported. Lower plasma cobalamin in heterozygotes compared to the other genotypes was observed in a study of healthy Spanish adults.	Whether the maternal *MTRR* c.66A>G SNP affects 1C analytes during pregnancy. Whether the maternal *MTRR* c.66A>G SNP affects cord blood 1C analytes. Whether the SNP has an effect on cobalamin and tHcy during pregnancy and whether this is affected by folate status.	The pregnancy-associated plasma folate decrease and tHcy increase start later in the *MTRR* c.66AG and GG compared to the AA genotype. There are no differences in cord plasma folate, tHcy and cobalamin among maternal genotypes. In early pregnancy tHcy is higher in GG compared to AA, and after stratification this effect is lost in the highest and lowest plasma folate tertiles. At 24-27 GW plasma cobalamin is higher in GG compared to AA, and plasma folate does not interact with this.

Placenta trace element study

What is known	What is unknown	What this thesis adds
There is controversy about the association between the placenta concentration of zinc, copper, selenium and iron, and birth weight. Placenta zinc or iron are not associated with IUGR. Inconsistent results have been reported for the association between placenta copper and selenium with IUGR.	Whether the placenta concentration of these 4 trace elements is associated with birth weight or IUGR.	Placenta zinc, copper and selenium are negatively correlated with birth weight. Placentas of IUGR neonates have higher copper and selenium concentrations, but after adjustment for possible confounders only copper is associated with birth weight and IUGR risk.
Maternal age and placenta selenium are not associated. Inconsistent results have been reported for the association between placenta zinc, copper and iron and maternal age. Maternal BMI is negatively associated with placenta zinc, and not associated with placenta selenium.	Whether placenta copper or iron are associated with maternal BMI.	Maternal age is negatively correlated with placenta zinc. Maternal BMI is a positive predictor of placenta copper concentrations after adjustment for possible confounders.
Neonate sex is not associated with placenta selenium. Higher or similar placenta zinc has been reported in male compared to female neonates. Lower or similar placenta copper has been reported in male compared to female neonates.	Whether neonate sex is associated with placenta iron.	Lower zinc concentration is found in placentas of male neonates, without differences in copper, selenium and iron.
Only deficient zinc intake is associated with low placenta zinc in animal studies. Zinc supplementation did not affect maternal blood zinc in a human pregnancy clinical trial. Copper and selenium intakes affect their placenta concentrations according to animal studies. Iron supplement use is not associated with iron concentration in human placentas.	Whether total zinc, copper, selenium and iron intakes are associated with their placenta concentrations in a human study.	2nd and 3rd trimester users of supplements containing any of the 4 trace elements have lower placenta copper concentration than non-users. Total intake of each trace element is not associated with their respective placenta concentrations.

What is known	What is unknown	What this thesis adds
There is controversy about whether folic acid impairs zinc absorption or lowers zinc status. According to *in vitro* experiments, intracellular zinc concentrations might increase under acetylcholine signalling stimulation.	Whether 1C metabolism affects zinc concentrations in human organs.	1C metabolism is not associated with zinc in human placentas.
Little evidence in human studies suggests no independent effect of folic acid on copper absorption and status in blood or amniotic fluid. Results in animal studies are controversial. Choline supplementation might increase hepatic copper according to an animal study.	Whether 1C metabolism affects copper concentrations in human organs.	Plasma cobalamin at ≤12 GW and tHcy at labour are negative and positive, respectively, predictors of copper concentrations in human placentas.
In rats, chronic folic acid reduces organic selenium absorption, and concomitant folic acid affects the selenium status of several compartments. Cobalamin but not choline is involved in selenium detoxification according to animal studies.	Whether 1C metabolism affects selenium concentrations in human organs.	1C metabolism is not associated with selenium concentrations in human placentas.
Concomitant folate does not affect non-haem iron uptake. Chronic folate can decrease non-haem iron absorption. Partially concomitant folic acid does not affect total iron absorption or status in blood and amniotic fluid. Concomitant and chronic choline does not affect non-haem iron absorption. Iron uptake is possibly higher in liver with chronic choline, but lower in RBC with concomitant acetylcholine.	Whether folate affects haem iron absorption. Whether 1C metabolism affects iron concentrations in human organs.	1C metabolism is not associated with iron concentrations in human placentas.

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

HYPOTHESES AND OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

Hypotheses and objectives

Hypotheses

Genetic study

General hypothesis

- Polymorphisms in 1C metabolism genes affect 1C analyte concentrations during pregnancy and pregnancy outcome.

Subhypotheses

- The effects of the polymorphisms (*MTHFR* c.665C>T, *BHMT* c.716G>A, *SLC19A1* c.80G>A and *MTRR* c.66A>G) on the analytes are modulated by folate status.

- These maternal and offspring polymorphisms negatively affect foetal growth and development, as higher risk of IUGR and miscarriage.

Placenta trace element study

General hypothesis

- 1C metabolism affects placenta trace element concentrations.

Objectives

Genetic study

General objective

- To analyse the associations between 1C metabolism polymorphisms and 1C metabolism analytes during pregnancy and pregnancy outcome.

Specific objectives

- To report the genotype frequency of the *MTHFR* c.665C>T, *BHMT* c.716G>A, *SLC19A1* c.80G>A, and *MTRR* c.66A>G polymorphisms in the RTBC.

- To investigate the effect of the *MTHFR* c.665C>T, *BHMT* c.716G>A, *SLC19A1* c.80G>A, and *MTRR* c.66A>G maternal polymorphisms on related 1C metabolism analytes, in the RTBC.

- To investigate whether folate status modulates the effect of the *MTHFR* c.665C>T, *BHMT* c.716G>A, *SLC19A1* c.80G>A, and *MTRR* c.66A>G maternal polymorphisms on 1C metabolism analytes other than plasma and RBC folate, in the RTBC.

- To assess whether the *MTHFR* c.665C>T, *BHMT* c.716G>A, *SLC19A1* c.80G>A, and *MTRR* c.66A>G maternal and offspring polymorphisms are associated with adverse pregnancy outcome (as IUGR and miscarriage), in the RTBC.

Placenta trace element study

General objective

- To analyse the associations between 1C metabolism and placenta trace element concentrations.

Specific objectives

- To report placenta zinc, copper, selenium and iron concentrations in the RTBC.

- To study the associations between environmental factors and placenta zinc, copper, selenium and iron concentrations and pregnancy outcome, in the RTBC.

- To investigate the associations of 1C metabolism analytes throughout pregnancy and placenta zinc, copper, selenium and iron concentrations, in the RTBC.

MATERIAL AND METHODS

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

Material and methods

1. The Reus-Tarragona Birth Cohort

1.1. Design and study population

The Reus-Tarragona Birth Cohort (RTBC; ClinicalTrials.gov identifier: NCT01778205) is an ongoing observational longitudinal cohort study. It is being carried out by the Area of Preventive Medicine and Public Health of the Universitat Rovira i Virgili (URV); and the Areas of Obstetrics and Gynaecology of the University Hospitals: Sant Joan, Reus and Joan XXIII, Tarragona (Spain). Ethics approval was granted by the Ethics Committees of both hospitals in accordance with the Helsinki Declaration (World Medical Association 2013). The global aim of the study is to investigate the associations between maternal and paternal nutritional, genetic, medical and other environmental factors and placental function, pregnancy outcome and offspring health and development. The pregnancy phase of the study is considered in this thesis.

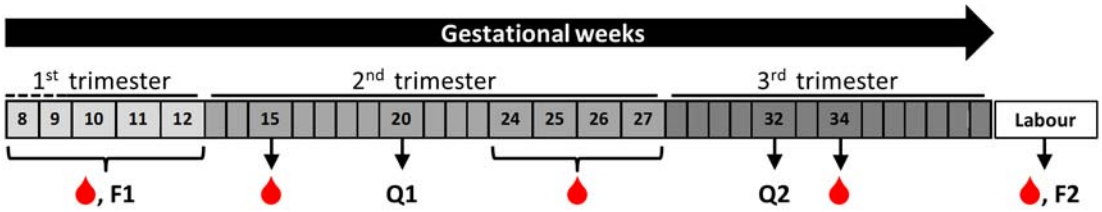
Participants are women whose pregnancies are monitored in the Sant Joan and Joan XIII hospitals. The population is not exposed to mandatory folic acid fortification as such a policy is absent in Spain. From January 2005 to October 2014, 626 women joined the study.

Participant recruitment was carried out by the obstetricians at the first antenatal check-up. Eligible participants were informed about the study and required to provide signed consent on acceptance to participate. Participants with a viable singleton pregnancy confirmed by ultrasonography and that provided their first prenatal blood sample before or at 12 GW were eligible to participate. Exclusion criteria included chronic diseases, surgical interventions and medical treatment affecting nutritional status or 1C metabolism.

In line with the recommendations of the Spanish Obstetrics and Gynaecology Society, women were recommended to take 400µg folic acid supplements daily until the end of the first trimester (Dirección General de Salud Pública (Ministerio de Sanidad y Consumo - Gobierno de España) 2001). The recommended supplements also contained 2µg cyanocobalamin. The regional pregnancy guidelines recommend folic acid supplements to be taken from four weeks before conception (Direcció General de Salut Pública (Departament de Salut - Generalitat de Catalunya) 2005). Daily iron supplements of 40mg were recommended from 12 GW onwards. It is worth noting the recommended supplements may be multivitamin, including in their composition trace elements such as zinc, and other vitamins that can be related to 1C metabolism, such as pyridoxine or riboflavin.

A summarising scheme of the RTBC is depicted in **Figure 12**.

Figure 12. RTBC study (pregnancy phase).



RTBC study (pregnancy phase) design. Red drops indicate the timing of blood sample collection. F1 and F2 are the first and second food frequency questionnaires. Q1 and Q2 are the first and second lifestyle questionnaires.

1.2. Biological sample collection

1.2.1. Blood samples

Maternal blood samples are collected from the antecubital vein. The umbilical cord blood sample is collected from the umbilical vein of the cleaned cord before placenta expulsion following the protocol for cord blood and stem cell sample collection of the participating hospitals. Dipotassium ethylenediaminetetraacetate (K_2EDTA) Vacutainer® tubes (Becton Dickinson) are used for all blood samples. Fasting blood samples are collected at ≤12, 24-27 and 34 GW. Initially another sample was collected at 15 GW but this has since been omitted due to changes in prenatal care protocols. Blood samples collected on admission to hospital with confirmed labour (or labour induction, or planned caesarean section) are not necessarily fasting (**Figure 12**).

The blood samples are collected into K_2EDTA vacutainers to be processed for whole blood, plasma, washed erythrocytes and leukocyte extraction and in to dry vacutainers for serum. Whole blood and erythrocytes are not isolated from labour or cord samples. Blood samples are kept at 4ºC after being drawn, and are processed in less than 1 hour of collection to avoid artefacts in the concentrations of 1C analytes. In the case of University Hospital Joan XXIII, blood samples are processed in the research laboratory of the hospital. In the case of University Hospital Sant Joan, blood samples are processed in the Institut d'Investigació Sanitària Pere Virgili (IISPV) Biobank.

For the RBC folate determination (≤12, 15, 24-27 and 34 GW), 50µl of whole blood is removed from the K_2EDTA tube before centrifugation and diluted in 450µl freshly prepared 1% (w/v) ascorbic acid (Panreac)(O'Broin and Kelleher 1992), mixed thoroughly and incubated at room temperature for 30 minutes. This procedure makes both the haemolysis of RBC for the release of gamma glutamyl hydrolase and cellular folates, and a slight acidification of the sample for the proper enzymatic activity of the

enzyme, making possible the conversion of the polyglutamate folates to the preferred assayable monoglutamate forms (Pfeiffer et al. 2010). Subsequently, this ascorbic acid lysate is stored at -80ºC in two separate aliquots. These aliquots and the plasma aliquots are stored at -80ºC in a freezer, as recommended for samples in which cell viability is not necessary (Vaught 2006).

Plasma aliquots (≤12, 15, 24-27, 34 GW, labour and cord) are obtained centrifuging the other whole blood K_2EDTA tube at 1500g for 15 minutes.

Aliquots of total leukocytes are obtained from the K_2EDTA tube samples and afterwards DNA is extracted from these. After the plasma has been removed, PBS [Phosphate Buffered Saline: 8g NaCl (Panreac), 0.2g KCl (Panreac), 0.24g KH_4PO_4 (Panreac), 1.44g Na_2HPO_4 (Panreac), 1L milliQ water; pH 7.4, autoclaved] is added to the remaining pellet of blood cells , which includes the buffy coat on top of it. It is thoroughly mixed with. The mixture is added to 30ml haemolysis solution [7.007g NH_4Cl (Fluka), 0.071g CH_5NO_3 (Fluka), 1L milliQ water] in a Falcon tube, thoroughly mixed and incubated at room temperature for 20-30 minutes. After the incubation the mixture is centrifuged at 2000g for 5 minutes and the supernatant is discarded. The removal of lysated RBC is also aided with several soft washings of the pellet edges with haemolysis solution. The pellet is then resuspended to 20ml haemolysis solution, and centrifuged and supernatant-discarded as previously described. This process yields a buffy pellet of total leukocytes, which is resuspended to 450µl PBS and finally placed in a Falcon tube with 10ml of Cell Lysis Solution (Qiagen). The Falcon tube is stored in dark at room temperature during a varying time ranging from one month to 18 months.

DNA is extracted from maternal and cord leukocytes with the Gentra Puregene Blood Kit (Qiagen). The following reagents of this kit are used: Protein Precipitation Solution and DNA Hydration Solution. First, 3.33ml of Protein Precipitation Solution are added

to the lysed leukocytes and mixed thoroughly with vortex for 20 seconds. Following 15 minutes incubation on ice, the mixture is centrifuged at 4ºC and at 2000g for 15 minutes. The supernatant is gently mixed with 10ml 100% isopropyl alcohol (Fluka) at 4ºC in a Falcon tube, discarding the pellet of precipitated proteins. When the DNA is visible, the tube is centrifuged at 4ºC and 2000g for 5 minutes. The supernatant is discarded before adding 10ml 70% ethanol (Merck) at 4ºC and mixing gently. Then the mixture is centrifuged at 4ºC and 2000g for 5 minutes discarding the supernatant, and the tube is left face down on blotting paper for the precipitated DNA pellet to dry during 30-40 minutes. The dried DNA pellet is rehydrated by incubation with 1200µl of DNA Hydration Solution in a shaker at room temperature for 3-4 days. The DNA extraction yield is assessed spectrophotometrically with the Nanodrop 1000 (Thermo Fisher Scientific): the ratio of sample absorbances at wave lengths 260nm and 280nm has been close or in the range 1.7-1.8 so far, therefore extra purification steps have not been needed. The DNA concentration of the solution is calculated for the 260nm absorbance peak with the nanodrop software, and should be 100-700ng/µL; if lower or greater, the solution is evaporated or diluted, respectively. This hydrated DNA is stored at 4ºC in a cryotube, with a chlorophorm drop to avoid fungal contaminations, until the genetic determinations.

The blood analytes assessed in this thesis may be affected by non-fasting conditions. It was reported that after a standard dinner (50g protein content) the concentration of plasma tHcy increases 14.6% (Guttormsen et al. 2004). In a small trial, women that took a ready-to-eat cereal-containing breakfast had an 11% increase in plasma folate concentrations after two hours (Noy et al. 2002); RBC folate was not assessed, although the authors deem its concentration could not change due to RBC folate being a long-term folate status indicator. A Norwegian study reported higher plasma betaine in a group of adult donors who took an unspecified light breakfast compared to a fasting group (Holm et al. 2003). However, there were more men in the non fasting group and this may have influenced the reported result. In a Northern Irish study only in

participants eating wheat bran-rich breakfast, postprandial plasma betaine was higher than at baseline (Keaveney et al. 2015). Plasma DMG was lower in fasting than in non-fasting individuals (Holm et al. 2003; Svingen et al. 2013). To the best of our knowledge, the only reports on plasma cobalamin changes after an unsupplemented meal were from an animal study. In this pig study, plasma cobalamin decreased after the meal, reaching at 15h after the meal a minimum value of 29.6% less than the premeal values (Matte et al. 2010). Therefore, concentrations in the non fasting samples at labour, may be so affected.

After blood sampling, if the blood fractions, i.e. blood cells and plasma or serum, are not separated by centrifugation and removed, plasma/serum tHcy has been reported to increase a 10% per hour at room temperature (Vester and Rasmussen 1991), or a ≈2.5% per hour in a cooler (Midttun et al. 2014). Another study reported lower tHcy increases, i.e. 1% or 3% after one hour at 4ºC or at 20ºC, respectively (Fiskerstrand et al. 1993). The possible explanation to this Hcy increase in whole blood has been most commonly homocysteine synthesis and release from blood cells (Ubbink et al. 1992). Indeed blood cells adenosylhomocysteinase activity has been reported to be necessary for this Hcy increase (Hill et al. 2002). However, several facts have led some authors to propose a supplementary mechanism for the temperature and time-dependent Hcy increase in whole blood. For example, non-protein bound iron mediates non-enzymatic synthesis of homocysteine, especially if chelated by EDTA (Baggott and Tamura 2015). This hypothesis was based on the different homocysteine increases in non-refrigerated whole blood depending on the anticoagulant of the extraction tube (Ubbink et al. 1992; Willems et al. 1998; Hübner et al. 2007), *in vitro* non-enzymatic synthesis of homocysteine from methionine, SAM and cystathionine in EDTA buffer with $FeSO_4$, and higher homocysteine in participants subjected to methionine load test with $FeSO_4$ compared to without it (Baggott and Tamura 2007). Therefore, the increases of tHcy in whole blood could be due to two non-excluding causes: synthesis and exportation in blood cells, and free iron-mediated non-enzymatic synthesis. In addition, tHcy has been

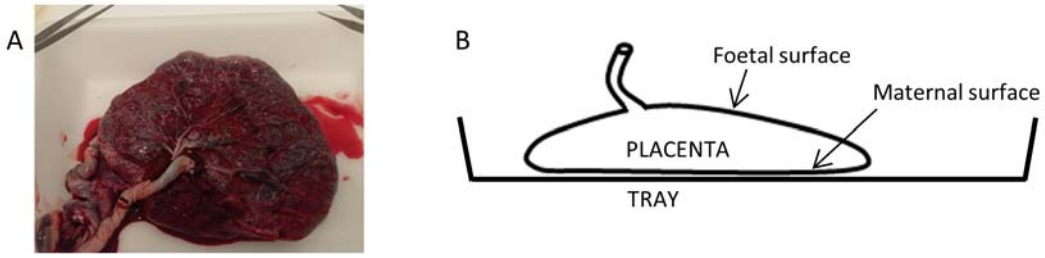
reported to be higher in serum than in plasma samples. This is attributed to the serum extraction tube needing an extra incubation time of at least 30 minutes for completion of coagulation before centrifugation (Rasmussen and Møller 2000). The protocol of RTBC blood samples collection emphasizes that the blood sample is refrigerated and plasma is isolated before one hour of the extraction.

1.2.2. Placenta samples

After expulsion, placentas are weighed and stored in black polyethylene bags at 4ºC until collected by a member of the RTBC team and transported by cooler to the Faculty of Medicine and Health Sciences (URV). Due to space constraints placentas from suspected IUGR pregnancies were initially targeted (see also *Material and methods* point *1.4*), and periodically placentas from control pregnancies were collected to represent collections across the timeframe of the field work of the RTBC study.

When starting the processing, the placenta is positioned in a plastic tray always in the same manner, as described next. The reason for this systematic positioning is to take triangular sections from the same region in all the analysed placentas. First the placenta is positioned foetal side up and maternal side down on the tray using stainless steel Adson and dissection forceps (**Figure 13**).

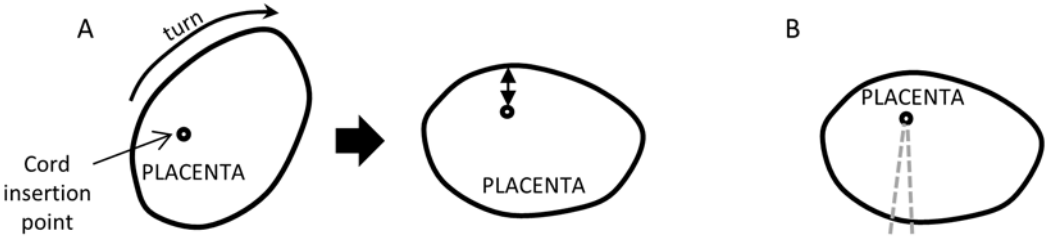
Figure 13. Placenta positioning I.



A. Top view photograph with foetal surface shown. Note the foetal surface has a violet colour, is scored with vessels and the cord protrudes from this side. B. Side view scheme with the positioning of the placenta in the tray.

From above, the placenta is positioned to include as the vertical axis the shortest distance from the protruding cord to the edge of the placenta. If the cord does not protrude from the centre of the foetal side of the placenta, which is the common situation, the positioning has to ensure that it is in the upper half (**Figure 14. A**).

Figure 14. Placenta positioning II and triangular section cut.



A. Top view scheme illustrating a turn necessary to place the organ in the reference position. Double-ended arrow indicates the shortest distance from the protruding cord to the edge of the placenta. B. Top view scheme of the triangular section.

With the placenta in the reference position and using a scalpel sleeve with a disposable stainless steel blade, two cross-sectional cuts are made to dissect a triangular section

from the cord insertion point to the edge of the placenta (**Figure 14. B**). As the triangular section contains the complete thickness of the organ, the chorionic plate with the amnion on top of it (foetal surface) is included, as well as the basal plate with the *decidua basalis* (maternal surface). The thickness of term placentas in the central region is usually about 2.5cm. The triangular section length from the cord insertion point to the edge of the placenta varies among placentas. The width varies according to the thickness and length of the triangular section, which are fixed parameters for each placenta placed in the reference position, and this width is the maximum possible for the specimen to be stored appropriately in a 20ml screw-capped flask (Deltalab). The width of the part of the triangular section that corresponds with the edge of the organ is not usually greater than 3cm. A triangular section contains several cotyledons or parts of them. The screw-capped flasks with the placenta triangular sections are stored at -80ºC in a freezer until further processing.

1.3. Lifestyle, dietary intake and clinical history collection

Lifestyle

Two lifestyle questionnaires are completed by the participants in personal interviews at 20 GW and 32 GW. In the first questionnaire information is collected to cover the period from preconception to 20 GW. The second questionnaire at 32 GW covers the time range between 20 GW to 32 GW. In these questionnaires information related to vitamin supplement use, breakfast cereal consumption, toxic habits, physical activity, sunbathing, socioeconomic level and planning of pregnancy is obtained (**Appendices**).

In relation to vitamin supplement use, commercial brand, product, timing of initiation, frequency and duration of use are asked. A designed catalogue with photographs and names of the vitamin supplement products which are sold in Spain is use to help the

participants recall and give reliable information. Used supplement composition, timing of initiation/cessation and supplement intake pattern vary widely among the participants. Intake of folic acid, zinc, copper, selenium and iron from supplements was estimated based on the information collected with these questionnaires.

Regarding toxic habits, information about cigarette smoking, alcohol consumption and illegal drug use during the pregnancy and 12 months before it, is recorded. This information includes frequency categories (cigarettes per day, drinks per week, and illegal drug use habitually or occasionally), timing of cessation before or during pregnancy, and type of illegal drug.

The recorded socioeconomic information refers to the combined education levels, occupations and net household income of the participant and the father of the expected child.

Dietary intake

A validated 45 item food frequency questionnaire (Rodríguez et al. 2008) is performed in two interviews, the first at ≤12 GW (referring to the year before pregnancy) and the second the day after delivery (referring to the pregnancy). The questionnaires also contain questions regarding diet, appetite during pregnancy, nausea and sickness, and how iron supplements were consumed (**Appendices**).

Clinical history

Age, height, weight at the first antenatal check-up and parity (number of term births, preterm births, abortions and living children) data from the participant are collected.

Systolic and diastolic blood pressure measurements taken at the prenatal check-ups are recorded.

The recommendations of the regional pregnancy guidelines (Direcció General de Salut Pública (Departament de Salut - Generalitat de Catalunya) 2005) state that pregnant women are tested for a suspicion of gestational diabetes by the glucose challenge test (O'Sullivan test) at 24-28 GW if they are not at diabetes risk. Pregnant women at risk are those of at least 35 years or with risk factors (family history of diabetes, previous gestational diabetes, previous obstetrical pathology, obesity), and are tested with glucose challenge tests at the first antenatal check-up, at 24-28 GW and at 30-32 GW; unless one of the tests give a positive result. Regardless of if the women are at risk or not, a confirmed suspicion of gestational diabetes is further checked to a confirmed diagnosis in the oral glucose tolerance test in the following check-up (cut-offs for positive results of both tests stated in *Material and methods* point *3.1*). The participant results of glucose challenge tests, and the oral glucose tolerance test if there is any, are recorded in databases.

Complete haematological profiles (including full blood count) from blood samples collected for routine prenatal check-ups are recorded.

Date of birth, gestational age at labour, birth weight, placenta weight and newborn sex, are recorded. Adverse pregnancy outcomes such as miscarriages, stillbirths and foetal malformations are also recorded.

1.4. IUGR cases recruited at labour

To increase the sample size in some of the placenta analyses, participants recruited at labour and that were suspected IUGR cases were included. Other differences from the usual RTBC participants recruited at ≤12 GW are the followings:

- Only one lifestyle questionnaire was made at the postpartum, and referred to the whole pregnancy time.

- Only the blood samples at labour were collected.

2. Biochemical and genetic determinations

2.1. Blood biochemical determinations

Plasma samples were sent frozen to BeVital A/S laboratories (Norway) as 0.5mL aliquots on dry ice for tHcy, folate, betaine, DMG, cobalamin and cotinine determinations as well as whole blood diluted with ascorbic acid solution for RBC folate determinations. All samples from the same pregnancy were analysed in the same batch in order to avoid the effects of inter assay variability on determinations that may affect changes in longitudinal samples. In order to minimise any potential preanalytical variability due to storage, the samples were kept at -80ºC and analysed, in batches, within 18 months after collection.

The determination of plasma creatinine was carried out at the Faculty of Medicine and Health Sciences, URV (Spain).

Plasma total homocysteine determination

Total homocysteine was determined by Gas Chromatography - Mass Spectrometry (GC-MS) in BeVital analytical platform A. Briefly, all forms of homocysteine bound to other molecules by disulphide bonds are reduced with dithioerythritol, which results in free reduced homocysteines. The mixture of originally free reduced homocysteine plus the dithioerythritol-reduced homocysteine is called total homocysteine, which is assayable in the present determination. Total homocysteine is derivatised and extracted in one step with methylchloroformate and toluene, respectively. Finally the derivatives are determined with GC-MS (Windelberg et al. 2005). The within-day and between-day coefficient of variation are 1% and 2%, respectively. The lower limit of detection is 0.1µmol/L (BeVital A/S 2016).

Plasma and red blood cell folate determination

Plasma and red blood cell folates were determined by automated microbiological assay with the chloramphenicol-resistant *Lactobacillus casei* strain NCIB 10463 (Molloy and Scott 1997) in BeVital analytical platform F. This assay allows the use of disposable laboratory ware and removes the need for autoclaving due to the resistance of the strain. The principle of this assay is the increase in turbidity in parallel to the *L. casei* growth, which depends on the concentration of folate forms in the media. As a clarification, for the case of RBC folate, the concentration obtained in this assay is indeed the ascorbic acid lysated whole blood folate concentration, and is converted to RBC folate concentration as specified in *Material and methods* point *3.2*. This assay is widespread used and considered superior than protein-binding assays, and the WHO has recommended it for RBC folate determination (World Health Organization 2015). The plasma folate within-day and between-day coefficient of variation are 4% and 5%, respectively, and the lower limit of detection is 2nmol/L (BeVital A/S 2016).

Plasma betaine, dimethylglycine and choline determination

Betaine, DMG and total choline were determined by Liquid Chromatography - tandem Mass Spectrometry (LC-MS/MS) in BeVital analytical platform C. Briefly, a protein precipitation step with acetonitrile is carried out. Then high performance liquid chromatography (HPLC) with a Fortis Phenyl column (Fortis Technologies) and a mobile phase of methanol and acetic acid is performed, prior to the tandem mass spectrometry (Midttun et al. 2013). The betaine within-day and between-day coefficient of variation are 6-7% and 8-10%, respectively and the lower limit of detection is 0.5µmol/L. The DMG within-day and between-day coefficient of variation are 2.8-3.4% and 4.5-5.6%, respectively and the lower limit of detection is 1.25µmol/L. The choline within-day and between-day coefficient of variation are 3% and 4%, respectively and the lower limit of detection is 1µmol/L (BeVital A/S 2016).

Plasma cobalamin determination

Plasma cobalamin was determined by automated microbiological assay with the colistin sulphate-resistant *Lactobacillus leichmannii* strain NCIB 12519 (Kelleher and Broin 1991) in BeVital analytical platform F. In this assay aseptic precautions are not necessary due to the resistance of the strain. The effect of antibiotic treated patient samples on the assay is removed by adding β-lactamase enzyme to the media. The principle of this assay is the increase in turbidity in parallel to the *L. leichmannii* growth, which depends on the concentration of cobalamin forms in the media. The within-day and between-day coefficient of variation are 4% and 5%, respectively. The lower limit of detection is 30pmol/L (BeVital A/S 2016).

Plasma cotinine determination

Plasma cotinine was determined by Liquid Chromatography - tandem Mass Spectrometry (LC-MS/MS) in BeVital analytical platform D. Cotinine is a marker of tobacco exposure after up to 3-4 days ago (Benowitz 1996). Briefly, after a protein precipitation step with trichloroacetic acid, a LC-MS/MS is performed with a mobile phase containing a high concentration of acetic acid (Midttun et al. 2009). The within-day and between-day coefficient of variation are 2-3% and 6%, respectively. The lower limit of detection is 1nmol/L (BeVital A/S 2016).

Plasma creatinine determination

Plasma creatinine was measured by Jaffé method with the Cobas Mira analyser (Roche) and a commercial kit (Química Clínica Aplicada). Creatinine is a widely used measure of renal function (Perrone et al. 1992). The principle of this assay is the increase in absorbance as the coloured compound is formed when creatinine and picric acid react. Inter-assay and intra-assay coefficients of variation are 2.11% and 1.72%, respectively.

2.2. Genetic determinations

Lyophilised aliquots of 120ng of total leukocyte DNA were sent to BeVital for polymorphism determinations.

The SNPs determined for this thesis were *MTHFR* c.665C>T (rs1801133), *BHMT* c.716G>A (rs3733890), *SLC19A1* c.80G>A (rs1051266) and *MTRR* c.66A>G (rs1801394). The technique is a high-level multiplex genotyping by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-TOF MS), as described (Meyer et al. 2004). Briefly, the DNA regions containing the SNPs of interest are amplified using

multiplex PCR in the first step. Then primers of one sense anneal immediately downstream from the polymorphic position to the amplicons of the previous step, and to these primers one more nucleotide (a dideoxynucleotide) is added in a DNA polymerase reaction (primer extension reaction with Pin-Point format). That is, for a specific SNP, a common homozygote participant will have one type of extended primers, a variant homozygote the other type of extended primers, and a heterozygote both types. These primers, which are purified prior to the MALDI-TOF MS, are soft ionized with a laser beam (MALDI). In the mass analyser, the time that these ionized primers take to cross a drift tube is measured to calculate the mass-to-charge ratio (TOF). Finally, the signal of each primer (with its own mass-to-charge ratio) is recorded and amplified in the detector, which sends this information to the data processor to calculate the abundance of each primer (Griffin and Smith 2000; Meyer et al. 2004).

2.3. Placenta chemical determinations

Prior to the trace element determinations by Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) or Inductively Coupled Plasma - Optic Emission Spectrometry (ICP-OES), placenta samples were washed and dissected to obtain clean and representative tissue blocks, and digested with nitric acid (HNO_3) and hydrogen peroxide (H_2O_2).

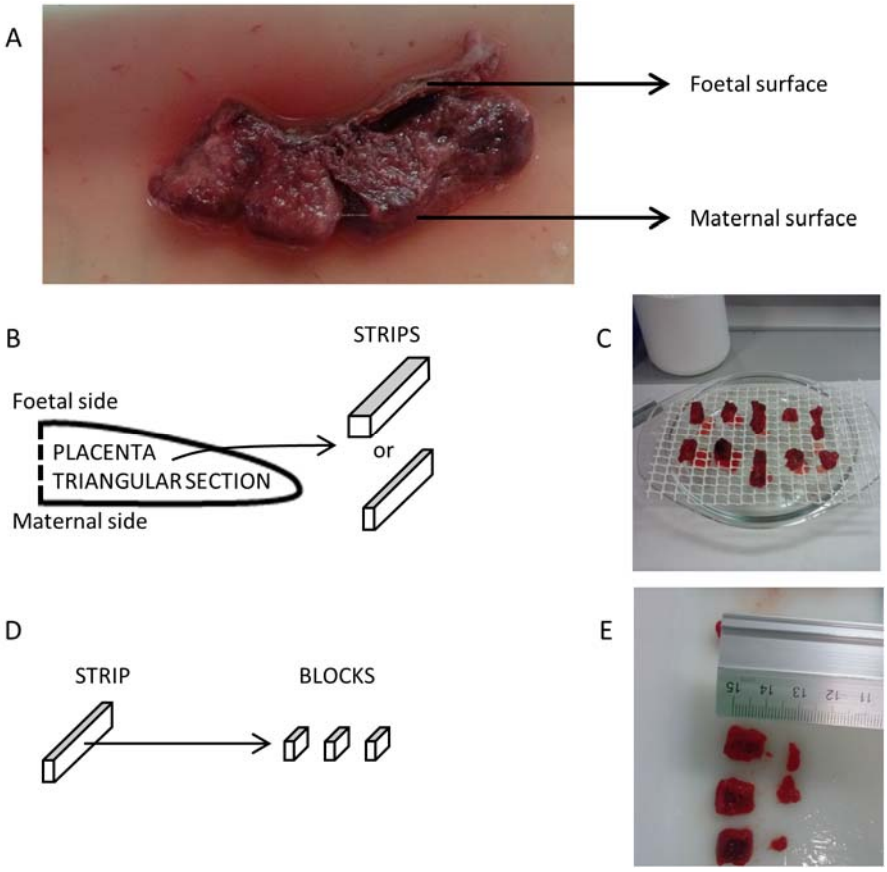
Washing and dissection of placenta samples

This step was carried out in the Faculty of Medicine and Health Sciences (URV). The triangular sections described in *Material and methods* point *1.2.2* were defrosted at room temperature in their closed screw-capped flask. The section was placed in a plastic tray and washed thoroughly with deionised water (H_2O) from a wash bottle, using stainless steel Adson and dissection forceps to handle the tissue (**Figure 15. A**).

With a scalpel sleeve with a disposable stainless steel blade, strips of 1cm thick, 1 or 0.5cm wide and a varying length were dissected from the foetal side (**Figure 15. B**). The longer the section, the bigger the possibilities of doing more representative blocks out of one triangular section, but this length depends on the triangular section (see point *1.2.2*) and on the suitable parts of this section, as explained below. Part containing features such as possible infarctions or calcifications, were avoided in an effort to keep the representativeness of the section for metal concentration determination. The strips were washed with deionised water and as much water as possible was removed shaking the strip off and with gentle taps in the borders of the tray. The strips were left to dry a little at room temperature being placed in a plastic grid over a glass plate for ≈40 minutes (**Figure 15. C**).

On the dry plastic tray, blocks of ≈0.5cm^3 volume and 0.5cm^2 foetal surface were cut from the strips: 0.5x1x1 or 1x0.5x1cm (long, wide, thick). This is shown in **Figure 15. D**. A ruler was used to measure the dimensions of the strips and blocks, but there was no contact between the ruler and the sample or work surfaces (**Figure 15. E**). The blocks were stored at -20ºC in acid-washed polypropylene tubes.

Figure 15. Washing and dissection of placenta samples.



A. Side view photograph of a placenta triangular section. B. Side view scheme of a placenta triangular section and the two possible types of foetal side strips (1 or 0.5cm wide). The amnion is illustrated in grey. C. Photograph of placenta strips drying on a plastic grid at room temperature for ≈40 minutes. D. Scheme of the representative blocks made from the placenta strip. E. Photograph of 3 blocks and discarded tissue.

All placentas were processed in this way in several batches of placenta triangular sections washing and dissecting, between 14/10/2015 and 19/01/2016. On average, 10 placenta triangular sections can be processed in one day in two batches of 5 placenta triangular sections. The forceps were washed with deionised H_2O between each placenta. The plastic grid, tray and other material is washed with deionised water between each batch, and acid-washed weekly. The procedure of this acid-washing,

which is also used in the polypropylene tubes for the storage of the blocks, is detailed in the following point *Acid digestion*. The washing of the material with deionised water and the positioning of the strips on the tray and grid ensure no contact between placentas from different participants occurs. Also, there was no use of paper in the procedure because paper can potentially contaminate the samples (Rankama 1941).

We consider that the placenta blocks obtained from this procedure are representative of the foetal side of the placenta and similar among them. They contain the chorionic plate with the amnion on top of it (foetal surface), plus the chorionic villi closest to the foetal side; and no *decidua basalis* (maternal surface). They have a similar proportion of foetal surface - volume of the inner part of the placenta; although the proportion of volume of the chorionic plate chorion - volume of chorionic villi could vary according to the thickness and development of each placenta. Keeping surface-volume proportion, selecting apparently healthy placenta areas and/or areas without features that could affect trace element content, excluding hollows, excluding *decidua basalis* in placentas of small thickness, and obtaining blocks which do not separate into smaller pieces; make this step hard, with usually only about 3 representative blocks obtained out of a triangular section. All the blocks came from placenta strips that have undergone a similar procedure, i.e. washing and time on the grid; though depending on the dimensions of the strip (1 or 0.5cm wide) more or less tissue is exposed to the washing and drying, and this could affect for example to the blood content of the subsequent blocks.

Acid digestion

This step was performed in the Faculty of Medicine and Health Sciences (URV). Each of the aforementioned clean and representative foetal side placenta blocks were defrosted at room temperature and weighed in the base of polytetrafluoroethylene (PTFE or

Teflon®) vessel on an analytical balance with a precision of 0.001g. In an fan extraction hood, 1.5ml 30% H_2O_2 (Merck) and 2ml 65% suprapure HNO_3 (Merck) were added and the vessels were closed with the screw lids. Certified reference material to digest were prepared with 1ml resuspended Trace Elements Whole Blood L-2 (Seronorm) and 1.5ml H_2O_2 and 2ml HNO_3. Digestion blanks were prepared with 1.5ml H_2O_2 and 2ml HNO_3. The characteristics of an average digestion batch are shown in **Table 8**. The total number of digestion batches was 9, between 30/11/2015 and 29/02/2016.

Table 8. Average digestion batch characteristics.

	Sample	**H_2O_2 (ml)**	**HNO_3 (ml)**	**Number**	**Temperature (ºC)**	**Time (h)**	**Final volume (ml)**
Placenta	≈0.5cm³	1.5	2	25-27	90	24	50
Reference material	1ml	1.5	2	3	90	24	50
Blank	-	1.5	2	4	90	24	50

The PTFE vessels were placed in an oven 24h at 90ºC. In all the batches a yellowish transparent digestate was obtained from the placenta blocks and reference material. When the vessels had cooled, the digestates were diluted with deionised water to a final volume of 50ml in volumetric flasks under the extraction hood (**Figure 16**). Two polypropylene tubes of 10ml from each diluted digestate were obtained. One was sent to the Scientific and Technological Centers - University of Barcelona (Centres Científics i Tecnològics - Universitat de Barcelona, CCiT-UB) for the trace element determination, and the other was kept at -20ºC in the Faculty of Medicine and Health Sciences (URV).

Figure 16. Digestate dilution.

Photograph of some of the material used in the acid digestion. At the front are four volumetric flasks with diluted placenta digestates. Behind the flasks there are the PTFE vessels. Behind the vessels there are several plastic containers for washing the material.

The surface of all plastic and glassware used in the acid digestion is acid washed by the following process: 2 washes with distilled H_2O > 2 washes with deionised H_2O > overnight in 10% diluted suprapure HNO_3 > 2 washes with distilled H_2O > 2 washes with deionised H_2O. The PTFE vessels cleaning is more thorough: hot tap water wash > 15 minutes sonication wash with distilled H_2O and phosphate free detergent > tap water wash > distilled H_2O wash > 8h room temperature and 8h 90ºC acid digestion with 10% diluted suprapure HNO_3 > distilled H_2O wash > deionised H_2O wash.

There are several forms of acid digestion depending on the acid or acids used with or without H_2O_2 as another oxidation reagent, and on the time and source of heat plus temperature degree. The purpose of all types of acid digestion is to eliminate the organic matter or convert it to simpler inorganic forms in solution (Bolann et al. 2007).

If this is achieved, interferences in the matrix (e.g. placenta) are expected to be minimised allowing the further trace element determination techniques to run properly, e.g. without differences in sample digestate and blank digestate transport through the inductively coupled plasma.

In relation to the reagents used (HNO_3 and H_2O_2) a comment can be made. Although analysing samples with a slightly different treatment (wet ashing, which uses open vessels), a study has reported better trace element recoveries from bovine liver analysed by inductively coupled plasma - optic emission spectrometry (ICP-OES) when using HNO_3 with perchloric acid ($HClO_4$) than HNO_3 with H_2O_2 (Ward et al. 1980). However, $HClO_4$ requires more specialised handling due to its higher hazard level; and when analysing via inductively coupled plasma - mass spectrometry (ICP-MS), $HClO_4$ can form the polyatomic ion ClO^+ causing spectral interferences in the technique (Subramanian 1996).

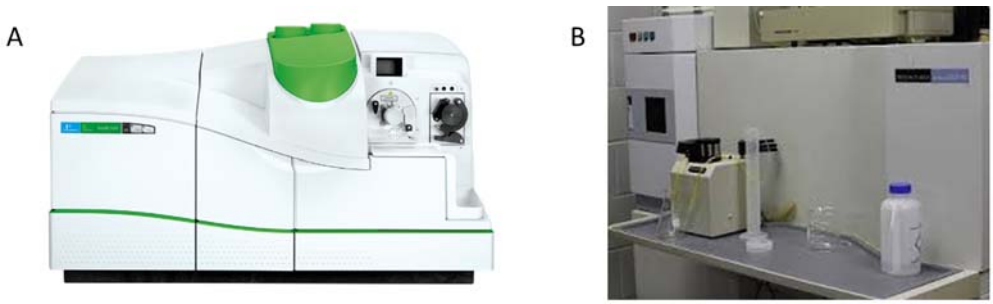
Regarding the used PTFE vessels, it is worth noting the closure is not completely hermetic, therefore a small loss of volatile elements such as mercury can take place. Also, although vessels made of PTFE are of widespread use, some authors consider quartz vessels can achieve better digestions due to its resistance to temperature and pressure (Subramanian 1996). However, quartz vessels were not available for the study.

As for the digestate dilution step, two main reasons account for it. First, a concentration of ≤5% HNO_3 was requested to rise the pH to non-detrimental levels for the ICP equipments. And second, matrix interferences of physical nature can occur in the trace element determination technique (especially in ICP-MS) because of a high concentration of dissolved solids (Bolann et al. 2007).

Inductively Coupled Plasma - Mass Spectrometry

This step was carried out in the CCiT-UB using a NexION 350D ICP-MS spectrometer (PerkinElmer) (**Figure 17. A**). Placenta zinc, copper, selenium, iron, cadmium, mercury, arsenic, vanadium, chromium, lead, nickel, and manganese concentrations are determined in the diluted digestates with this technique. The instrument detection limits and the method detection limits for each element are shown in **Table 9**. *Material and methods* point *3.2* includes the calculations to obtain: method detection limit, placenta trace element concentrations (in wet weight, ww), reference material trace element concentrations and recovery percentage. The trace element analysis by ICP-MS was in two consecutive days (08/03/2016 and 09/03/2016).

Figure 17. Trace element analysis equipment.



A. NexION 350D ICP-MS spectrometer. B. Optima 3200RL ICP-OES spectrometer.

Table 9. Instrument and method lower detection limits.

	Zn	Cu	Se	Fe	Cd	Hg	As	V	Cr	Pb	Ni	Mn
IDL[1]	1	1	1	0.01	0.05	0.2	0.2	1	0.5	0.1	0.2	0.5
MDL[2]	100	100	100	1	5	20	20	100	50	10	20	50

[1]IDL. Instrument detection limit (ng/ml; except for iron µg/ml).
[2]MDL. Method detection limit (ng/cm^3 wet placenta; except for iron µg/cm^3).

The sample was hard ionized, i.e. sample molecules gained charge but were also broken into ionic products; by hot argon-plasma activated by a high-voltage field (ICP). The generated sample ions were accelerated to the mass analyser using an electric field. The mass analyser separated each ion type according to its mass-to-charge ratio, and a detector recorded and amplified the signal of each of the sample ions (MS) (Klotz et al. 2013). With the intensity of the signal, a data processor can calculate the abundance for each trace element. It is worth noting the NexION 350D ICP-MS spectrometer has a helium collision reaction cell to avoid spectral interferences; e.g. the polyatomic interferences of chlorine with arsenic and vanadium, and of calcium with nickel. ICP-MS is considered a recommended method to analyse in placenta zinc, copper, selenium, cadmium, mercury, arsenic, vanadium, chromium, lead, and nickel, but not manganese (Iyengar and Rapp 2001a).

Inductively Coupled Plasma - Optic Emission Spectrometry

This step was carried out in CCiT-UB using an Optima 3200RL ICP-OES spectrometer (PerkinElmer) (**Figure 17. B**). Diluted digestates iron concentration was determined with this technique. The Instrument Detection Limit and the Method Detection Limit for iron is shown in **Table 9**. As stated for ICP-MS, the calculations are included in *Material and methods* point *3.2*. The iron analysis by ICP-OES was in two consecutive days (14/03/2016 and 15/03/2016).

The sample was hard ionized by hot argon-plasma activated by a high-voltage field (ICP). Then, each excited ion emits several wave length light signals depending on which element it is. This optic emission was measured and with the intensity of the signal, the abundance of each element was calculated (OES) (Klotz et al. 2013). ICP-OES (also called ICP-AES, Inductively Coupled Plasma - Atomic Emission Spectrometry) is considered a recommended method to analyse iron in placenta (Iyengar and Rapp 2001a).

3. Data handling and analysis

3.1. Definitions of exposures and outcomes

Socioeconomic status

Maternal and paternal occupations are grouped into categories as described (Álvarez-Dardet et al. 1995). Index pregnancies are classified in low, mid and high socioeconomic level based on the maternal and paternal occupation categories and the household income.

Smoking status

Participants were categorised as first trimester smokers if plasma cotinine was >10ng/mL in the ≤12 GW blood sample, if smoking during the first trimester was declared in the habits and lifestyle questionnaires or if it was recorded in their obstetrical history. Women that continued to smoke throughout pregnancy were identified by plasma cotinine >10ng/mL in the ≤24-27 GW blood sample or in the cord. Declaration of continued smoking in the second lifestyle questionnaire or a record of

continued smoking on the obstetrical history were also used to identify smokers throughout pregnancy.

Maternal first term BMI

Maternal first term BMI (kg/m^2) was calculated as first term weight (kg) divided by height squared (m^2).

Pregnancy induced hypertension

Pregnancy induced hypertension was defined in the study with the following criteria: a systolic blood pressure measure greater or equal to 140mmHg and/or a diastolic blood pressure greater or equal to 90mmHg at least in two check-ups (6 hours apart) after the 20^{th} GW.

Gestational diabetes

Gestational diabetes participants was defined in the study as those with a positive result in any of the glucose challenge tests (serum glucose ≥140mg/dl) and positive results in at least two of the four time points of the oral glucose tolerance test (plasma glucose ≥105mg/dl at 0', ≥190mg/dl at 60', ≥165mg/dl at 120', ≥145mg/dl at 180').

Late pregnancy anaemia

Late pregnancy anaemia was defined as haemoglobin concentration <11g/dl at 34 GW and/or at labour.

Birth weight percentiles and IUGR

Birth weight percentile was recorded using Spanish growth curves and based on sex, gestational age at births in completed weeks (Santamaría et al. 1998). Newborns with birth weight below the P_{10}, i.e. SGA (World Health Organization 2016), were considered as IUGR cases for the study. In the placenta trace element study, the controls compared to the IUGR cases were those whose birth weight is above the P_{25}.

Preterm newborns

Preterm newborns were defined as those born before the 37 GW (World Health Organization 2016). As gestational age is an estimated variable, the controls compared to the preterm cases in the placenta trace element study were those of ≥38 GW.

Miscarriages and stillbirths

Miscarriage is the loss of pregnancy due to natural causes before the 20th week of pregnancy (National Institutes of Health 2016a). If this loss is at ≥20GW, and also by natural causes, it is termed stillbirth (National Institutes of Health 2016a).

3.2. Applied formulae

RBC folate

RBC folate concentrations are calculated as ascorbic acid lysated whole blood folate concentration multiplied by the dilution factor (10) and divided by haematocrit (as a decimal) (Molloy and Scott 1997).

Trace element analysis method detection limit

The method detection limit (MDL) is estimated for each trace element as follows:

$$MDL = IDL \times \frac{dilution}{block\ volume} = IDL \times \frac{50ml}{0.5cm^3}$$

The instrument detection limit (IDL) is provided by the CCiT-UB team. The dilution is the final volume to which the digestates have been diluted in all the batches. To relate the detection limit to the placenta samples, the aimed volume of one representative block is used because the block weight was more variable.

Placenta trace element concentration

First, in each acid digestion batch the arithmetic mean of each trace element concentration in the diluted blanks digestates is calculated. Whenever this blank mean is below zero because of negative signals, the blank mean is imputed as zero (upper bound).

For each digestion batch, placenta and trace element, a background subtraction is done: the diluted blank digestate mean is subtracted from the placenta diluted digestate trace element concentration. Whenever this difference is below the detection limit (as IDL), the value is imputed as half the detection limit (middle bound), and the placenta is categorised as below the detection limit for a specific trace element.

To get the placenta trace element concentration in function of the tissue wet weight (ng/g ww, except for iron µg/g ww), the result of the background subtraction is

multiplied by the final volume to which the digestates have been diluted, and divided by the digested block wet weight:

$$Placenta\ element\ concentration = (diluted\ digestate\ element\ concentration - blank\ mean) \times \frac{dilution}{block\ wet\ weight}$$

Certified reference material trace element concentration and percentage recovery

To get the reference material trace element concentration in function of the digested volume (ng/ml, except for iron µg/ml), the calculation is done as in the placenta trace element concentration, including the imputation as half the detection limit (middle bound) when the background subtraction result is below the detection limit; but dividing by the reference material digested volume (1ml) instead of the placenta weight:

$$Reference\ material\ element\ concentration = (diluted\ digestate\ element\ concentration - blank\ mean) \times \frac{dilution}{1ml}$$

For each digestion batch, the mean of the three reference material trace element concentrations is calculated. Then the recovery percentage of each batch is calculated as the obtained reference material trace element concentration mean divided by the expected reference material trace element concentration according to the manufacturer, and multiplied for 100:

$$Percentage\ recovery\ (\%) = \frac{obtained\ reference\ material\ element\ concentration}{expected\ reference\ material\ element\ concentration} \times 100$$

The mean recovery percentage is calculated as the arithmetic mean of the recovery percentages of all the acid digestion batches.

Criteria for the selection of trace elements to address in this thesis

Out of the twelve trace elements analysed in placenta, i.e. zinc, copper, selenium, iron, cadmium, mercury, arsenic, vanadium, chromium, lead, nickel and manganese, a screening according to two methodological criteria was used. For each trace element, these criteria were: percentage of placentas below limit of detection (LOD, as MDL) lower than 40%, and recovery percentage within a range of 80-110%. RTBC placenta zinc, copper, selenium, iron, cadmium, mercury, arsenic, vanadium, chromium, lead, nickel and manganese concentrations, the percentage of placentas below LOD for each trace element, and the percentage recoveries for each trace element; are reported in **Table 10**. In the screening, cadmium, mercury, arsenic, vanadium, chromium, lead and nickel concentrations were below the LOD in a percentage greater than 40% of the placentas; while vanadium and manganese had a percentage recovery too high. The selected trace elements in the screening were zinc, copper, selenium and iron. These four trace elements were present in high concentrations in placenta and might be modulated by one carbon metabolism analytes as detailed in *Introduction* point *5*. It is worth noting that trace elements known to affect foetal development and/or to be affected by one carbon metabolism, such as arsenic, were also excluded from this selection. Arsenic can be harmful in the placenta and foetus because it is at higher concentrations in placentas of LBW neonates (Llanos and Ronco 2009), associated with lower birth weight in mice studies (Gutiérrez-Torres et al. 2015), and known to lower SAM concentration and DNA methylation *in vitro* (Zhao et al. 1997; Reichard et al. 2007). The arsenic-induced 1C metabolism imbalances can be due to the necessity of methylate the metal for a faster excretion (Gebel 2002). In line with this, homocysteine, folate and cobalamin are associated with arsenic and its methylated products, according to human studies in pregnancy (Hall et al. 2007), adulthood (Gamble et al.

2005; Gamble et al. 2006; Gamble et al. 2007; Hall et al. 2009a; Peters et al. 2015) and childhood (Hall et al. 2009b).

Table 10. RTBC placenta trace element concentrations, percentage of placentas below limit of detection (n=212) and percentage recovery.

Trace element	Median[1] (P_{25}, P_{75})	Arithmetic mean[1] (95% CI)	Percentage below LOD[2]	Percentage recovery[3]
Zinc	6809 (6036, 7378)	6802 (6670, 6935)	0.0	84.8
Copper	534 (476, 596)	552 (534, 569)	0.0	89.6
Selenium	82.2 (52.1, 117.8)	88.7 (82.7, 94.6)	37.7	86.7
Iron	62.9 (49.2, 77.2)	64.8 (61.9, 67.8)	0.5	90.5
Cadmium	2.9 (1.9, 4.1)	3.3 (3.0, 3.5)	61.3	91.5
Mercury	7.8 (6.1, 10.7)	8.7 (8.2, 9.3)	93.9	96.9
Arsenic	7.4 (6.0, 9.8)	8.1 (7.7, 8.5)	99.5	89.2
Vanadium	37.0 (29.9, 48.1)	40.3 (38.3, 42.3)	100.0	426.1
Chromium	18.8 (15.0, 26.1)	22.5 (20.6, 24.3)	94.8	107.1
Lead	4.8 (3.3, 10.6)	16.0 (10.9, 21.1)	67.0	90.7
Nickel	8.5 (6.2, 21.0)	40.4 (15.3, 65.5)	70.8	103.8
Manganese	54.3 (46.5, 66.0)	55.8 (53.4, 58.2)	17.9	207.1

[1]All placenta median (P_{25}, P_{75}) and arithmetic mean (95% confidence interval) trace element concentrations are reported as ng/g of placenta wet weight (ww); except for iron: µg/g of placenta ww. [2]LOD, Limit Of Detection, as lower instrument detection limit. [3]Trace element recovery percentages of the certified reference materials (arithmetic mean of the 9 batches where the RTBC placenta samples were digested).

Dietary energy and trace element intake

The second 45-item food frequency questionnaire was used to evaluate dietary habits and energy intake during pregnancy (Jean-Claude et al. 1995; Rodríguez et al. 2008). As the reference database used for this estimation includes data on iron food composition, dietary iron intake was estimated according to it. However, it does not contain data on zinc, copper and selenium food composition (Jean-Claude et al. 1995; Rodríguez et al. 2008). For estimating zinc, copper and selenium intake the French Agency for Food, Environmental and Occupational Health & Safety/Ciqual French food composition table version 2013 was used (ANSES 2013).

3.3. Statistical analysis

Specific details on the statistical tests applied are reported where relevant throughout the results section. This section describes the general statistical tests used and analytical strategies.

Variables with skewed distributions were natural log transformed for analyses using parametric tests (ANOVA, ANCOVA, Student's t-test, the dependent variables in multiple linear regression analysis). In these cases geometric means (95% CI) are reported. Frequencies are reported as % (95% CI). Hardy-Weinberg equilibrium of the observed allele frequencies was determined by CubeX (Gaunt et al. 2007). Frequencies were compared between groups using the chi square test. Plasma concentrations of 1C metabolism nutrients or metabolites were compared between different genotypes using ANCOVA. Covariables included in the models were gestational age at the time of blood draw. Interactions between genotypes and plasma concentration of 1C metabolism nutrients or metabolites were determined by including the product genotype*plasma concentration as a covariable in the model. When a significant interaction was observed, a stratified analysis was carried out and the effect reported for individual genotypes or concentration level (for example tertiles). Plasma nutrient /

metabolite concentrations and RBC folate were compared throughout pregnancy with the earliest determinations at ≤12 GW or with the previous time point using 2-factor ANOVA for repeated measures (intersubject factor: gestational age at blood draw; intrasubject factor: genotype). Post-hoc Bonferoni correction of P values for multiple comparisons was applied.

When examining the *BHMT* c.716G>A polymorphism, the betaine-DMG pathway and the *SLC19A1* c.80G>A polymorphism according to folate status, folate status was categorised at 2 different points of pregnancy to allow for changes in status due to the cessation of folic acid supplement use. When testing the effects of low or high folate status during the most intense phase of supplement use (at ≤12 GW and at 15 GW), it was classified as low when plasma folate was in the lowest tertile at either of these time points and was classified as high when it was in the highest tertile at either of these time points. Similar criteria were applied in mid-late pregnancy to plasma folate determined at 24-27 or 34 GW.

The effects of folate status or *BHMT* c.716G>A genotypes on changes in plasma DMG throughout pregnancy were determined using multiple linear regression analysis. The dependent variables were natural log transformed so the model showed the percentage variability in the dependent variable per unit change in the independent variable. The effects of maternal characteristics on placental trace element concentrations were also determined using multiple linear regression analysis. In all multiple lineal regression models tested, individual effects of independent variables were only considered valid if the global model was significant.

Correlations between 2 quantitative variables were determined using Spearman correlation coefficients.

Non-parametric tests, such as the Mann Whitney U test, were used to compare placenta trace element concentrations between groups of small size, such as between preterm cases and controls.

Multiple logistic regression analysis was used to test the probability of an IUGR affected pregnancy when placenta TE concentration was high. The dependent variable (IUGR) was categorical (case or control) whereas the independent variables were categorical (highest placenta trace element tertiles versus the other tertiles).

All analyses were performed using SPSS version 23.0 for windows and significance level was set at $P<0.05$.

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

GENETIC STUDY RESULTS

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

Genetic study results

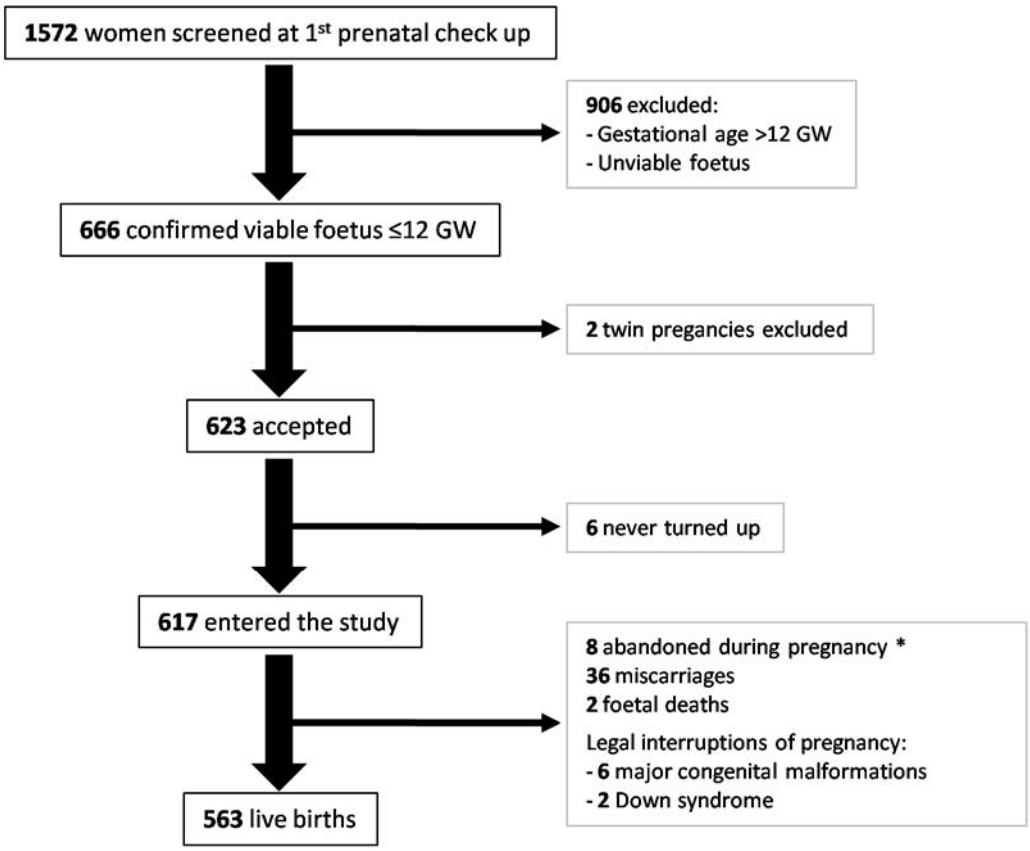
1. Participation in the study and descriptive characteristics of participants and offspring

Participation in the study by eligible candidates and completion (as live birth) is illustrated in **Figure 18**. Of the women screened at their first prenatal check-up, 42.4% were eligible to participate in the study. Of the 623 participants that accepted to enter the study (93.8% of those eligible) first trimester data was collected from 617 and 563 went on to have live births. Legal terminations of pregnancy were performed in 8 cases due to 1 neural tube defect, 3 congenital heart defects, 2 other foetal malformations and 2 Down syndromes. There were 2 foetal deaths.

Information regarding lifestyle, habits, supplement use and other basic participant characteristics is reported in **Table 11**. The prevalence of low socioeconomic status was 7.2% (95% CI: 5.3, 9.5). Although 80.8% (95% CI: 77.2, 83.9) of the pregnancies were declared as planned, only 34.2% (95% CI: 30.2, 38.3) of the women reported taking preconception folic acid supplements. Folic acid supplement use in the first trimester was reported by 92.4% (95% CI: 90.0, 94.2) of participants but only 53.1% (95% CI: 48.9, 57.3) continued taking folic acid supplements throughout pregnancy.

The median plasma concentrations of 1C metabolism nutrients and metabolites relevant to the thesis are shown in **Table 11**.

Figure 18. Participation in the study



RTBC flow chart of the pregnancy phase in women recruited between January 2005 and October 2014.
*Did not show up for expected prenatal check-ups for unknown reasons.

Table 11. Lifestyle, obstetrical, clinical and 1C metabolism status characteristics (n=617).

Age (y) [612][1]		32.1 (31.7, 32.5)
Body mass index at 1st trimester (kg/m^2) [578][1]		23.8 (23.5, 24.1)
Planned pregnancy [536][2]		80.8 (77.2, 83.9)
Previous pregnancy [607][2]		52.6 (48.6, 56.5)
Socioeconomic status [586][2,3]	High	43.5 (39.6, 47.6)
	Mid	49.3 (45.3, 53.4)
	Low	7.2 (5.3, 9.5)
Folic acid supplement use[2]	Preconception [530]	34.2 (30.2, 38.3)
	First trimester [604]	92.4 (90.0, 94.2)
	Throughout pregnancy [540]	53.1 (48.9, 57.3)
Smoking during pregnancy[2]	Periconception [612]	28.1 (24.7, 31.8)
	Throughout pregnancy [599]	17.0 (14.2, 20.2)
Regular alcohol consumption[2]	Periconception [526]	52.9 (48.6, 57.1)
	Throughout pregnancy [536]	13.6 (11.0, 16.8)
Illegal drug use[2]	Periconception [547]	2.6 (1.5, 4.2)
	Throughout pregnancy [547]	0.4 (0.1, 1.3)
1st trimester 1C nutrient status		
Plasma folate (nmol/L) [605][1]		33.8 (31.0, 36.6)
Plasma cobalamin (pmol/L) [605][1]		398.2 (374.0, 404.3)
Plasma total homocysteine (µmol/L) [605][1]		5.5 (5.3, 5.6)
Plasma choline (µmol/L) [603][1]		7.9 (7.7, 8.0)
Plasma betaine (µmol/L) [603][1]		22.4 (21.8, 23.0)
Pregnancy induced hypertension [535][2]		5.6 (4.0, 7.9)
Gestational diabetes [565][2]		6.9 (5.1, 9.3)
Late pregnancy anaemia [517][2]		26.7 (23.1, 30.7)
Gestational age at labour (weeks) [563][1]		39.0 (38.9, 39.1)
Birth weight (g) [563][1]		3218.3 (3180.6, 3256.0)
Offspring male sex [563][2]		48.8 (44.7, 53.0)
Preterm delivery [573][2]		4.4 (3.0, 6.4)
IUGR [544][2]		8.1 (6.1, 10.7)
Miscarriage [617][2]		5.9 (4.3, 8.1)

Values are [1]arithmetic means or [2]percentages (95% confidence interval) [sample size]; [3]based on total income, occupation and education level of both parents.

2. Genotype and allele frequencies of the polymorphisms

The genotype and allele frequencies of the *MTHFR* c.665C>T, *BHMT* c.716G>A, *SLC19A1* c.80G>A, *MTRR* c.66A>G polymorphisms for the mothers and neonates (by cord blood) are reported in **Table 12**. The reported genotype frequencies were in Hardy-Weinberg equilibrium (P>0.05).

Table 12. Genotype and allele frequencies in the RTBC study.

		Mother	Cord
***MTHFR* c.665C>T**		603[1]	433
	CC	34.0 (30.3, 37.9)[2]	36.3 (31.9, 40.9)
	CT	48.6 (44.6, 52.6)	45.4 (40.9, 50.2)
	TT	17.4 (14.6, 20.6)	18.2 (14.9, 22.2)
	C	58.3 (55.5, 61.0)	59.0 (55.7, 62.2)
	T	41.7 (39.0, 44.5)	41.0 (37.8, 44.3)
***BHMT* c.716G>A**		603	434
	GG	48.9 (45.0, 52.9)	43.1 (38.5, 47.8)
	GA	40.5 (36.6, 44.4)	48.6 (43.9, 53.3)
	AA	10.6 (8.4, 13.3)	8.3 (6.1, 11.3)
	G	69.2 (66.5, 71.7)	67.4 (64.2, 70.4)
	A	30.8 (28.3, 33.5)	32.6 (29.6, 35.8)
***SLC19A1* c.80G>A**		600	429
	GG	27.7 (24.2, 31.4)	27.0 (23.1, 31.4)
	GA	45.3 (41.4, 49.3)	46.2 (41.5, 50.9)
	AA	27.0 (23.6, 30.7)	26.8 (22.8, 31.2)
	G	50.3 (47.5, 53.2)	50.1 (46.8, 53.5)
	A	49.7 (46.8, 52.5)	49.9 (46.5, 53.2)
***MTRR* c.66A>G**		599	431
	AA	28.9 (25.4, 32.6)	27.8 (23.8, 32.3)
	AG	44.9 (41.0, 48.9)	45.5 (40.8, 50.2)
	GG	26.2 (22.8, 29.9)	26.7 (22.7, 31.1)
	A	51.3 (48.5, 54.2)	50.6 (47.2, 53.9)
	G	48.7 (45.8, 51.5)	49.4 (46.1, 52.8)

Values are [1]sample size; [2]percentages (95% confidence interval). Variations in sample size for SNP determinations in some cases is due to insufficient DNA for the genotyping of multiple SNPs.

3. *MTHFR* c.665C>T polymorphism, plasma and RBC folate, and plasma tHcy

Plasma and RBC folate and tHcy throughout pregnancy and in cord blood were compared among the maternal *MTHFR* c.665C>T genotypes (**Table 13**). As explained in *Material and Methods* point *1.2*, RBC folate was not determined for samples collected at labour. There were no differences in plasma folate among genotypes at any time of pregnancy. RBC folate was lower in participants with the *MTHFR* c.665TT genotype throughout pregnancy with the exception of at 15 GW. THcy was higher in the *MTHFR* c.665TT genotype compared to the other genotypes throughout pregnancy and in cord. It was also higher in the *MTHFR* c.665CT genotype compared to the *MTHFR* c.665CC genotype at ≤12GW.

There were no differences in the longitudinal changes of plasma and RBC folate, and tHcy among the *MTHFR* c.665CT genotypes. Plasma folate decreased between 15 and 24-27, and between 24-27 and 34 GW. RBC folate increased between ≤12 and 15 GW, and decreased between 15 to 24-27, and between 24-27 and 34 GW. Plasma tHcy decreased between ≤12 to 15 GW, and increased between 24-27 and 34 GW, and between 34 GW and labour.

RBC folate modified the association between the *MTHFR* c.665C>T genotype and tHcy at ≤12 GW (P for interaction: 0.013); plasma folate modified it at 15, 24-27 and 34 GW, although at 34 GW the significance was borderline (P for interaction: 0.058). Maternal plasma folate at labour also modified the association between *MTHFR* c.665C>T genotype and cord tHcy (P for interaction: 0.043).

Table 13. Plasma and RBC folate, and tHcy during pregnancy and in the cord according to maternal *MTHFR* c.665C>T genotypes.

	***MTHFR* c.665C>T genotype**	**≤12 GW**	**15 GW**	**24-27 GW**	**34 GW**	**Labour**	**Cord**
Plasma folate (nmol/L)	**CC**	26.7[1] (24.2, 29.3)	25.6 (23.0, 28.6)	13.4[a] (12.0, 14.9)	11.2[a] (9.9, 12.6)	11.4 (10.0, 12.9)	24.3 (22.3, 26.5)
	CT	24.6 (22.6, 26.8)	24.5 (22.4, 26.8)	13.0[a] (11.9, 14.2)	10.9[a] (10.0, 12.0)	10.4 (9.4, 11.5)	22.7 (21.1, 24.4)
	TT	27.1 (23.2, 31.6)	26.2 (22.4, 30.6)	13.3[a] (11.3, 15.6)	11.0[a] (9.2, 13.1)	10.4 (8.6, 12.6)	26.5 (23.4, 30.0)
ANCOVA[2] models		NS	NS	NS	NS	NS	NS
RBC folate (nmol/L)	**CC**	1025 (947, 1106)	1269[a] (1176, 1369)	1165[b] (1088, 1248)	943[a] (864, 1029)		
	CT	981 (920, 1046)	1267[a] (1188, 1353)	1104[a] (1035, 1177)	938[a] (876, 1005)		
	TT	863# (763, 976)	1200[a] (1053, 1367)	993* [b] (875, 1127)	806** † [a] (699, 929)		
ANCOVA[2] models		P=0.062	NS	P<0.05	P=0.073		

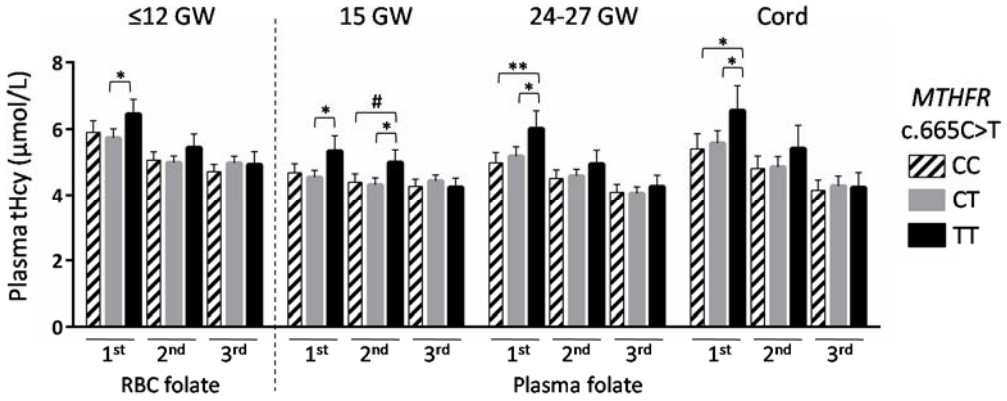
Table 13. Plasma and RBC folate, and tHcy during pregnancy and in the cord according to maternal *MTHFR* c.665C>T genotypes (continued).

	***MTHFR* c.665C>T genotype**	**≤12 GW**	**15 GW**	**24-27 GW**	**34 GW**	**Labour**	**Cord**
Plasma tHcy (µmol/L)	**CC**	5.12 (4.98, 5.27)	4.44[a] (4.32, 4.57)	4.51 (4.38, 4.66)	5.12[a] (4.94, 5.32)	5.98[a] (5.72, 6.25)	4.70 (4.50, 4.91)
	CT	5.24** (5.11, 5.39)	4.43[a] (4.31, 4.56)	4.58 (4.45, 4.73)	5.24[a] (5.08, 5.41)	6.12[a] (5.91, 6.34)	4.87 (4.70, 5.06)
	TT	5.83** †† (5.53, 6.14)	4.83** †† [a] (4.59, 5.07)	5.04*** †† (4.73, 5.37)	5.78*** †† [a] (5.40, 6.20)	6.83** †† [a] (6.33, 7.37)	5.30* (4.87, 5.76)
ANCOVA[3] models		P<0.01	P<0.001	P<0.001	P<0.001	P<0.001	P<0.05
Plasma folate-genotype interaction[4]		NS	P<0.001	P<0.001	P=0.058	NS	P<0.05
RBC folate-genotype interaction		P<0.05	NS	NS	NS		

GW: gestational weeks; NS: non-significant. Sample size: plasma folate (n = 598, 443, 512, 496, 479, 465; for ≤12, 15, 24-27, 34 GW, labour and cord, respectively), RBC folate (n = 559, 418, 499, 484; for ≤12, 15, 24-27, 34 GW, respectively) and plasma tHcy (n = 598, 443, 512, 496, 480, 465; for ≤12, 15, 24-27, 34 GW, labour and cord, respectively). Sample size varies between time points due to participant loss due to complications, non-attendance of programmed blood draw or delivery elsewhere or failure to collect blood samples. [1]Values are geometrical means (95% confidence interval). [2]Adjusting for gestational age at time of blood draw. [3]Adjusting for maternal folate status (RBC folate concentration at ≤12 GW and plasma folate concentration at the remaining time points) and gestational age at time of blood draw. ANCOVA Bonferroni posthoc: ***p<0.001, **p<0.01, *p<0.05, #p=0.06, versus CC; †††p<0.001, ††p<0.01, versus CT. [4]Based on maternal plasma folate concentration at labour for the cord tHcy-genotype interaction. Two-factor repeated measures ANOVA (intrasubject factor: gestational age; intersubject factor: *MTHFR* c.665C>T genotype) followed by post hoc Bonferroni correction for multiple comparisons: [a]p<0.001, [b]p<0.01, versus previous time point.

A stratified analysis of tHcy for each *MTHFR* c.665C>T genotype according to RBC folate or plasma folate tertiles is shown in those pregnancy time points where significant folate interactions on genotype-tHcy were observed (**Figure 19**). No differences in tHcy among genotypes were found in the highest tertile of RBC folate for ≤12 GW, or of plasma folate for 15, 24-27 GW and cord tHcy. Higher tHcy was observed in variant homozygotes than in normal homozygotes in the lowest plasma folate tertiles, at 24-27 GW and in the cord. Higher tHcy in variant homozygotes compared to heterozygotes was observed in the lowest tertile of RBC folate for ≤12 GW, or of plasma folate for 15, 24-27 GW and cord.

Figure 19. Plasma tHcy according to maternal *MTHFR* c.665C>T genotype and folate status tertiles.



Plasma tHcy at ≤12, 15, 24-27 GW and in cord, according to maternal *MTHFR* c.665C>T genotype and folate status: RBC folate tertiles at ≤12 GW, and plasma folate tertiles at 15, 24-27 GW and labour. GW: Gestational weeks; 1st, first tertile; 2nd, second tertile; 3rd, third tertile. At ≤12 GW, 1st: CC (n=56), CT (n=83), TT (n=42); 2nd: CC (n=60), CT (n=99), TT (n=27); 3rd: CC (n=70), CT (n=92), TT (n=26). At 15 GW, 1st: CC (n=50), CT (n=72), TT (n=23); 2nd: CC (n=49), CT (n=68), TT (n=30); 3rd: CC (n=47), CT (n=72), TT (n=29). At 24-27 GW, 1st: CC (n=62), CT (n=77), TT (n=31); 2nd: CC (n=55), CT (n=87), TT (n=22); 3rd: CC (n=56), CT (n=83), TT (n=31). With cord blood samples, 1st: CC (n=47), CT (n=72), TT (n=27); 2nd: CC (n=55), CT (n=76), TT (n=21); 3rd: CC (n=55), CT (n=70), TT (n=28). Values are geometrical means. Error bars represent 95% confidence interval. Comparison between genotypes were made using ANCOVA adjusting for gestational age at time of blood draw, and RBC folate at ≤12 GW or plasma folate concentration at 15, 24-27 GW and labour; with posthoc Bonferroni correction for multiple comparisons: *p<0.05, **p<0.01, #p=0.06.

4. *BHMT* c.716G>A polymorphism, plasma betaine, dimethylglycine, dimethylglycine/betaine ratio and tHcy

Plasma betaine, dimethylglycine (DMG), tHcy and DMG/betaine ratio according to maternal *BHMT* c.716G>A genotype throughout pregnancy and in cord blood are reported in **Table 14**. Betaine and tHcy did not differ among genotypes at any time of pregnancy. Lower plasma DMG was observed in the *BHMT* c.716AA compared to GG genotype in late pregnancy and in the cord. This was also true for the heterozygote genotype at ≤12, 15 GW and at labour. A lower DMG/betaine ratio was also observed in the heterozygote compared to homozygote common genotype at 15 GW and in the homozygote variant compared to homozygote common genotype at 34 GW.

In all of the *BHMT* c.716 genotypes, plasma betaine decreased between ≤12 and 24-27 GW and subsequently, remained unchanged for the rest of pregnancy. Plasma DMG initially decreased between ≤12 and 15 GW, plateaued and started increasing from 24-27 GW in the *BHMT* c.716GG and GA genotypes but not until 34GW in the AA genotype. The DMG/betaine ratio increased steadily throughout pregnancy, except in the case of the *BHMT* c.716AA genotype where there was no change between 15 and 34 GW. The pattern of change in tHcy followed the same U shape in all *BHMT* c.716 genotypes.

Interactions between plasma folate tertile based category and the effect of *BHMT* c.716G>A genotype on DMG were observed at ≤12 and 15 GW and on the DMG/betaine ratio at ≤12 GW. Plasma folate category also modified the association between the *BHMT* c.716G>A genotype and plasma tHcy at ≤12, 34 GW, labour and in the cord (P for interactions: 0.037, 0.056, 0.057 and 0.019, respectively).

Table 14. Plasma betaine, dimethylglycine, tHcy and dimethylglycine/betaine ratio during pregnancy and in the cord according to maternal *BHMT* c.716G>A genotypes.

	***BHMT* c.716G>A genotype**	**≤12 GW**	**15 GW**	**24-27 GW**	**34 GW**	**Labour**	**Cord**
Plasma betaine (µmol/L)	**GG**	20.85[1] (20.10, 21.63)	14.62[a] (14.21, 15.04)	12.78[a] (12.42, 13.07)	12.83 (12.51, 13.16)	13.13 (12.78, 13.50)	24.65 (23.93, 25.40)
	GA	21.93 (21.04, 22.85)	15.28[a] (14.81, 15.76)	13.09[a] (12.69, 13.50)	13.28 (12.92, 13.64)	13.37 (12.94, 13.83)	25.13 (24.42, 25.85)
	AA	20.88 (19.24, 22.66)	14.57[a] (13.59, 15.61)	12.66[a] (11.98, 13.39)	13.04 (12.31, 13.82)	12.71 (11.97, 13.50)	25.33 (23.75, 27.02)
ANCOVA[2] models		NS	NS	NS	NS	NS	NS
Plasma folate-genotype interaction[4]		NS	NS	NS	NS	NS	NS
Plasma dimethylglycine (µmol/L)	**GG**	2.56 (2.45, 2.66)	2.27[a] (2.16, 2.38)	2.23 (2.13, 2.34)	2.53[a] (2.39, 2.67)	2.99[a] (2.83, 3.16)	3.72 (3.54, 3.92)
	GA	2.42* (2.31, 2.53)	2.14* [a] (2.04, 2.24)	2.12 (2.01, 2.34)	2.41[a] (2.27, 2.55)	2.72* [a] (2.54, 2.91)	3.58 (3.41, 3.76)
	AA	2.47 (2.21, 2.76)	2.03[b] (1.82, 2.27)	2.00 (1.79, 2.24)	2.01*** † (1.83, 2.21)	2.37*** [c] (2.06, 2.73)	3.24* (2.89, 3.62)
ANCOVA[3] models		P<0.05	P<0.05	NS	P<0.01	P<0.001	P<0.05
Plasma folate-genotype interaction[4]		P<0.05	P<0.05	NS	NS	NS	NS

Table 14. Plasma betaine, dimethylglycine, tHcy and dimethylglycine/betaine ratio during pregnancy and in the cord according to maternal *BHMT* c.716G>A genotypes (continued).

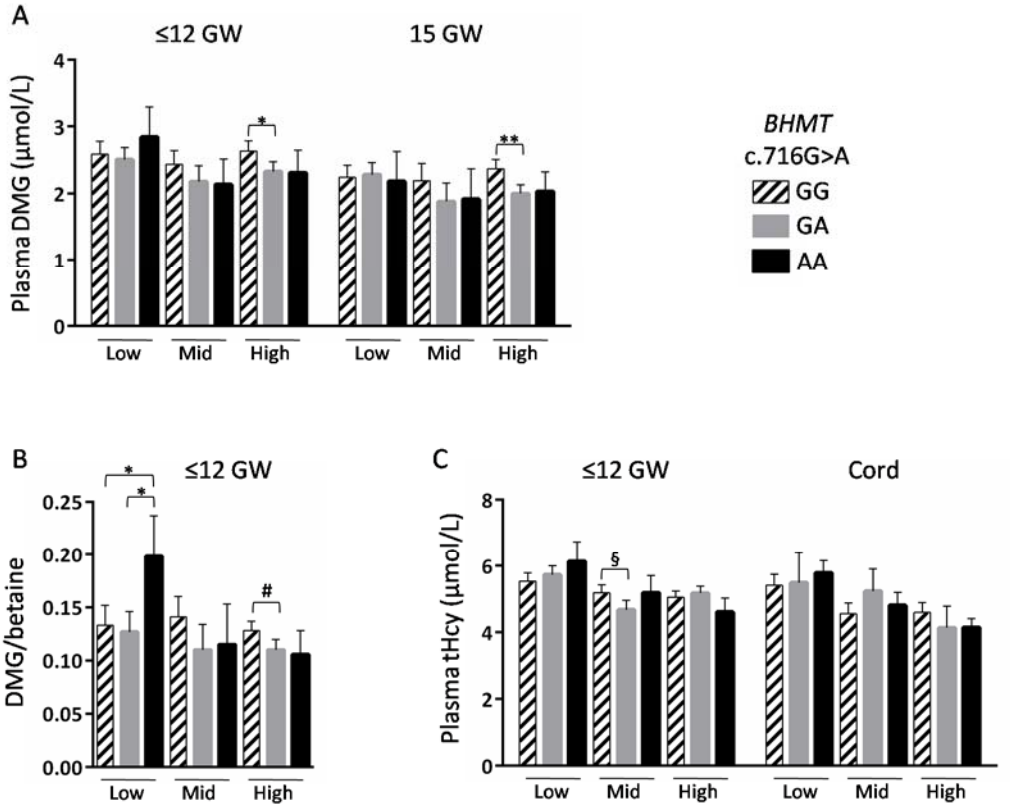
	***BHMT* c.716G>A genotype**	**≤12 GW**	**15 GW**	**24-27 GW**	**34 GW**	**Labour**	**Cord**
Dimethylglycine /betaine	**GG**	0.13 (0.13, 0.14)	0.17[a] (0.16, 0.18)	0.20[a] (0.18, 0.21)	0.22[a] (0.20, 0.24)	0.26[a] (0.23, 0.28)	0.17 (0.15, 0.18)
	GA	0.12 (0.11, 0.12)	0.15*[a] (0.14, 0.16)	0.17[a] (0.16, 0.19)	0.20[a] (0.18, 0.21)	0.23[a] (0.20, 0.26)	0.15 (0.14, 0.16)
	AA	0.15[§] (0.10, 0.19)	0.15[a] (0.13, 0.17)	0.18 (0.13, 0.23)	0.16* (0.15, 0.18)	0.22[c] (0.16, 0.28)	0.14 (0.12, 0.16)
ANCOVA[2] models		P<0.05	P<0.05	NS	P<0.01	NS	P=0.053
Plasma folate-genotype interaction[4]		P<0.01	NS	NS	NS	NS	NS
Plasma tHcy (µmol/L)	**GG**	5.27 (5.14, 5.40)	4.54[a] (4.42, 4.67)	4.63 (4.50, 4.77)	5.29[a] (5.13, 5.46)	6.25[a] (6.02, 6.48)	4.86 (4.68, 5.05)
	GA	5.35 (5.18, 5.52)	4.48[a] (4.35, 4.61)	4.65 (4.48, 4.82)	5.31[a] (5.11, 5.52)	6.13[a] (5.88, 6.40)	4.90 (4.69, 5.13)
	AA	5.31 (4.97, 5.67)	4.50[a] (4.21, 4.81)	4.64 (4.32, 4.99)	5.20[a] (4.83, 5.60)	6.07[a] (5.60, 6.58)	4.91 (4.52, 5.33)
ANCOVA[2] models		NS	NS	NS	NS	NS	NS
Plasma folate-genotype interaction[4]		P<0.05	NS	NS	P=0.056	P=0.057	P<0.05

GW: gestational weeks; NS: non-significant. Sample size: plasma betaine (n = 603, 454, 517, 501, 480, 463; for ≤12, 15, 24-27, 34 GW, labour and cord, respectively), dimethylglycine and dimethylglycine/betaine ratio (n = 603, 439, 516, 501, 480, 463; for ≤12, 15, 24-27, 34 GW, labour and cord, respectively) and plasma tHcy (n = 605, 449, 516, 501, 480, 466; for ≤12, 15, 24-27, 34 GW, labour and cord, respectively). Sample size varies between time points due to participant loss due to complications, non-attendance of programmed blood draw or delivery elsewhere or failure to collect blood samples. [1]Values are geometrical means (95% confidence interval). [2]Adjusting for maternal folate status (plasma folate tertile-based

categories at early and mid-late pregnancy) and gestational age at time of blood draw. [3]Adjusting for maternal folate status (plasma folate tertile-based categories at early and mid-late pregnancy), plasma betaine and gestational age at time of blood draw. ANCOVA Bonferroni posthoc: ***p<0.001, *p<0.05, versus GG; †p<0.05, §p=0.055, versus GA. [4]Maternal plasma folate tertile-based categories at mid-late pregnancy for cord analyte-genotype interactions. Two-factor repeated measures ANOVA (intrasubject factor: gestational age; intersubject factor: *BHMT* c.716G>A genotype) followed by post hoc Bonferroni correction for multiple comparisons: [a]p<0.001, [b]p<0.05, [c]p<0.01 versus previous time point.

A stratified analysis of plasma DMG, DMG/betaine ratio and tHcy for each *BHMT* c.716G>A genotype according to plasma folate status (tertile based categories) in early or mid-late pregnancy was conducted in those pregnancy time points where significant plasma folate status interactions on genotype-analyte were observed (**Figure 20**). In the high folate status category, lower DMG was observed in *BHMT* c.716GA compared to GG genotype at both ≤12 and 15 GW (**Figure 20.A**). In the low folate status category the DMG/betaine ratio was higher in participants with the *BHMT* c.716AA compared to GG or GA genotypes at ≤12 GW. However, in the high folate status category, the DMG/betaine ratio was lower in GA than GG, though not significant (P=0.082) (**Figure 20.B**).

Figure 20. Plasma dimethylglycine, dimethylglycine/betaine ratio and tHcy according to maternal *BHMT* c.716G>A genotype and plasma folate status[1].



Plasma analytes according to maternal *BHMT* c.716G>A genotype and folate status (plasma folate tertile-based categories). A. Plasma dimethylglycine at ≤12 and 15 GW. B. Dimethylglycine/betaine ratio at ≤12 GW. C. tHcy at ≤12 GW and in cord. [1]Relating to corresponding time of pregnancy: ≤12 and 15 GW (low: ≤18.6 or 20.5nmol/L at ≤12 or 15 GW respectively; high: ≥35.8 or 33.4nmol/L, at ≤12 or 15 GW respectively; mid) or in the cord (low: ≤8.9 or 7.1nmol/L, at 24-27 or 34 GW respectively; high: ≥17.1 or 13.4nmol/L, at 24-27 or 34 GW respectively). At ≤12 GW, for dimethylglycine and dimethylglyicine/betaine ratio, Low: GG (n=110), GA (n=110), AA (n=26); Mid: GG (n=65), GA (n=40), AA (n=16); High: GG (n=114), GA (n=91), AA (n=20); for tHcy, Low: GG (n=110), GA (n=110), AA (n=26); Mid: GG (n=66), GA (n=40), AA (n=16); High: GG (n=114), GA (n=91), AA (n=20). At 15 GW, Low: GG (n=81), GA (n=87), AA (n=14); Mid: GG (n=36), GA (n=24), AA (n=10); High: GG (n=89), GA (n=70), AA (n=16). With cord blood samples, Low: GG (n=84), GA (n=79), AA (n=13); Mid: GG (n=48), GA (n=39), AA (n=16); High: GG (n=75), GA (n=76), AA (n=14). Values are geometrical means. Error bars represent 95% confidence interval. Comparison between genotypes were made using ANCOVA adjusting for gestational age at time of blood draw, plasma folate concentration at ≤12, 15 GW and labour, and in the case of dimethylglycine analysis also for plasma betaine at ≤12 or 15 GW; with posthoc Bonferroni correction for multiple comparisons: *p<0.05, **p<0.01, #p=0.08, §p=0.07.

How maternal plasma folate status (tertile-based categories) and *BHMT* c.716G>A genotype are associated with plasma DMG throughout pregnancy and in the cord is reported in **Table 15**. Low compared to mid (reference tertile based category) plasma folate was associated with higher plasma DMG throughout pregnancy and in the cord. High compared to mid plasma folate status was associated with lower plasma DMG from mid pregnancy throughout the rest of pregnancy although the association was only significant at 34 GW. Compared to the normal homozygote *BHMT* c.716 genotype (GG), both variant GA and AA genotypes were associated with lower plasma DMG throughout pregnancy. The observed associations are stronger in the case of the variant homozygote genotype (AA) during late pregnancy and in the cord.

Table 15. Change in plasma dimethylglycine according to plasma folate category and maternal *BHMT* c.716G>A genotype.

	Model		Plasma folate category[1]		*BHMT* c.716G>A genotype	
	R^2	**F [n]**	**Low vs Mid**	**High vs Mid**	**GA vs GG**	**AA vs GG**
≤12 GW	18.3	12.3 [455]***	0.08 (0.04)[2]	-0.01 (0.04)	-0.09 (0.03)**	-0.07 (0.06)
15 GW	13.9	8.4 [420]***	0.10 (0.05)*	0.03 (0.05)	-0.08 (0.03)*	-0.10 (0.06)#
24-27 GW	13.8	9.8 [494]***	0.13 (0.04)**	-0.03 (0.05)	-0.06 (0.04)	-0.09 (0.06)
34 GW	21.9	15.8 [476]***	0.15 (0.05)**	-0.09 (0.05)*	-0.07 (0.04)	-0.20 (0.06)***
Labour	19.0	13.1 [463]***	0.16 (0.05)**	-0.04 (0.05)	-0.10 (0.04)*	-0.22 (0.07)**
Cord	10.6	6.7 [436]***	0.16 (0.05)**	0.06 (0.04)	-0.06 (0.04)§	-0.12 (0.06)*

GW: Gestational weeks. Multiple linear regression analysis: dependent variable plasma dimethylglycine (μmol/L). Adjusted for plasma betaine and gestational age at time of blood draw. [1]Relating to corresponding time of pregnancy: ≤12 and 15 GW (low: ≤18.6 or 20.5nmol/L at ≤12 or 15 GW respectively; high: ≥35.8 or 33.4nmol/L, at ≤12 or 15 GW respectively) or 24-27 GW throughout the rest of pregnancy and in the cord (low: ≤8.9 or 7.1nmol/L, at 24-27 or 34 GW respectively; high: ≥17.1 or 13.4nmol/L, at 24-27 or 34 GW respectively). [2]B coefficient (standard error). *p<0.05, **p<0.01, ***p<0.001, #p=0.065, §p=0.071.

5. *SLC19A1* c.80G>A polymorphism, plasma and RBC folate, and plasma tHcy

Plasma folate, RBC folate, and tHcy were compared between the different maternal *SLC19A1* c.80G>A genotypes throughout pregnancy and in the cord (**Table 16**). No differences were observed.

In all of the *SLC19A1* c.80 genotypes, plasma folate decreased between 15 and 24-27, and between 24-27 and 34 GW. In all of the *SLC19A1* c.80 genotypes, RBC folate increased between ≤12 and 15 GW, and decreased between 15 to 24-27, and between 24-27 and 34 GW. THcy had a U shape pattern throughout pregnancy; but in GG the tHcy increase was observed earlier (between 15 and 24-27 GW) compared to GA and AA genotypes (between 24-27 and 34 GW).

The effect of folate status (as plasma folate tertile-based categories) on the relationship between *SLC19A1* c.80G>A genotype and tHcy was tested and no folate-genotype interaction throughout pregnancy or in the cord was observed.

Table 16. Plasma and RBC folate and tHcy during pregnancy and in the cord according to maternal *SLC19A1* c.80G>A genotypes.

	***SLC19A1* c.80G>A genotype**	**≤12 GW**	**15 GW**	**24-27 GW**	**34 GW**	**Labour**	**Cord**
Plasma folate (nmol/L)	**GG**	25.7[1] (22.9, 29.0)	25.9 (23.0, 29.1)	13.2[a] (11.7, 14.9)	10.4[a] (9.2, 11.8)	10.2 (8.9, 11.6)	23.6 (21.5, 26.0)
	GA	26.2 (24.0, 28.5)	25.0 (22.7, 27.5)	13.5[a] (12.3, 14.8)	11.7[a] (10.5, 13.0)	11.0 (9.9, 12.3)	23.9 (22.1, 25.8)
	AA	25.0 (22.4, 27.9)	24.7 (21.8, 28.0)	12.6[a] (11.1, 14.1)	10.6[a] (9.4, 12.0)	10.9 (9.3, 12.7)	24.3 (22.0, 26.8)
ANCOVA[2] models		NS	NS	NS	NS	NS	NS
RBC folate (nmol/L)	**GG**	1015 (929, 1110)	1340[a] (1232, 1456)	1123[a] (1035, 1221)	884[a] (814, 959)		
	GA	962 (901, 1027)	1224[a] (1138, 1316)	1117[b] (1044, 1196)	964[a] (888, 1047)		
	AA	949 (864, 1044)	1225[a] (1117, 1344)	1058[b] (971, 1153)	873[a] (789, 965)		
ANCOVA[2] models		NS	NS	NS	NS		

Table 16. Plasma and RBC folate and tHcy during pregnancy and in the cord according to maternal *SLC19A1* c.80G>A genotypes (continued).

	***SLC19A1* c.80G>A genotype**	**≤12 GW**	**15 GW**	**24-27 GW**	**34 GW**	**Labour**	**Cord**
Plasma tHcy (µmol/L)	**GG**	5.40 (5.20, 5.60)	4.50[a] (4.34, 4.67)	4.76[b] (4.57, 4.96)	5.48[a] (5.23, 5.74)	6.38[a] (6.07, 6.71)	4.87 (4.62, 5.13)
	GA	5.28 (5.13, 5.42)	4.47[a] (4.35, 4.60)	4.55 (4.41, 4.70)	5.21[a] (5.03, 5.39)	6.10[a] (5.87, 6.34)	4.96 (4.77, 5.16)
	AA	5.26 (5.07, 5.45)	4.58[a] (4.40, 4.77)	4.67 (4.47, 4.88)	5.23[a] (5.00, 5.47)	6.14[a] (5.83, 6.46)	4.82 (4.55, 5.10)
ANCOVA[3] models		NS	NS	NS	NS	NS	NS
Plasma folate-genotype interaction[4]		NS	NS	NS	NS	NS	NS

GW: gestational weeks; NS: non-significant. Sample size: plasma folate (n = 605, 449, 516, 501, 479, 466; for ≤12, 15, 24-27, 34 GW, labour and cord, respectively), RBC folate (n = 563, 423, 503, 489; for ≤12, 15, 24-27, 34 GW, respectively) and plasma tHcy (n = 605, 449, 516, 501, 480, 466; for ≤12, 15, 24-27, 34 GW, labour and cord, respectively). Sample size varies between time points due to participant loss due to complications, non-attendance of programmed blood draw or delivery elsewhere or failure to collect blood samples. [1]Values are geometrical means (95% confidence interval). [2]Adjusting for gestational age at time of blood draw. [3]Adjusting for maternal folate status (plasma folate tertile-based categories at early and mid-late pregnancy) and gestational age at time of blood draw. [4]Maternal plasma folate tertile-based categories at mid-late pregnancy for cord tHcy-genotype interactions. Two-factor repeated measures ANOVA (intrasubject factor: gestational age; intersubject factor: *SLC19A1* c.80G>A genotype) followed by post hoc Bonferroni correction for multiple comparisons: [a]$p<0.001$, [b]$p<0.01$, versus previous time point.

6. *MTRR* c.66A>G polymorphism, plasma and RBC folate, plasma cobalamin and tHcy

Plasma and RBC folate, plasma cobalamin and tHcy throughout pregnancy and in the cord are compared between the different maternal *MTRR* c.66A>G genotypes in **Table 17**. Plasma folate was lower in heterozygotes at ≤12 GW compared to normal homozygotes, but no other differences were observed between the different *MTRR* c.66 genotypes for the rest of pregnancy or in the cord. RBC folate did not differ between the genotypes. Higher plasma tHcy and cobalamin were observed in the *MTRR* c.66GG compared to AA genotype at ≤12 and 15 GW, and at 24-27 GW, respectively.

In the three *MTRR* c.66A>G genotypes, plasma folate decreased between 15 and 24-27, and between 24-27 and 34 GW. RBC folate decreased from 15 GW throughout pregnancy in the three *MTRR* c.66 genotypes. Plasma tHcy decreased in the three genotypes at early pregnancy, started increasing after 15 GW for AA genotype, and after 24-27 GW for AG and GG genotypes. Plasma cobalamin decreased throughout pregnancy in the three genotypes, but in AG the decrease from 34 GW to labour was borderline significant (P=0.062).

The relationship between *MTRR* c.66A>G and tHcy was modified by plasma folate at ≤12, 15 and 24-27 GW. Plasma folate also affected the genotype effect on plasma cobalamin at ≤12, 15 and 24-27 and 34 GW; although at 34 GW the interaction did not reach significance (P for interaction: 0.070).

Table 17. Plasma and RBC folate, plasma cobalamin and tHcy during pregnancy and in the cord according to maternal *MTRR* c.66A>G genotypes.

	***MTRR* c.66A>G genotype**	**≤12 GW**	**15 GW**	**24-27 GW**	**34 GW**	**Labour**	**Cord**
Plasma folate (nmol/L)	**AA**	28.5[1] (25.5, 31.8)	25.2[a] (22.1, 28.8)	13.7[b] (12.1, 15.6)	10.8[b] (9.4, 12.4)	10.7 (9.3, 12.4)	24.1 (21.7, 26.8)
	AG	24.0* (21.9, 26.1)	24.8 (22.8, 27.0)	12.9[b] (11.8, 14.2)	10.9[b] (9.9, 12.0)	10.6 (9.5, 11.7)	23.4 (21.8, 25.1)
	GG	26.3 (23.4, 29.6)	25.7 (22.6, 29.3)	13.0[b] (11.6, 14.6)	11.5[a] (10.1, 13.1)	11.0 (9.5, 12.7)	24.5 (22.2, 27.1)
ANCOVA[2] models		P=0.051	NS	NS	NS	NS	NS
RBC folate (nmol/L)	**AA**	1017 (930, 1112)	1329[b] (1212, 1456)	1160[c] (1057, 1272)	959[b] (860, 1068)		
	AG	954 (890, 1022)	1228[b] (1147, 1314)	1083[b] (1015, 1155)	894[b] (832, 960)		
	GG	963 (884, 1048)	1224[b] (1112, 1346)	1078[b] (994, 1170)	906[b] (823, 997)		
ANCOVA[2] models		NS	NS	NS	NS		

Table 17. Plasma and RBC folate, plasma cobalamin and tHcy during pregnancy and in the cord according to maternal *MTRR* c.66A>G genotypes (continued).

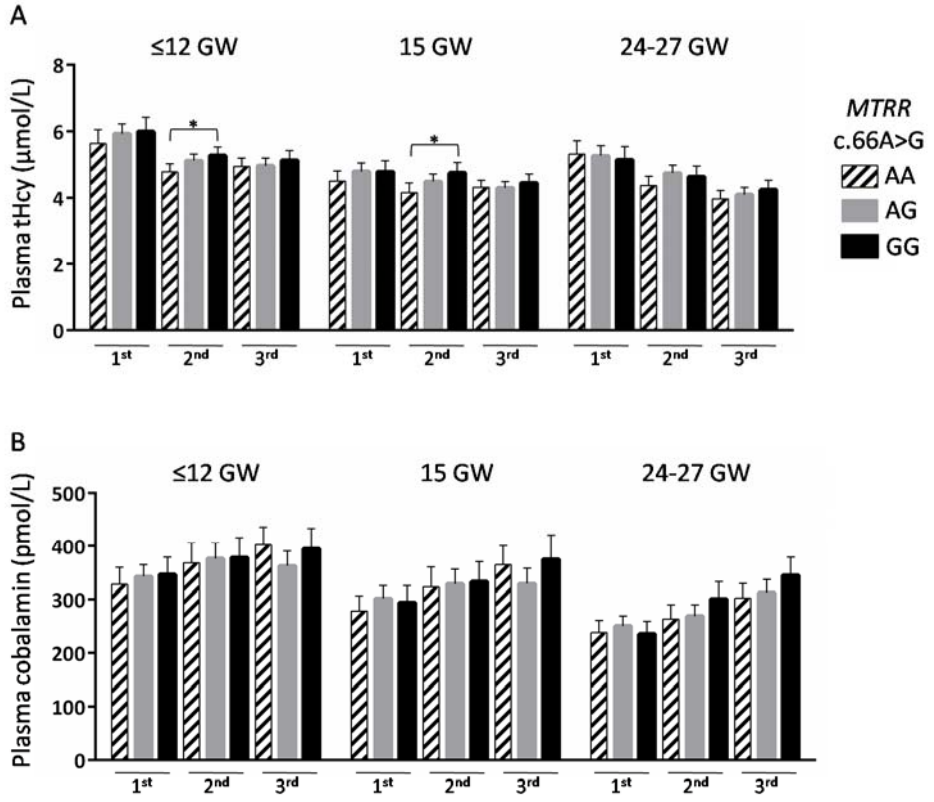
	***MTRR* c.66A>G genotype**	**≤12 GW**	**15 GW**	**24-27 GW**	**34 GW**	**Labour**	**Cord**
tHcy (µmol/L)	**AA**	5.04 (4.87, 5.21)	4.34[b] (4.19, 4.50)	4.50[a] (4.32, 4.70)	5.22[b] (4.97, 5.49)	6.19[b] (5.88, 6.53)	5.10 (4.85, 5.37)
	AG	5.37 (5.21, 5.53)	4.53[b] (4.39, 4.67)	4.72 (4.57, 4.87)	5.34[b] (5.17, 5.52)	6.29[b] (6.06, 6.53)	4.76 (4.56, 4.96)
	GG	5.46* (5.28, 5.65)	4.67* [b] (4.53, 4.82)	4.66 (4.48, 4.85)	5.27[b] (5.04, 5.51)	6.01[b] (5.71, 6.32)	4.88 (4.65, 5.13)
ANCOVA[3] models		P<0.05	P<0.05	NS	NS	NS	NS
Plasma folate-genotype interaction[5]		P<0.001	P<0.05	P<0.001	NS	NS	NS
Plasma cobalamin (pmol/L)	**AA**	371 (352, 391)	318[b] (298, 340)	268[b] (254, 283)	246[b] (232, 261)	231[a] (216, 247)	319 (288, 353)
	AG	359 (346, 373)	322[b] (308, 337)	275[b] (264, 287)	246[b] (235, 258)	233 (221, 245)	328 (303, 356)
	GG	373 (352, 396)	332[b] (312, 354)	291* [b] (273, 310)	261[b] (245, 278)	241[c] (225, 260)	332 (291, 378)
ANCOVA[4] models		NS	NS	P<0.05	NS	NS	NS
Plasma folate-genotype interaction[5]		P<0.05	P<0.05	P<0.01	P=0.07	NS	NS

GW: gestational weeks; NS: non-significant. Sample size: plasma folate (n = 605, 449, 516, 501, 479, 466; for ≤12, 15, 24-27, 34 GW, labour and cord, respectively), RBC folate (n = 563, 423, 503, 489; for ≤12, 15, 24-27, 34 GW, respectively), plasma tHcy (n = 605, 449, 516, 501, 480, 466; for ≤12, 15, 24-27, 34 GW, labour and cord, respectively) and plasma cobalamin (n = 605, 449, 516, 501, 477, 466; for ≤12, 15, 24-27, 34 GW, labour and cord, respectively). Sample size varies between time points due to participant loss due to complications, non-attendance of programmed blood

draw or delivery elsewhere or failure to collect blood samples. [1]Values are geometrical means (95% confidence interval). [2]Adjusting for gestational age at time of blood draw. [3]Adjusting for maternal folate status (plasma folate concentration at each time point), plasma cobalamin tertile and gestational age at time of blood draw. [4]Adjusting for maternal folate status (plasma folate concentration at each time point) and gestational age at time of blood draw. ANCOVA Bonferroni posthoc: *p<0.05, versus AA. [5]Based on maternal plasma folate concentration at labour for cord analyte-genotype interactions. Two-factor repeated measures ANOVA (intrasubject factor: gestational age; intersubject factor: *MTRR* c.66A>G genotype) followed by post hoc Bonferroni correction for multiple comparisons: [a]p<0.05, [b]p<0.001, [c]p<0.01, versus previous time point.

A stratified analysis of plasma tHcy and cobalamin for each *MTRR* c.66A>G genotype according to plasma folate tertiles was conducted in those pregnancy time points where significant folate interactions on genotype-analyte were observed. There were higher plasma tHcy concentrations in *MTRR* c.66GG than AA at ≤12 and 15 GW only in the mid plasma folate tertiles (**Figure 21.A**). No differences in plasma cobalamin were observed among *MTRR* c.66A>G genotypes at ≤12, 15 and 24-27 GW after stratification by plasma folate tertiles (**Figure 21.B**).

Figure 21. Plasma tHcy and cobalamin according to maternal *MTRR* c.66A>G genotype and plasma folate tertiles.

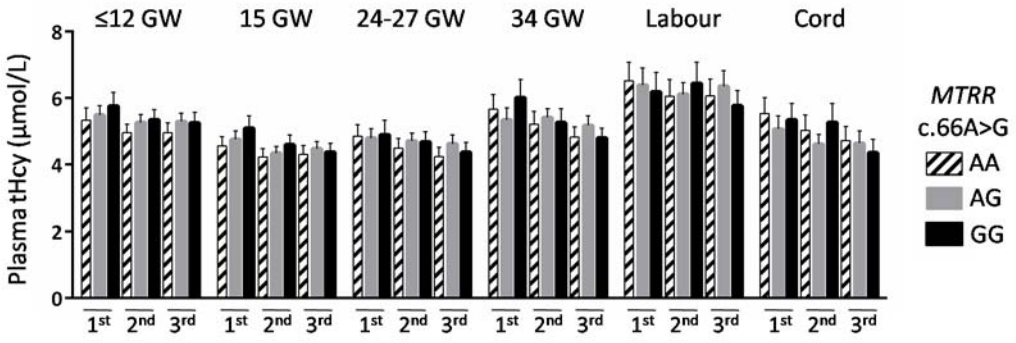


Plasma analytes according to maternal *MTRR* c.66A>G genotype and plasma folate tertile. A. Plasma tHcy at ≤12, 15 and 24-27 GW. B. Plasma cobalamin at ≤12, 15 and 24-27 GW. GW: Gestational weeks; 1st, first tertile; 2nd, second tertile; 3rd, third tertile. At ≤12 GW, 1st: AA (n=45), AG (n=96), GG (n=48); 2nd: AA (n=54), AG (n=88), GG (n=56); 3rd: AA (n=69), AG (n=81), GG (n=52). At 15 GW, 1st: AA (n=43), AG (n=64), GG (n=38); 2nd: AA (n=37), AG (n=69), GG (n=40); 3rd: AA (n=51), AG (n=62), GG (n=35). At 24-27 GW, 1st:

AA (n=47), AG (n=78), GG (n=45); 2nd: AA (n=49), AG (n=77), GG (n=43); 3rd: AA (n=55), AG (n=68), GG (n=46). Values are geometrical means. Error bars represent 95% confidence interval. Comparison between genotypes were made using ANCOVA adjusting for gestational age at time of blood draw, plasma folate concentration at ≤12, 15 and 24-27 GW, and in the case of tHcy analysis also for plasma cobalamin tertile at ≤12, 15 and 24-27 GW; with posthoc Bonferroni correction for multiple comparisons: *p<0.05.

Due to the known relationship between cobalamin and tHcy, and cobalamin being a substrate for MTRR, a stratified analysis of plasma tHcy for each *MTRR* c.66A>G genotype according to plasma cobalamin tertiles was conducted throughout pregnancy and in the cord. No differences in plasma tHcy were observed among *MTRR* c.66A>G genotypes throughout pregnancy and in the cord, in any of the maternal plasma cobalamin tertiles (**Figure 22**).

Figure 22. Plasma tHcy according to maternal *MTRR* c.66A>G genotype and plasma cobalamin tertiles.



Plasma tHcy throughout pregnancy and in the cord according to maternal *MTRR* c.66A>G genotype and plasma cobalamin tertile. GW: Gestational weeks; 1st, first tertile; 2nd, second tertile; 3rd, third tertile. At ≤12 GW, 1st: AA (n=49), AG (n=96), GG (n=50); 2nd: AA (n=63), AG (n=81), GG (n=50); 3rd: AA (n=56), AG (n=88), GG (n=56). At 15 GW, 1st: AA (n=49), AG (n=61), GG (n=35); 2nd: AA (n=42), AG (n=70), GG (n=35); 3rd: AA (n=40), AG (n=64), GG (n=43). At 24-27 GW, 1st: AA (n=56), AG (n=78), GG (n=35); 2nd: AA (n=44), AG (n=77), GG (n=48); 3rd: AA (n=51), AG (n=68), GG (n=51). At 34 GW, 1st: AA (n=53), AG (n=70), GG (n=41); 2nd: AA (n=40), AG (n=90), GG (n=36); 3rd: AA (n=47), AG (n=62), GG (n=54). At labour, 1st: AA (n=52), AG (n=61), GG (n=45); 2nd: AA (n=42), AG (n=86), GG (n=31); 3rd: AA (n=46), AG (n=60), GG (n=52). In the cord, 1st: AA (n=48), AG (n=60), GG (n=42); 2nd: AA (n=38), AG (n=81), GG (n=29); 3rd: AA (n=46), AG (n=59), GG (n=45). Values are geometrical means. Error bars represent 95% confidence interval. Comparison between genotypes were made using ANCOVA adjusting for gestational age at time of blood

draw, plasma folate and cobalamin concentrations at ≤12, 15, 24-27, 34 GW and labour; with posthoc Bonferroni correction for multiple comparisons.

7. Maternal and neonate genotypes, miscarriage and intrauterine growth restriction

There were 36 cases of miscarriage and 43 cases of IUGR. Due to the small number of cases of congenital malformations previously commented; further analyses of the associations between these pregnancy complications and *MTHFR* c.665C>T, *BHMT* c.716G>A, *SLC19A1* c.80G>A, *MTRR* c.66A>G polymorphisms were not carried out. The occurrence of miscarriage according to maternal genotype of the aforementioned polymorphisms is reported in **Table 18**. The prevalence of miscarriage varied between the maternal *SLC19A1* c.80G>A genotypes, although with borderline significance (p=0.055). More miscarriages were observed in mothers with the *SLC19A1* c.80AA genotype (variant homozygote) compared to the other genotypes. No other associations were observed for miscarriage prevalence with any of the other maternal SNPs. The associations between maternal or cord genotypes and IUGR prevalence are reported in **Table 18**. The prevalence of IUGR was only observed to vary for the *MTHFR* c.665C>T polymorphism in the cord. It was highest in the presence of the *MTHFR* c.665CC genotype (normal homozygote).

Table 18. Miscarriage and intrauterine growth restriction (IUGR) cases percentages in each maternal and neonate genotypes of the *MTHFR* c.665C>T, *BHMT* c.716G>A, *SLC19A1* c.80G>A, *MTRR* c.66A>G polymorphisms.

		Miscarriage		IUGR			IUGR	
Mother						**Neonate**		
***MTHFR* c.665C>T**	**CC**	5.4 (3.1, 9.4)[1]	[203][2]	7.9 (4.9 12.7)	[189]	**CC**	10.8 (6.9, 16.7)*	[157]
	CT	5.2 (3.2, 8.4)	[290]	7.4 (4.8, 11.1)	[272]	**CT**	6.1 (3.5, 10.3)	[197]
	TT	7.6 (3.9, 14.3)	[105]	8.5 (4.4, 15.9)	[94]	**TT**	1.3 (0.2, 6.8)	[79]
***BHMT* c.716G>A**	**GG**	5.8 (3.7, 9.1)	[293]	8.1 (5.4, 11.9)	[272]	**GG**	5.3 (2.9, 9.6)	[187]
	GA	5.0 (2.9, 8.5)	[242]	6.7 (4.1, 10.7)	[225]	**GA**	8.1 (5.1, 12.5)	[211]
	AA	7.9 (3.4, 17.3)	[63]	10.3 (4.8, 20.8)	[58]	**AA**	8.3 (2.9, 21.8)	[36]
***SLC19A1* c.80G>A**	**GG**	5.5 (2.9, 10.1)#	[164]	7.8 (4.5, 13.2)	[153]	**GG**	7.8 (4.1, 14.1)	[116]
	GA	3.7 (2.0, 6.7)	[269]	7.8 (5.1, 11.8)	[256]	**GA**	6.6 (3.9, 10.9)	[198]
	AA	9.3 (5.7 14.7)	[162]	7.7 (4.3, 3.2)	[143]	**AA**	6.1 (3.0, 12.0)	[115]
***MTRR* c.66A>G**	**AA**	3.5 (1.6, 7.4)	[172]	5.6 (3.0, 10.2)	[162]	**AA**	4.2 (1.8, 9.4)	[120]
	AG	5.6 (3.4, 9.1)	[266]	6.9 (4.3, 10.7)	[247]	**AG**	8.2 (5.1, 12.8)	[196]
	GG	6.4 (3.5, 11.4)	[156]	11.7 (7.5, 18.0)	[245]	**GG**	7.8 (4.2, 14.2)	[115]

[1]Percentage of cases (95% confidence interval). [2]Total sample size of the specific genotype. Chi square test, different frequencies between genotypes: *p<0.05; #p=0.055.

GENETIC STUDY DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

Genetic study discussion

1. *MTHFR* c.665C>T polymorphism

The observed genotype frequencies (34.0, 48.6 and 17.4%, for CC, CT and TT, respectively) were similar to previous studies in Spain (Guillén et al. 2001; García-Minguillán et al. 2014; Bueno et al. 2016) although another reported a lower frequency (7.1%) of the variant homozygote genotype (Ubeda et al. 2011). No apparent differences regarding ethnicity, age, health state and sampling date were observed between Ubeda *et al*. study and the RTBC or the other previous Spanish studies. However, the region of the country was different, i.e. centre (Ubeda et al. 2011) or east/northeast (Guillén et al. 2001; Fernàndez-Roig et al. 2013; García-Minguillán et al. 2014; Bueno et al. 2016). As in the RTBC study, high variant homozygote frequencies (17-18%) occur in Europe in populations from France (Chango et al. 2000c) and Italy (Botto and Yang 2000), but not in other European countries (Harmon et al. 1996; Molloy et al. 1997; Geisel et al. 2001; Castro et al. 2003; Relton et al. 2005; Devlin et al. 2006; Fredriksen et al. 2007; Stanislawska-Sachadyn et al. 2009; de Lau et al. 2010; Pavlíková et al. 2012).

Plasma/serum folate has been reported to be lower in *MTHFR* c.665C>T variant homozygote and/or heterozygotes in pregnancy studies in the absence of folic acid supplement use (Parle-McDermott et al. 2006; Barbosa et al. 2008a), or when there is little folic acid use (Molloy et al. 1997). The effect of the polymorphism on plasma folate was no longer observable when folic acid supplements were used in late pregnancy (Molloy et al. 2002). In a Spanish study conducted in 2000, when the folic acid supplement intake recommendation to prevent NTD was beginning, plasma folate was lower in CT than CC in the second and third trimester (Ubeda et al. 2011). The recommendations are to use folic acid supplements before the conception and in the first trimester to prevent NTDs (Dirección General de Salud Pública (Ministerio de Sanidad y Consumo - Gobierno de España) 1998; Dirección General de Salud Pública (Ministerio de Sanidad y Consumo - Gobierno de España) 2001). The use of folic acid

supplements during the second and third trimester may have been lower than in the first trimester in Ubeda *et al*'s. study. We have previously shown that coinciding with the decrease in folic acid supplement use after the first trimester, plasma folate falls sharply (although RBC folate increases during supplementation and then is maintained more or less stable at least until 34 GW) (Fernàndez-Roig et al. 2013). In line with this, in Ubeda *et al*. first and second trimester serum folate were 35.0 and 19.8nmol/L (Ubeda et al. 2011). In our study most of the participants took folic acid supplements in the first trimester, and half on them continued in the rest of pregnancy. We did not observe the effect of the maternal polymorphism on plasma folate throughout pregnancy and in the cord, possibly due to the widespread use of supplements. In previous studies from the same geographical region with a non pregnant population, where vitamin supplement users were excluded, and with 11.5nmol/L plasma folate mean; lower plasma folate was associated with the variant allele (García-Minguillán et al. 2014; Bueno et al. 2016). In a postfortification Brazilian pregnancy study where half the sample were supplement users (multivitamin or folic acid alone), 24.2nmol/L serum folate was the median and 25%, 50% and 25% of the participants in first, second and third trimester, respectively; lower serum folate was observed in TT participants (Barnabé et al. 2015). But in South Korean pregnant women at 24-28 GW, without medications and with 17.1nmol/L serum folate mean; the effect on serum folate was not observable (Kim et al. 2004).

In the RTBC plasma folate decreased throughout pregnancy, with similar pattern in the three *MTHFR* c.665C>T genotypes. Previously our group had reported this decrease pattern in RTBC participants below and above the plasma folate median (Fernàndez-Roig et al. 2013). As detailed in *Introduction* point *2.2*, there is controversy about this plasma/serum folate change throughout pregnancy, although most studies found a decrease. In a clinical trial of folic acid supplement intake (400μg/d) continued in the second and third trimester, the intervention group had similar serum folate at 14 and 36 GW while in the placebo group serum folate decreased (McNulty et al. 2013). McNulty *et al*.'s study where folic acid intake compliance was controlled, may explain

the different results in the observational studies. In McNulty *et* al.'s greater folic acid supplement intake in the second and third trimester (intervention group) not occurring in the RTBC could prevent the pregnancy induced plasma/serum folate changes.

Regardless of the use of folic acid supplements during pregnancy, lower RBC folate was reported in *MTHFR* c.665TT participants in Irish studies (Molloy et al. 1997; Molloy et al. 2002; Parle-McDermott et al. 2006). Unlike in the Irish studies, using microbiological assay for folate determination, a UK pregnancy study using a protein-binding assay found higher RBC folate in TT participants (Relton et al. 2005). We found lower RBC folate by the microbiological assay in TT pregnant women at mid-late pregnancy; but at 15 GW this was not significant, and at ≤12 GW it was borderline significant. RBC folate indicates long term folate status (the previous 120 days or ≈17 weeks) and plasma folate indicates recent folate intake. According to the questionnaires, in spite of the 81% RTBC participants planning pregnancy, 34% took folic acid supplements preconceptionally and 92% in the first trimester. At the prenatal visit at ≤12 GW a blood sample is collected for the study, and many participants may have been taking supplements during little time before the visit for this to be reflected in RBC folate. We consider probable that at ≤12 GW RBC folate reflected folate status before folic acid use was fully established. However at 15 GW when the effect was established, there was no difference. After folic acid supplement use ceased the effect gradually became evident again. Our group previously found lower RBC folate in TT non-pregnant population with no use of vitamin supplements (Bueno et al. 2016). The differences between both assays in the literature demonstrate that the SNP affect the proportion of RBC folate forms; and according to the microbiological assay, which is considered the most recommended, the SNP also lowers the RBC total folate (Molloy et al. 1998; Pfeiffer et al. 2010). Small studies analysing RBC from USA Caucasian population have found:

- More THF and 5,10-methyleneTHF, and less 5-methylTHF in TT than in CC women (Huang et al. 2008) and adults (Summers et al. 2010).

- Detectable amounts of formylTHF, and less 5-methylTHF in TT than in CC women (Davis et al. 2005).

The MTHFR variant enzyme is thermolabile and has lower activity than the normal enzyme (Frosst et al. 1995; Daly et al. 1999), maybe due to its higher homodimer dissociation rate with the subsequent release of its FAD cofactor (Yamada et al. 2001). An impairment in the MTHFR irreversible reaction can increase 5,10-methyleneTHF which is poorly polyglutamated by the FPGS in comparison to other folate forms (Cichowicz and Shane 1987; Chen et al. 1996). Non-polyglutamated folate would not be retained in RBC and would pass to the blood plasma, although we did not observe higher plasma folate, and indeed other studies reported lower plasma concentrations in TT individuals not taking folic acid supplements, as previously commented. DHF and 10-formylTHF are assumed to be the starting compounds for folate catabolism (Suh et al. 2001), and both can be formed from 5,10-methyleneTHF. In line with this, authors have suggested increased folate catabolism as the mechanism by which lower RBC folate is observed in TT individuals (Parle-McDermott et al. 2006), and this might explain also the lower plasma/serum folate associated with the variant allele in some studies. Our finding of a RBC folate lowering effect with the variant homozygote genotype only reaching significance at mid-late pregnancy, suggests that folic acid supplementation use pattern in the RTBC, and folate status (decrease in folate throughout pregnancy) can affect the effect of the SNP on RBC folate. However, in Australian gastroenterology patients the variant allele was associated with lower RBC folate, but this effect was lost when stratifying by RBC folate in the subgroup below the median (Lucock et al. 2013).

We observed a similar RBC folate pattern in the three genotypes in the RTBC: an increase from ≤12 to 15 GW, and a decrease from 15 GW on. This change was also found previously in RTBC below and above the plasma folate median (Fernàndez-Roig et al. 2013). When comparing RBC folate at 24-27 or 34 GW to ≤12 GW there were no differences (data not shown), i.e. there was no net decrease. As with plasma folate there

is controversy in observational pregnancy studies, with some reporting a decrease (Ek and Magnus 1981; Qvist et al. 1986; Fernàndez-Roig et al. 2013) and others an increase in RBC folate throughout pregnancy (Cikot et al. 2001; Takimoto et al. 2007). In the aforementioned clinical trial of folic acid supplement intake continued in the second and third trimester, a RBC folate increase from 14 to 36 GW was found in the intervention group while in the placebo group RBC folate decreased (McNulty et al. 2013). We suggest in McNulty *et* al.'s trial greater folic acid supplement intake in the second and third trimester (intervention group) not occurring in the RTBC could prevent or reverse the pregnancy induced RBC folate changes.

Pregnancy studies, in the absence of folic acid supplementation, reported higher plasma/serum tHcy in TT participants in the first trimester (Parle-McDermott et al. 2006), between 11-25 GW (Liang et al. 2014) or 24-28 GW (Kim et al. 2004), at varying times of pregnancy (Barnabé et al. 2015) and in variant allele carriers at labour (Lopreato et al. 2008; Barbosa et al. 2008a). Similar but non significant trends were reported in two smaller studies including folic acid supplement users (Molloy et al. 2002; Ubeda et al. 2011). We observed higher plasma tHcy in TT than in CC or CT participants throughout pregnancy; and higher cord plasma tHcy in TT than CC participants. The Irish study of Molloy *et al*. in 2002, found non significant higher cord plasma tHcy with maternal TT genotype as well as a non significant effect on maternal plasma tHcy at labour (Molloy et al. 2002). As we found an effect of the SNP on maternal tHcy, an effect on cord tHcy was expected given the foeto-maternal tHcy correlations (Guerra-Shinohara et al. 2002; Murphy et al. 2004; Molloy et al. 2005; Obeid et al. 2005; Wallace et al. 2008), that maternal tHcy is the most important determinant of foetal tHcy (Molloy et al. 2002), and the existence of tHcy transport systems in human placentas (Tsitsiou et al. 2011). An impairment in the MTHFR reaction leads to lower 5-methylTHF and hence less folate-dependent homocysteine remethylation. The *MTHFR* c.665C>T polymorphism is the most important genetic factor that determines blood tHcy, as meta analysed (van Meurs et al. 2013). According

to our results and most pregnancy studies, the homocysteine enhancing effect of the polymorphism occurs also during pregnancy.

In our study, maternal RBC folate affected the relationship SNP-tHcy at ≤12 GW, and so did plasma folate at 15, 24-27 GW and in cord tHcy. Stratifying the analysis according to RBC or plasma folate tertiles at these time points, showed that the differences in plasma tHcy among the genotypes were lost in the highest tertiles. The tHcy-enhancing effect of the *MTHFR* variant genotype or allele has been reported to be lost in higher plasma/serum folate status categories in non-pregnant populations (Jacques et al. 1996; Harmon et al. 1996; Geisel et al. 2001; Kluijtmans et al. 2003; Brilakis et al. 2003; de Lau et al. 2010). There is some controversy about this in pregnancy. The effect was lost in North Korean women at 24-28 GW with serum folate above the median (Kim et al. 2004). However, it was also lost below the serum folate median in Brazilian women of multiple ethnicities at labour (Barbosa et al. 2008a). Differences between both studies are ethnicity and ethnicity homogeneity, fasting conditions, and folate determination method (although both are types of protein-binding assay). At ≤12 GW higher tHcy was observed in variant homozygotes compared to heterozygotes, but this only occurred in the low RBC folate tertile. RBC and plasma folate status indicate long term folate status and recent folate intake, respectively. We suppose the effect of *MTHFR* c.665C>T polymorphism on tHcy at ≤12 GW is affected by the folate status that was present at the time the effect has occurred, i.e. folate status before taking folic acid supplements, which RBC folate can reflect better than plasma folate at ≤12 GW.

At 15 and 24-27 GW plasma folate modulated the association between the *MTHFR* c.665C>T polymorphism and tHcy. THcy was higher in TT compared to CC genotype at 24-27 GW and in cord, only in the first plasma folate tertile. THcy was higher in TT compared to CT genotype at 15, 24-27 GW and in cord in the first plasma folate tertile, and also in the second plasma folate tertile at 15 GW. At 34 GW, RBC folate did not modulate the effect of the SNP on tHcy, and plasma folate did with borderline significance. At labour no modulation by plasma folate was observed; and RBC folate

was not determined at this point. We speculate plasma folate must be a better determinant of the effect of the SNP than RBC at 15 and 24-27 GW. We deem two factors can be contributing to this pattern of folate interaction from 15 GW on. The first is that half the RTBC participants stopped taking folic acid supplements after the first trimester. The second is that the tHcy change at late pregnancy compared to earlier stages is less determined by folate status. In the RTBC study, plasma betaine but not folate was a significant predictor of the change in tHcy at labour in participants above the plasma folate median (Fernàndez-Roig et al. 2013). In participants below the plasma folate median, plasma folate and betaine became equally strong determinants of the tHcy change at labour (Fernàndez-Roig et al. 2013). As RTBC participants started taking folic acid supplements in the first trimester at different times, the RBC folate pool at 15 GW might be at different stages of "filling". In such situation, plasma folate would be a better indicator than RBC folate of folate status at 15 GW and hence plasma but not RBC folate interaction was observed. At 34 GW and labour no interactions by folate status were observed and we speculate this is due to reduced effect of folate status on tHcy at late pregnancy. However, the interpretation of the interaction by plasma but not RBC folate at 24-27 GW on SNP-tHcy, and of the interaction by plasma folate at labour on SNP-tHcy in cord; is unclear.

According to most observational studies, tHcy follows a U-shape pattern during pregnancy, decreasing from early to mid pregnancy and then increasing towards labour (Cikot et al. 2001; Murphy et al. 2002; Murphy et al. 2004; Velzing-Aarts et al. 2005; Ubeda et al. 2011). As previously reported for the RTBC (Fernàndez-Roig et al. 2013), we found the same pattern of tHcy change throughout pregnancy, without differences among *MTHFR* c.665C>T genotypes.

We found no differences in the proportion of miscarriage cases among *MTHFR* c.665C>T genotypes. Hyperhomocysteinaemia is a risk factor for recurrent miscarriage (Nelen et al. 2000), and the *MTHFR* c.665 variant allele has a known homocysteine-enhancing effect. However, the maternal genotype has not been associated with

recurrent miscarriage risk in studies of Caucasians (Cao et al. 2013); nor specifically in the Spanish population, even in the presence of low RBC folate (Creus et al. 2013). Regarding sporadic miscarriage or unspecified type of miscarriage, no association between genotypes or allele and miscarriage risk was found in a big Irish study with undocumented use of supplements (Parle-McDermott et al. 2005). However, maternal TT genotype increased the risk in a postfortification Mexican study with smaller sample size and little use of folic acid supplements (Rodríguez-Guillén et al. 2009). Our results seem to be in line with the idea of no effect of maternal genotype on miscarriage risk, although the number of miscarriage cases is small to consider this result as evidence.

No differences in the proportion of IUGR offspring among maternal *MTHFR* c.665C>T genotypes were found either. The existing studies assessing the effect of the maternal genotype on IUGR, which were in non fortified or non fully exposed to fortification populations, have found no association (Facco et al. 2009). Moderately elevated maternal tHcy (7.1μmol/L) at 8 GW was associated with a threefold increased risk of reduced birth weight in the offspring in a Spanish study (Murphy et al. 2004). The independent effect of the maternal variant allele on tHcy may not be strong enough for the studies to find an association between the maternal SNP and miscarriage or IUGR risk. Also, the variant MTHFR affects directly folate metabolism pathways related with methylation, but possibly to a lesser extent DNA synthesis and repair. The observed association between high tHcy and miscarriage or IUGR risk may actually be reflecting low folate status. Low early pregnancy plasma folate concentration has been associated with miscarriage (George et al. 2002), and folic acid supplementation with higher birth weight (Lassi et al. 2013). Given the absence of association between the maternal *MTHFR* c.665C>T genotypes and miscarriage or IUGR risk, the pathological mechanisms for the effect of low folate status on these outcomes might be more related to impaired DNA synthesis than to methylation. As a nucleoside transport system has been reported in human placentas (Dancis et al. 1993; Acevedo et al. 1995), a flow of nucleosides from the mother to the foetus can occur. This is in line with the speculation of lower DNA synthesis in the presence of low maternal folate status. However, given the multiple

roles of homocysteine as a toxic agent (*Introduction* point *2.1*), an effector role of homocysteine in miscarriage and IUGR risk cannot be completely ruled out.

The proportion of IUGR cases were different according to *MTHFR* c.665C>T genotypes in the offspring. A lower number of TT neonates had IUGR (1.3%) compared to the other genotypes. And higher number of CC neonates had IUGR (10.8%) compared to the other genotypes. In a Canadian study the TT offspring genotype was less prevalent in IUGR cases than controls (Infante-Rivard et al. 2002). Given that the pregnancies at 24 GW were between May 1998 and June 2000, and the start of the mandatory policy in Canada (November 1998), in Infante-Rivard *et al*.'s study the sample was partially fortified. Also in this study 79% of the participants took multivitamin supplements in the third trimester (Infante-Rivard et al. 2002). The authors stated a protective effect of the variant allele had been observed previously in specific leukaemias, and a potential mechanism for the protection of them and IUGR could be increased availability of 5,10-methyleneTHF for thymidylate synthesis (Infante-Rivard et al. 2002). In smaller UK study a trend toward less variant homozygotes in IUGR cases was reported (Glanville et al. 2006). The authors suggested decreased foetal survival with the T allele in situations of deficient folate status, although no data on folic acid supplement use or folate status was provided (Glanville et al. 2006). In addition to the sample size difference, it could be possible that the likely lower folate status of the UK study (Glanville et al. 2006) compared to the Canadian study (Infante-Rivard et al. 2002) was the reason for the effect of the foetal SNP on IUGR not reaching significance in Glanville *et al*. No associations between the offspring genotype and birth weight were observed in a prefortification Mexican study were 36% of the participants took prenatal supplements (Kordas et al. 2009). Our results are in line with the studies assessing IUGR (Infante-Rivard et al. 2002; Glanville et al. 2006). As our group has reported higher risk of the neonate being in the lower birth weight tertile when cord plasma tHcy is moderately elevated (Murphy et al. 2004), it could be expected that the variant allele would increase IUGR risk. But in spite of the homocysteine-enhancing effect of the *MTHFR* c.665C>T polymorphism in pregnant and non pregnant populations, this effect has not

been observed between neonate genotype and cord tHcy (Molloy et al. 2002; Lopreato et al. 2008; Plumptre et al. 2015). In regards of the foetus survival speculation, a Spanish study found higher proportion of TT in aborted foetuses than in controls (Callejón et al. 2007); but no association (Zetterberg et al. 2002) or the contrary has also being reported (Isotalo et al. 2000; Bae et al. 2007). As in Callejon *et al*. the upper limit of gestational age of the miscarriage foetuses was earlier (10 GW) than in the other studies, it is possible that the abortions due to the deleterious effects of the TT genotype took place earlier. A high number of embryo/foetal losses can also occur at pregnancy times where they cannot be identified, such as in embryo resorptions. Therefore, in addition to what other authors have proposed as the mechanisms of the "protective" effect of the T allele, we suggest the variant genotype embryos have reduced survival at very early stages of pregnancy, and that variant genotype surviving embryos has less other polymorphisms and mutations affecting foetal growth than the surviving CC embryos. Experiments with human embryos suggest the CT genotype has a higher risk of developmental failure between fertilisation and blastocyst stage, and the TT genotype from later on in the implantation process (Enciso et al. 2016).

2. *BHMT* c.716G>A polymorphism

The RTBC genotype frequencies (48.9, 40.5 and 10.6%, for GG, GA and AA, respectively) were similar to those reported in studies of healthy population in Norway (Fredriksen et al. 2007) and China (Liang et al. 2014). A slightly higher frequency of variant homozygotes (14.5%) was reported in Italy (Giusti et al. 2008).

To the best of our knowledge, no study has investigated the maternal association between the *BHMT* c.716G>A polymorphism and plasma betaine or DMG concentrations in pregnancy. Cord plasma betaine was unaffected by the offspring genotype in a postfortification Canadian study (Visentin et al. 2015). We found no effect in the RTBC study of the maternal SNP on plasma betaine throughout pregnancy and in the cord. These results are similar to those reported in a Norwegian older adult cohort (Fredriksen et al. 2007). Our result is not surprising because a stable betaine pool is

assumed to be maintained even when the BHMT pathway is upregulated (Allen et al. 1993a; Dominguez-Salas et al. 2013), possibly to spare betaine for its principal functions as an osmolyte (Yancey et al. 1982) and in protein stabilisation (Sheikh-Hamad et al. 1994).

In the three genotypes plasma betaine decreased from ≤12 to 24-27 GW, and remained unchanged afterwards. This longitudinal pattern has been reported previously in other studies (Velzing-Aarts et al. 2005; Wallace et al. 2008; Visentin et al. 2015) and in the RTBC (Fernàndez-Roig et al. 2013). *In vitro* and *in vivo* experiments of early embryo development in mice suggest betaine is important at this stage, as an osmoprotector (Biggers et al. 1993; Dawson and Baltz 1997; Anas et al. 2007) and also as a methyl donor (Lee et al. 2012). Phosphatidylcholine is preferentially produced/taken up by the human foetus when it is derived from choline entering the PEMT pathway, where betaine donates methyl groups (Yan et al. 2013).

Lower plasma DMG concentrations were found with variant allele increase in Norwegian cohorts of older adults (Fredriksen et al. 2007) or stable angina pectoris patients (Svingen et al. 2013). Our results in pregnancy are in line with them at most time points with lower concentration in GA than in GG at early pregnancy, and in AA than in GG at late pregnancy and in the cord. In spite of the betaine pool not being affected by the *BHMT* c.716G>A polymorphism, the DMG/betaine ratio did not mirror exactly the differences among genotypes reported for DMG. In some time points the differences in the ratio might not be big enough to be statistically detectable, given that the ratio depends on the concentration of DMG and betaine, and only the former was different among genotypes. Also, small differences among genotypes for betaine and DMG at the same time points and not reaching significance are possible. If these differences are in contrary sense for betaine and DMG, the combination of both could lead to significant differences in the DMG/betaine ratio

Plasma DMG had a U-shape pattern throughout pregnancy, as described in the RTBC (Fernàndez-Roig et al. 2013) and other studies (Velzing-Aarts et al. 2005; Wu et al.

2013; Visentin et al. 2015). In the case of the *BHMT* c.716AA genotype, the late pregnancy DMG increase started later than in GG and GA. The DMG/betaine ratio increased throughout pregnancy, but in AA no increase between 15 to 34 GW was observed. This is in agreement with human observational studies suggesting downregulation of the BHMT pathway with the variant enzyme (Fredriksen et al. 2007; Svingen et al. 2013). However, it does not agree with *in vitro* experiments where no effect (Weisberg et al. 2003; Feng et al. 2011), or higher affinity for the substrates (Li et al. 2008) have been reported in the variant enzyme.

Maternal plasma folate interacted with the effect of the *BHMT* c.716G>A polymorphism on plasma DMG at ≤12 and 15 GW, and on DMG/betaine ratio at ≤12 GW. Stratifying by plasma folate tertile based categories at these time points showed that lower DMG (and borderline lower ratio) was observed in the GA than in GG genotype in the high folate status category. This suggests a less active BHMT pathway in the presence of the variant allele. The fact that at mid-late pregnancy where folate status is lower, no folate interaction was found may be due to less variation in folate status at these time points. In the unstratified analysis lower DMG was observed in AA than in GG at late pregnancy, and in GA only in labour. These results point toward less BHMT activity with the variant allele in the extreme ranges of plasma folate status distribution during gestation; i.e. in the high folate status category at early pregnancy, and at late pregnancy where folate status is homogeneously low. Higher risk of NTD in offspring with the variant allele was reported in pregnancies of postfortification American folic acid supplement users (Boyles et al. 2006), and in Chinese non users (Liu et al. 2014). This could be a clinical effect of the polymorphism at folate status extremes. As exposed in *Introduction* point *3.2,* experiments with hepatic physiological concentrations of DMG, methionine, SAM and SAH suggest all of them can be inhibitors of BHMT *in vivo*. We have previously shown in the RTBC an apparent upregulation of the BHMT pathway at mid-late pregnancy when folate status is lower (Fernàndez-Roig et al. 2013). Also in micropigs fed a folate deficient diet there was more hepatic BHMT specific activity than in the control group (Halsted et al. 2002). A speculative suggestion from these and our

data is that, on one hand high folate status leads to BHMT inhibition, with the inhibitory effect being stronger in the variant enzyme; and on the other hand low folate status leads to BHMT activation, with a weaker effect in the presence of the variant enzyme. Whether different folate forms can affect BHMT activity has not been studied, so a direct *in vivo* effect occurring cannot be discarded. The concentration of the aforementioned inhibitors can be affected by folate status. We speculate the following hypotheses:

- During early pregnancy with high folate status, the variant enzyme is more inhibited than the normal enzyme due to a molecular environment with high SAM and folate.

- During late pregnancy, that is when low folate status is leading to upregulation of the BHMT pathway, the resulting higher DMG is inhibiting the variant enzyme to a greater extent than the normal enzyme.

We exclude methionine as one of the potential effectors given the stability of its plasma concentration throughout pregnancy and in the subgroups (data not shown), although differences in the hepatic concentrations of the folate status groups cannot be discarded. Other compounds affected by folate status may be unknown inhibitors of BHMT with a potentially higher effect on the variant enzyme. It is worth noting that SAM is assumed not to interact directly with BHMT according to experiments with the purified enzyme (Szegedi et al. 2008), and the inhibition in Finkelstein *et al*. rat liver extract experiments could therefore be mediated by other proteins bound to BHMT *in vivo* (Finkelstein et al. 1972). According to *in vitro* experiments, SAM but not methionine inhibits *BHMT* mRNA expression in a dose-dependent manner (Castro et al. 2002; Ou et al. 2007). However, the harsh conditions of the assay in the aforementioned rat liver extract study would likely prevent the cells from surviving and be transcriptionally/translationally active. Also, the *BHMT* c.716G>A polymorphism is non-synonymous and no effect on protein expression has been reported (Li et al. 2008; Feng et al. 2011). Further knowledge of the yet unknown

properties of the BHMT tetramers occurring in the heterozygote genotype might be relevant for understanding the observed effects.

In the stratified analysis, a higher DMG/betaine ratio at ≤12 GW with low folate status was observed in AA than in GG and GA genotypes. This finding is hard to interpret and contrary to previous human studies reporting less BHMT activity because DMG concentration was lower in the presence of the variant allele (Fredriksen et al. 2007; Svingen et al. 2013). In addition to the molecular environment according to folate status, other unknown factors related with gestational age may affect the variant enzyme differently to the normal enzyme. However, this might be a chance finding. We carried out a stratified analysis of the sample in two plasma folate status categories (deficient/possibly deficient and normal/high) using WHO criteria in a recent publication (Colomina et al. 2016), and we observed differences only in the normal/high group and in the same sense as the results of this thesis (data not shown, and **Appendices**).

No differences in the frequency of miscarriage or IUGR cases were found among the three maternal *BHMT* c.716 genotypes. A higher rate of embryo resorption was reported in mouse experiments where embryonic *BHMT* transcription was inhibited (Lee et al. 2012). However, there is controversy in human and *in vitro* studies about the implications of the *BHMT* c.716G>A polymorphism on the BHMT pathway, and in Lee *et al*.'s study, inhibition of maternal *BHMT* expression was not investigated. We found lower cord plasma DMG concentration in AA participants; and cord plasma DMG has been reported to be a positive predictor of birth weight (Hogeveen et al. 2013). The lack of association between maternal genotype and IUGR may be due to birth weight and IUGR risk not being fully comparable. Also, the association found in Hogeveen *et al*. does not indicate cause, and unknown factors could be affecting DMG concentration and birth weight independently. For example, as DMG through its full oxidation to glycine in the mitochondria contributes to glutathione synthesis in pregnancy (Friesen

et al. 2007), higher cord DMG concentration could result from less oxidative stress, and thus be associated with higher birth weight.

No association was found between IUGR and offspring *BHMT* c.716 genotypes. In the aforementioned *in vitro* mouse embryo study, the posttransfer growth of the surviving embryos whose *BHMT* expression had been inhibited was the same than with no inhibition (Lee et al. 2012). Plasma DMG and the DMG/betaine ratio increased in late pregnancy, as shown here and previously (Fernàndez-Roig et al. 2013), suggesting more importance of the BHMT pathway at that stage where the foetus has higher growth rate. BHMT activity and expression in foetal livers has been reported to increase with gestational age in human (Gaull et al. 1973; Feng et al. 2011) and pig studies (Ganu et al. 2013). However, the contribution of the foetal BHMT to 1C metabolism is unknown, and other authors have found no differences in cord plasma betaine and DMG among the three foetal *BHMT* c.716 genotypes (Visentin et al. 2015). It seems the maternal *BHMT* c.716G>A polymorphism could be relevant to small changes in birth weight not reaching differences in IUGR risk in our study; while the foetal BHMT could be more involved in early stages of development related with miscarriage (as embryo resorption) that we were unable to study here.

3. *SLC19A1* c.80G>A polymorphism

The genotype frequencies in the RTBC were 27.7, 45.3 and 27.0%, for GG, GA and AA, respectively. The genotype frequencies of the *SLC19A1* c.80G>A polymorphism in healthy populations are highly variable in Europe (Chango et al. 2000b; Relton et al. 2005; Devlin et al. 2006; Stanislawska-Sachadyn et al. 2009; de Lau et al. 2010; Pavlíková et al. 2012) and in the rest of the world (Barbosa et al. 2008b; Liang et al. 2014), but usually there are more normal homozygotes than variant homozygotes and the heterozygotes are the most common. In a big Norwegian study there were more normal homozygotes than heterozygotes though (Fredriksen et al. 2007). To our knowledge, we have found the highest variant homozygote frequency (27.0%) among other studies in healthy populations (17.2-24.1%).

SLC19A1 is the major route of folate transport into systemic tissues (Zhao and Goldman 2013), but the molecular implications of the *SLC19A1* c.80G>A polymorphism are unclear. The variant transporter might be more prone to be in the wrong cellular location, but this is based on a study where an amino acid residue segment containing the polymorphic position is ablated (Marchant et al. 2002), and a similar investigation specifically with the variant transporter is lacking. Higher affinity for formylTHF in the variant transporter has been reported (Whetstine et al. 2001), but its affinity and other kinetic parameters for 5-methylTHF (most prevalent folate form in the body) are unknown. Moreover, depending on cell type different expression, affinity and transport efficacy for its transportable molecules have been described in normal and variant SLC19A1s (Whetstine et al. 2001; Baslund et al. 2008).

We found no effect of the variant genotypes on plasma folate, which is in line with most human studies exposed in *Introduction* point *3.3*. If the variant transporter performed less folate transport, higher plasma folate concentration would be expected. However, according to these results, the potential effect of the SNP is not strong enough to observe differences in plasma folate. Another possibility is that other folate transporters could compensate the potential effect of this polymorphism, e.g. folate receptors can be up-regulated in the intestine in situations of folate deficiency (Liu et al. 2005).

The absence of erythropoiesis in mouse foetus and surviving pups that are knock-out mutants for the reduced folate carrier (Zhao et al. 2001; Gelineau-van Waes et al. 2008), points to this transporter being important in folate uptake during the development of RBCs. As with plasma folate, most human studies have not found an association between the variant genotypes and RBC folate concentration. It is important to note that many of the studies in healthy populations used protein binding assays for RBC folate determination (Relton et al. 2005; Vesela et al. 2005; Barbosa et al. 2008b), which are less recommended than the microbiological assay (Pfeiffer et al. 2010). Two studies in healthy populations that used microbiological assay reported

contrary results. In young Northern Irish adults including multivitamin supplement users (22.6% of sample) higher RBC folate was observed with variant allele increase (Stanislawska-Sachadyn et al. 2009). In Spanish adults with a broader age range and supplement users excluded lower RBC folate was found in variant homozygotes (Bueno et al. 2016). We did not observe any effect of the *SLC19A1* c.80G>A polymorphism on RBC folate in the RTBC. Our result is in line with another study in a healthy population, using the microbiological assay, excluding folic acid supplement users and conducted in France (Chango et al. 2000b). The RTBC sample is from the same Spanish geographical region as in Bueno *et al*., but they differ in that in Bueno *et* al.'s study participants had to have lived in the region for at least 2 generations. Therefore, according these studies in non-pregnant populations, it can be hypothesised that with no supplement use the variant allele has no effect (Chango et al. 2000b) or a lowering effect on RBC folate (Bueno et al. 2016). However, with supplement users included and younger individuals the effect is in the sense of enhancing RBC folate (Stanislawska-Sachadyn et al. 2009). On the contrary, taking into account folate status of these three studies do not shed light in the comparison of the different results. Chango *et al*.'s study plasma and RBC folate medians were 13.4 and 547nmol/L, respectively. Stanislawska-Sachadyn *et al*.'s study plasma and RBC folate medians were 12.8 and 644nmol/L, respectively. And Bueno *et al*.'s study plasma and RBC folate means were 11.5 and 810nmol/L, respectively. On average, the RTBC participants were young and folic acid supplement users, but as they are pregnant they cannot be compared to the Northern Irish study (Stanislawska-Sachadyn et al. 2009). If the hypothesis of higher RBC folate with variant allele and supplement use was true, pregnancy-associated unknown factors might hamper this effect in the RTBC. Also the genetic background may be relevant, as haplotypes including the variant allele could be different in Northern Ireland than in Spain.

We found no differences in plasma tHcy among the *SLC19A1* c.80G>A genotypes throughout pregnancy and in the cord, which is in agreement with most studies (*Introduction* point *3.3*) and the previous results on plasma and RBC folate. The absence

of interaction of maternal folate status on polymorphism-tHcy relationship is also in line with the literature (Devlin et al. 2006). However, in the characteristic U-shape pattern of tHcy throughout pregnancy, the increase in mid-late pregnancy started earlier in the *SLC19A1* c.80GG genotype (normal homozygote). Given the lack of information regarding this polymorphism at the molecular level, the controversy in human studies, and that in Spanish non-pregnant non-supplemented population the variant transporter is associated with apparently less folate uptake (Bueno et al. 2016); we deem this as a likely chance finding.

The proportion of miscarriage cases was borderline higher in participants with the *SLC19A1* c.80AA genotype. Previous studies have not found this association, but all were conducted in Asian populations and the outcome specifically included or was recurrent miscarriage (Rah et al. 2012; Luo et al. 2015; Mohtaram et al. 2016). Due to the low significance, small number of cases and the absence of effect on maternal and cord 1C metabolism analytes, this finding has to be regarded cautiously. As differences in the effect of the variant transporter according to cell types has been described (Whetstine et al. 2001; Baslund et al. 2008), it can be speculated whether the variant transporter in maternal cells involved in folate transport toward the foetus at early pregnancy is functioning worse than the normal transporter.

The maternal or foetal *SLC19A1* c.80G>A polymorphism was not associated with IUGR in the RTBC. To our knowledge there are no reports about this in the literature. There is an English study finding no effect of the maternal or foetal SNP on birth weight (Relton et al. 2005), and despite different outcomes our result agrees with it.

As a final remark on SLC19A1, the effect of the addressed polymorphism is largely unknown and our results (with the exception of a possible effect on miscarriage risk) support the idea of this polymorphism having no effect or a slight effect.

4. *MTRR* c.66A>G polymorphism

The genotype frequencies in the RTBC were 28.9, 44.9 and 26.2%, for AA, AG and GG, respectively (with similar frequencies in RTBC offspring). Among healthy population studies, the heterozygote genotype is the most common genotype, except in a Chinese study, where also the lowest variant homozygote frequency (5.4%) was reported (Liang et al. 2014). Normal homozygotes are more common than variant homozygotes in studies from Northern Ireland (Gaughan et al. 2001), Germany (Geisel et al. 2001), Brazil (Barbosa et al. 2008a) and China (Liang et al. 2014); but not from Norway (Fredriksen et al. 2007) and the USA (Vaughn et al. 2004; Yang et al. 2008).

According to *in vitro* experiments, the variant MTRR has less affinity for MTR resulting in lower formation of the MTRR-MTR complex (Olteanu et al. 2002; Wolthers and Scrutton 2007). This would mean that after the MTR cobalamin cofactor is oxidised to the inactive form cob(II)alamin, the variant MTRR would activate the cofactor at a lower rate than the normal enzyme, leading to impaired MTR reaction. Other implications leading to the same effect would be lower reduction of MTR-bound aquacob(III)alamin to cob(II)alamin, lower conversion of free cob(II)alamin to MTR-bound methylcob(III)alamin, as well as less stability of the MTR apoenzyme, in the variant compared to normal MTRR (Yamada et al. 2006). A hampered MTR reaction would result in a higher proportion of 5-methylTHF that cannot be recycled back to other folate forms by enzymes other than MTR. As 5-methylTHF is poorly polyglutamated by the FPGS in comparison to other folate forms (Cichowicz and Shane 1987; Chen et al. 1996), it can be expected that folate is not retained in cell. According to this idea, in variant homozygotes higher plasma folate and lower RBC folate concentrations are expected.

Plasma folate was lower in AG than AA (normal homozygote) participants at ≤12 GW. In spite of the aforementioned molecular implications of the polymorphism, most human studies report no effect of the polymorphism on plasma/serum folate (*Introduction* point *3.4*). Our result in the heterozygote is counterintuitive because

MTRR is a monomer (Olteanu and Banerjee 2001), so differences in the analyte concentrations among the three genotypes would be expected to follow a pattern where the heterozygote has intermediate concentrations than the variant and normal homozygotes. However, there are reports of differences in plasma cobalamin in heterozygotes than in homozygotes (Botto et al. 2003; García-Minguillán et al. 2014), suggesting a different effect specific to heterozygotes. As the difference in plasma folate in RTBC heterozygotes only occurred at one time point and the model was borderline significant, this result could be a chance finding.

Most human studies also find no effect of the variant MTRR on RBC folate concentrations, and our result in the RTBC was in line with them. The longitudinal changes in RBC folate followed the pattern of an increase between ≤12 and 15 GW, and a decrease from 15 GW on, but with the concentrations at 24-27 or 34 GW non different to ≤12 GW (data not shown). This RBC folate pattern was not different among the *MTRR* c.66A>G genotypes.

Higher plasma tHcy was observed in variant homozygotes than normal homozygotes at ≤12 and 15 GW, which is line with the commented putative metabolic outcomes of lower affinity of variant MTRR for MTR, but not with most human studies that find no effect. In a non-pregnant population from the same geographical region as the RTBC, a trend towards higher plasma tHcy in variant allele carriers was found when plasma cobalamin status was low (≤273pmol/L) and riboflavin optimal (García-Minguillán et al. 2014). However, after stratifying by plasma cobalamin tertiles, we did not find an effect on tHcy in the RTBC. Plasma folate interacted with the SNP effect on tHcy at early and mid pregnancy; and when stratifying by plasma folate tertiles, higher plasma tHcy was found in variant homozygotes than normal homozygotes at ≤12 and 15 GW only in the second tertile.

We hypothesise that in the first plasma folate tertile of early pregnancy, the small effect of the variant MTRR on tHcy is covered by the upregulation of the BHMT pathway that occur under low folate status conditions. This would explain also why the effect of the

SNP on tHcy is not observed at mid and late pregnancy when folate status is lower than at early pregnancy, and homogenously low compared to the more variable values of early pregnancy. In line with this in a Spanish non pregnant population with no supplement use and 11.5nmol/L plasma folate mean (possibly deficient), no independent effect of the *MTRR* c.66A>G polymorphism on tHcy was reported (García-Minguillán et al. 2014; Bueno et al. 2016). We speculate that although the folate statuses of the first tertiles at ≤12 or 15 GW, of the mid-late pregnancy period and of the Spanish non pregnant population are not similarly low; all of them are below a threshold at which the effect of the SNP on tHcy is not observed.

As for the absence of effect on plasma tHcy in the highest plasma folate tertile of early pregnancy, we hypothesise that the very high folate status at this pregnancy stage leads to higher SAM, SAM/SAH ratio and lower SAH concentrations resulting in the remediability of the SNP defect in the enzyme. There is some evidence that folate status can affect these metabolites. In an Alzheimer disease patients sample, those with supplemental treatment of folic acid (1.25 mg/d) for 6 months had higher plasma SAM and SAM/SAH ratio than the untreated group (Chen et al. 2016a). In healthy individuals following the same diet, dietary folate and RBC SAM concentration and SAM/SAH ratio were positively correlated (Poirier et al. 2001). In micropigs fed a folate deficient diet higher hepatic SAH concentration and lower SAM/SAH ratio (Halsted et al. 2002), and lower expression and activity of hepatic methionine adenosyltransferase 1A and AHCY enzymes were found (Villanueva and Halsted 2004). Given that SAM is the methyl donor of the reaction catalysed by MTRR (Foster et al. 1964; Ludwig and Matthews 1997), it can be expected that higher SAM levels occurring with high folate status correct to a certain extent a defect in the variant MTRR. Lower SAH concentration could also add to this putative correction as SAH inhibits many methyltransferases using SAM (Clarke and Banfield 2001), but whether SAH can inhibit MTRR has not been investigated yet to the best of our knowledge.

Plasma folate and tHcy throughout pregnancy had the longitudinal patterns described previously, but in normal homozygotes (AA) the folate decrease and tHcy increase were observed earlier. The interpretation of this finding is unclear because in the analyses at each time point whether no effect, or lower plasma folate in AG compared to AA, or higher tHcy in GG compared to AA, were observed. A possibility is that normal MTRR is more susceptible to the pregnancy-induced changes in plasma folate and tHcy than the variant MTRR; but this idea does not correspond with the reported higher affinity for MTR in the normal compared to the variant MTRR (Olteanu et al. 2002; Wolthers and Scrutton 2007).

According to the differences in affinity for MTR, the *MTRR* c.66A>G polymorphism could imply impaired conversion of free or MTR-bound cob(II)alamin to MTR-bound methylcob(III)alamin, and of MTR-bound aquacob(III)alamin to MTR-bound cob(II)alamin. However, the conversion of free aquacob(III)alamin to free cob(II)alamin is probably unaffected. Plasma cobalamin mainly occurs as methylcobalamin with less as adenosylcobalamin and the rest as other forms (Linnell et al. 1974; Gimsing et al. 1983). Methylcobalamin and adenosylcobalamin are mostly bound to transporter proteins in blood (Nexo 1977). It is currently assumed that non-protein bound cobalamin forms are exported from the cell by the ATP-binding cassette transporter C1 (ABCC1) and other unknown transporters (Beedholm-Ebsen et al. 2010; Nielsen et al. 2012). The transport mediated by ABCC1 involves the formation of glutathionyl-cobalamin (Beedholm-Ebsen et al. 2010), suggesting this that methylcobalamin or adenosylcobalamin cannot be exported by this mechanism. However, other unknown export mechanisms of free cobalamin are assumed to exist, and this could account for the high prevalence of methyl and adenosylcobalamin in plasma. As not much is known about cobalamin metabolism, the potential implications of the *MTRR* c.66A>G polymorphism on plasma cobalamin concentrations are difficult to elucidate. It is possible that a higher proportion of free cob(II)alamin instead of MTR-bound methylcob(III)alamin occurs with variant MTRR, leading to extracellular cobalamin release at a higher rate than with the normal MTRR. We indeed found higher

plasma cobalamin in variant homozygote participants than in normal homozygotes. However this was only found at 24-27 GW and could be a chance finding.

Plasma cobalamin decreased throughout pregnancy in the three *MTRR* c.66 genotypes. This pattern has been previously reported in the RTBC (Fernàndez-Roig et al. 2013) and other studies (Bartels et al. 1989; Cikot et al. 2001; Koebnick et al. 2002; Velzing-Aarts et al. 2005; Murphy et al. 2007; Wallace et al. 2008; Ubeda et al. 2011; Wu et al. 2013; McNulty et al. 2013).

The concentration of these analytes in cord plasma was not affected by the maternal *MTRR* c.66A>G polymorphism. This finding cannot be compared to other studies as such investigation has never been carried out to the best of our knowledge.

In MTRR deficient mutant mice, the dams had a higher frequency of embryo resorption and lower birth weight of the offspring than in wild type dams (Deng et al. 2008). There were no differences in the frequency of miscarriage or IUGR cases among the three maternal *MTRR* c.66A>G genotypes in the RTBC, and these results are in line with previous human studies (Furness et al. 2008; Kim et al. 2011). Also the MTRR deficient mutant mice offspring of Deng *et al*. had lower birth weight than wild type offspring (Deng et al. 2008). We found no association either between IUGR and offspring *MTRR* c.66A>G genotypes, which agrees with a previous human study (Furness et al. 2008). The *MTRR* c.66A>G polymorphism effect is probably not as severe as the mutation in the aforementioned animal study.

5. Strengths and limitations

A strength of this study is its longitudinal design and follow up from very early pregnancy. It was carried out in the absence of mandatory fortification with folic acid and therefore the effects of changing folate status due to prenatal folic acid supplement use were observed. Blood samples were collected under fasting conditions (except at delivery), and processed according to specific protocols to prevent artefacts in plasma analyte concentrations caused by temperature and time before separation of blood

cells. Pregnant women with chronic diseases and surgical interventions affecting nutritional status, and medical treatment known to affect 1C metabolism were not included in the RTBC.

Plasma/serum creatinine is a marker of glomerular filtration rate which can affect plasma metabolites and nutrient concentrations, such as tHcy. As a result of the pregnancy induced hyperfiltration state, plasma/serum creatinine decreases during early pregnancy (de Weerd et al. 2003; Ogueh et al. 2011), being lower at 10 GW and remaining at this low concentration 6 weeks postpartum (Ogueh et al. 2011). Rather than specifically adjusting for changes in renal function, haemodilution and albumin decline we adjusted the analyses by gestational age at the time of blood draw. This allowed us to reduce the number of covariables included in the mutivariate models. Further adjustment by plasma creatinine did not change the results or conclusions (data not shown).

As MTHFR and MTRR have riboflavin cofactors, plasma riboflavin or riboflavin status markers might affect the SNPs effects. In *in vitro* studies, the normal and variant MTHFR enzymes are protected against heat inactivation when incubated with FAD (Yamada et al. 2001). In South Korean pregnant women at 24-28 GW higher serum tHcy in *MTHFR* variant allele carriers was only found below serum FAD median (Kim et al. 2004). We have previously reported in the non pregnant population of Catalonia that the effect of *MTHFR* c.665C>T polymorphism on homocysteine was increased with deficient or marginally deficient riboflavin status (García-Minguillán et al. 2014). In García-Minguillán *et al*. there was a trend toward higher plasma tHcy in *MTRR* variant allele carriers when plasma cobalamin was low and riboflavin status was not deficient nor marginally deficient (García-Minguillán et al. 2014). Adjusting by riboflavin status, as Erythrocyte Glutathione Reductase Activation Coefficient, did not change the results or conclusions on *MTHFR* and *MTRR* polymorphisms (data not shown).

Smoking has been associated with the concentration of 1C analytes in some studies. In non pregnant subjects, there are studies reporting slightly higher serum/plasma tHcy

in smokers while others did not find an effect, as discussed (Pagán et al. 2001). In pregnant smokers plasma tHcy was higher at 18 GW in a Norwegian study (Bjørke-Monsen et al. 2013) and at 11-16 GW in a Dutch study (Bergen et al. 2012). However, no differences were reported at 18 and 30 GW in prefortification USA (Pagán et al. 2001), and in a Canadian study between the first and second trimester (McDonald et al. 2002). In some studies plasma/serum folate was lower in pregnant smoker (Pagán et al. 2001; McDonald et al. 2002; Bergen et al. 2012; Bjørke-Monsen et al. 2013; Krikke et al. 2016), but others reported no effect of smoking (Pagán et al. 2001; Shen et al. 2016). Similarly, in whole blood/RBC folate a lowering effect in pregnant smokers has been found in more studies (Furness et al. 2012; Vandevijvere et al. 2012; Shen et al. 2016) than the studies reporting no effect (McDonald et al. 2002; Prasodjo et al. 2014). In our analyses a further adjustment by smoking habit did not alter the results for *MTHFR*, *BHMT*, *SLC19A1* and *MTRR* polymorphisms (data not shown).

The foetal genotypes of the addressed SNPs might be relevant for the cord plasma concentration of 1C analytes. Foetal *MTHFR* c.665 genotype was reported to affect cord plasma folate (Lopreato et al. 2008; Plumptre et al. 2015), RBC folate (Relton et al. 2005; Plumptre et al. 2015), but not tHcy (Molloy et al. 2002; Lopreato et al. 2008; Plumptre et al. 2015). Cord plasma concentrations of betaine and DMG were not different among foetal genotypes of *BHMT* c.716G>A (Visentin et al. 2015), as was reported for amniotic fluid tHcy among *MTRR* c.66A>G genotypes (Brouns et al. 2008). Also, the foetal *SLC19A1* c.80G>A polymorphism did not affect cord RBC folate (Relton et al. 2005), nor placenta plasma tHcy, serum folate or cobalamin (Lopreato et al. 2008). No significant independent effect of the foetal genotypes for the four SNPs on cord plasma analytes concentration was found, and adjusting by foetal genotype did not change the results observed for the maternal genotypes (data not shown).

Cobalamin intake has not been taken into account in *MTRR* c.66A>G analyses and could have affected the results on plasma cobalamin concentration. We do not expect big

differences in dietary cobalamin among participants as the vegetarian dietary pattern is very rare in the RTBC study.

Many hypotheses arising from the results of this thesis involve SAM and SAH; and different folate forms in contrast to the used "total" folate. These analytes are highly unstable and due to logistic and methodological reasons their determination is not possible in this large scale study. With the existing limitations to date, it would be more feasible to explore these hypotheses in animal models. Also, the concentrations of these and other 1C analytes in cells and tissues such as the liver, could help to understand the effects of the four polymorphisms.

PLACENTA TRACE ELEMENT STUDY RESULTS

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

Placenta trace element study results

1. Overview of specimen collection and trace element concentrations

Placenta samples from 212 out of the 562 RTBC pregnancies that went on to have live births were collected and stored at -80ºC. Placentas were also collected from 6 other participants that were recruited on admission to hospital with confirmed labour and therefore are not included in the section on the genetic study. Tissue blocks from the foetal side were cut, rinsed and digested in acid prior to determining the trace element concentrations.

The characteristics of these participants are described in **Table 19** and were similar to the whole RTBC. The median plasma concentrations of 1C metabolism nutrients and metabolites relevant to the thesis are also shown in **Table 19** .

Placenta zinc, copper, selenium and iron concentrations were determined. About thirty eight percent of placentas had selenium below the limit of detection (see *Material and methods* point *3.2.*, **Table 10**). Placenta selenium had a non-parametric distribution (data not shown), so ln transformation was applied to all of the trace element variables for the following analyses. Geometric mean concentrations are reported in **Table 20**.

Table 19. Lifestyle, obstetrical, clinical and 1C metabolism status characteristics of participants with collected placenta sample (n=218).

Age (y) [218][1]		32.1 (31.5, 32.7)
Body mass index at 1st trimester (kg/m^2) [209][1]		24.0 (23.4, 24.6)
Planned pregnancy [206][2]		79.1 (73.1, 84.1)
Previous pregnancy [218][2]		56.4 (49.8, 62.8)
Socioeconomic status [215][2,3]	High	40.0 (33.7, 46.7)
	Mid	52.1 (45.4, 58.7)
	Low	7.9 (5.0, 12.3)
Folic acid supplement use[2]	Preconception [203]	37.9 (31.5, 44.8)
	First trimester [177]	98.9 (96.0, 99.7)
	Throughout pregnancy [200]	50.0 (43.1, 56.9)
Smoking during pregnancy[2]	Periconception [218]	28.4 (22.9, 34.8)
	Throughout pregnancy [218]	19.7 (15.0, 25.5)
Regular alcohol consumption[2]	Periconception [204]	42.6 (36.1, 49.5)
	Throughout pregnancy [205]	11.7 (8.0, 16.8)
Illegal drug use[2]	Periconception [207]	3.4 (1.6, 6.8)
	Throughout pregnancy [207]	0.0 (0.0, 1.8)
1st trimester 1C nutrient status		
Plasma folate (nmol/L) [209][1]		32.8 (28.0, 37.7)
Plasma cobalamin (pmol/L) [209][1]		378.7 (363.6, 394.1)
Plasma total homocysteine (µmol/L) [209][1]		5.3 (5.2, 5.5)
Plasma choline (µmol/L) [209][1]		7.4 (7.2, 7.6)
Plasma betaine (µmol/L) [209][1]		22.1 (21.1, 23.1)
Pregnancy induced hypertension [165][2]		7.9 (4.7, 13.0)
Gestational diabetes [176][2]		6.8 (3.9, 11.5)
Late pregnancy anaemia [205][2]		31.7 (25.7, 38.4)
Gestational age at labour (weeks) [218][1]		38.9 (38.7, 39.1)
Birth weight (g) [218][1]		3164.7 (3105.7, 3223.7)
Offspring male sex [218][2]		47.2 (40.7, 53.9)
Preterm delivery [218][2]		3.7 (1.9, 7.1)
IUGR [218][2]		9.2 (6.0, 13.7)

Values are [1]arithmetic means or [2]percentages (95% confidence interval) [sample size]; [3]based on total income, occupation and education level of both parents.

Table 20. Placenta trace element geometric mean concentrations (n=212).

Trace element	Geometric Mean	(95% CI)
Zinc (ng/g ww)	6732	(6602, 6866)
Copper (ng/g ww)	540	(526, 555)
Selenium (ng/g ww)	77.8	(72.5, 83.6)
Iron (µg/g ww)	60.5	(57.0, 64.3)

2. Correlations among placenta trace element concentrations

There were weak but significant positive correlations between placenta zinc, copper and selenium (**Table 21**). Placenta iron concentration was not associated with any of these.

Table 21. Placenta trace element concentration correlations (n=218).

	Zinc	Copper	Selenium	Iron
Zinc	-	0.36[1]**	0.16*	0.13
Copper		-	0.28**	0.03
Selenium			-	0.02

[1]Spearman correlation coefficients. Significant correlations: *p<0.05, **p<0.01.

3. Maternal and foetal characteristics and placenta trace element concentrations

3.1. Pregnancy smoking patterns and placenta trace element concentrations

Zinc, copper, selenium and iron concentrations were compared between never smokers, first trimester only smokers or smokers throughout pregnancy (**Table 22**).

Table 22. Pregnancy smoking patterns and placenta trace element concentrations.

	Non-smokers [156][1]	Smokers in the first trimester only [19]	Smokers throughout pregnancy [43]
Zinc (ng/g ww)	6761 (6619, 6906)[2]	6620 (6051, 7230)	6785 (6452, 7135)
Copper (ng/g ww)	530 (516, 546)	568 (515, 626)	565 (526, 606)
Selenium (ng/g ww)	75.0 (69.0, 81.6)	84.8 (63.2, 114.0)	89.7* (77.8, 103.4)
Iron (µg/g ww)	60.8 (56.5, 65.5)	60.4 (50.5, 72.3)	59.7 (53.5, 66.5)

[1]Sample size. [2]Trace element concentration geometric mean (95% confidence interval). T-test: *p<0.05 smokers throughout pregnancy vs non-smokers.

Placenta selenium concentration was higher in smokers throughout pregnancy than in non-smokers. Limiting the results to first trimester smoking versus never smoking, both copper and selenium concentrations were higher in first trimester smokers than non-smokers: 566 vs 530 ng/g ww (P<0.05) and 88.2 vs 75.0 ng/g ww (P<0.05), respectively.

3.2. Correlations between obstetric, lifestyle and demographic parameters, and placenta trace element concentrations

Correlations between birth weight, gestational age at birth, maternal (at ≤12 and 24-27 GW), cord plasma cotinine concentrations, maternal age and BMI at first antenatal check-up, and each trace element concentration are reported in **Table 23**. Several weak but significant correlations were detected. Birth weight and placental copper, zinc and selenium concentrations were negatively correlated. Plasma cotinine at 24-27 GW was positively correlated with placenta copper. Gestational age and maternal age were negatively correlated with placenta zinc. Placenta iron was not correlated with any of the analysed parameters.

Table 23. Correlations between obstetric, lifestyle and demographic parameters, and placenta trace element concentrations.

	Birth weight	Gestational age	Plasma cotinine concentrations			Maternal age	First trimester maternal BMI
			≤12 GW	24-27 GW	Cord		
	[218][1]	[218]	[209]	[169]	[204]	[218]	[209]
Zinc	-0.27[2]***	-0.14*	0.12	0.08	0.02	-0.19**	-0.03
Copper	-0.23***	0.01	0.09	0.17*	0.09	0.01	0.04
Selenium	-0.17**	-0.06	0.09	0.08	0.09	-0.001	-0.05
Iron	-0.03	0.04	-0.05	-0.05	-0.05	0.04	-0.10

GW, gestational weeks. BMI, body mass index. [1]Sample size varies due to missing information, blood sample or plasma cotinine determination. [2]Spearman correlation coefficients. Significant correlations: *p<0.05, **p<0.01, ***p<0.001.

3.3. Socioeconomic level and placenta trace element concentrations

No differences in placenta trace element concentrations were detected between low and mid socioeconomic status levels (**Table 24**). However, placenta iron concentration was higher in participants with high compared to low socioeconomic status

Table 24. Socioeconomic level and placenta trace element concentrations.

	Low [17][1]	**Mid [112]**	**High [86]**
Zinc (ng/g ww)	6762 (6302, 7257)[2]	6749 (6560, 6943)	6717 (6523, 6917)
Copper (ng/g ww)	548 (497, 604)	547 (524, 570)	530 (513, 549)
Selenium (ng/g ww)	80.8 (64.4, 101.4)	75.1 (67.8, 83.1)	80.8 (72.3, 90.4)
Iron (µg/g ww)	51.2 (41.0, 63.8)	59.8 (54.4, 65.8)	63.4* (59.1, 68.1)

[1]Sample size. Missing socioeconomic data in 3 pregnancies. [2]Trace element concentration geometric mean (95% confidence interval). T-test: *p<0.05 high vs low socioeconomic level.

3.4. Neonate sex and placenta trace element concentrations

Trace element concentrations from placentas of male or female neonates were compared (**Table 25**). Placenta copper concentration was higher in female neonates, although this did not reach significance (P=0.058). Zinc concentration was higher in placentas from female neonates. No differences were detected for selenium and iron concentrations.

Table 25. Neonate sex and placenta trace element concentrations.

	Male [103][1]	**Female [115]**
Zinc (ng/g ww)	6591 (6411, 6776)[2]	6902* (6720, 7089)
Copper (ng/g ww)	526 (506, 547)	553# (534, 573)
Selenium (ng/g ww)	75.7 (68.3, 84.0)	81.1 (73.6, 89.4)
Iron (µg/g ww)	57.9 (52.3, 64.2)	63.0 (59.1, 67.2)

[1]Sample size. [2]Trace element concentration geometric mean (95% confidence interval). T-test: *p<0.05, #p=0.058 female vs male neonate placenta.

3.5. Intake of trace elements from supplements and food during pregnancy

Data on supplement intake during the first trimester of pregnancy was available from 204 participants. Of these, 14.2% (95% CI: 10.1, 19.7%) took supplements containing copper, zinc, selenium or iron and of these, 55.2% (95% CI: 37.5, 71.6%) took iron only. In the second and third trimester of pregnancy based on data from 200 participants, 74.5% (95% CI: 68.0, 80.0%) took supplements containing the aforementioned trace elements and 77.9% (95% CI: 70.5, 83.8%) of these took iron only. The patterns of use of supplements containing each of the addressed trace elements are shown in **Table 26**.

Table 26. Trace element supplement use.

	First trimester users [204][1]	Second and third trimester users [200]
Zinc containing supplements	6.4 (3.8, 10.6)[2]	16.5 (12.0, 22.3)
Copper containing supplements	0.5 (0.1, 2.7)	3.0 (1.4, 6.4)
Selenium containing supplements	3.9 (2.0, 7.5)	12.0 (8.2, 17.2)
Iron containing supplements	12.7 (8.8, 18.0)	74.5 (68.0, 80.0)

[1]Sample size. [2]Percentages (95% confidence interval).

Trace element intake from supplements, food and the combination of both, throughout pregnancy and the percentage of the sample that met the RDA or was above the UL; are reported in **Table 27**. Most participants met the RDA for selenium, but this was not the case for zinc, copper and iron. About 44.6% (95% CI: 37.9, 51.5%) of participants did not meet the RDA for iron. Intakes above the UL were only observed for iron.

3.6. Trace element supplement use and placenta trace element concentrations

Users of supplements containing trace elements in the first trimester had borderline lower placenta selenium concentrations than non users (**Table 28**). And users of these supplements in the second and third trimesters had lower placenta copper and borderline higher iron concentrations.

Table 27. Trace element intake from supplements, food, and the combination of both, during the complete pregnancy and participant with intakes meeting the RDAs and above the ULs.

	Intake from supplements	Intake from food	Total intake	Intake meeting RDA	Intake above UL
	[200][1]	[204]	[204]	[204]	[204]
Zinc (mg)	270 (170, 370)[2]	2574 (2454, 2694)[2]	2839 (2676, 3001)[2]	51.0 (44.2, 57.8)[3]	0.0 (0.0, 1.8)[3]
Copper (mg)	3.1 (0.2, 6.0)[2]	293.4 (274.9, 311.9)[2]	296.5 (277.8, 315.1)[2]	76.5 (70.2, 81.8)[3]	0.0 (0.0, 1.8)[3]
Selenium (µg)	905 (503, 1306)[2]	26715 (25439, 27991)[2]	27602 (26231, 28972)[2]	98.0 (95.1, 99.2)[3]	0.0 (0.0, 1.8)[3]
Iron (mg)	3992 (3860, 5260)[2]	2264 (2160, 2368)[2]	6480 (5786, 7175)[2]	44.6 (37.9, 51.5)[3]	11.3 (7.6, 16.3)[3]

RDA, recommended dietary allowance (zinc: 11mg/d, copper: 1mg/d, selenium: 60µg/d, iron: 27mg/d); UL, tolerable upper intake level (zinc: 40mg/d, copper: 10mg/d, selenium: 400µg/d, iron: 45mg/d) (Institute of Medicine - Panel on Dietary Antioxidant and Related Compounds 2000; Institute of Medicine - Panel on Micronutrients 2001). [1]Sample size. [2]Arithmetic mean intakes (95% confidence interval). [3]Percentage of participants (95% confidence interval).

Table 28. Trace element supplement use and placenta trace element concentrations.

	First trimester		Second and third trimester	
	Non-users [175][1]	**Users [28]**	**Non-users [51]**	**Users [148]**
Zinc (ng/g ww)	6746.82 (6607.42, 6889.16)[2]	6500.95 (6132.19, 6891.89)	6753.30 (6509.72, 7005.99)	6715.87 (6556.74, 6878.85)
Copper (ng/g ww)	544.98 (528.76, 561.70)	509.01 (479.09, 540.80)	570.12 (544.68, 596.76)	530.27* (512.74, 548.40)
Selenium (ng/g ww)	81.15 (74.98, 87.82)	66.50# (55.57, 79.56)	85.03 (73.10, 98.90)	76.12 (70.04, 82.74)
Iron (µg/g ww)	59.26 (55.32, 63.48)	66.40 (58.24, 75.71)	52.13 (42.89, 63.35)	63.41§ (60.18, 66.82)

[1]Sample size. [2]Trace element concentration geometric mean (95% confidence interval). T-test: *p<0.05, §p=0.056, #p=0.051 user vs non-user placenta.

3.7. Lifestyle, demographic and pregnancy predictors of placenta trace element concentrations

Birth weight, gestational age at time of delivery, pregnancy smoking patterns, maternal age, first trimester maternal BMI, and total trace element intake (for each specific trace element) were studied as predictors of placenta trace element concentrations in the RTBC by multiple linear regression models. The models were not significant for zinc, selenium and iron (data not shown). Placenta copper was positively predicted by gestational age and maternal BMI, and negatively by birth weight (**Table 29**).

Table 29. Lifestyle, demographic and pregnancy predictors of placenta copper concentrations (n=200)[1].

Independent variables	B coefficient (SE)	p value
Birth weight (g)	-0.18×10^{-3} (0.04×10^{-3})	<0.001
Gestational age (GW)	0.03 (0.01)	<0.01
Smoking in the first trimester	0.06 (0.05)	0.241
Smoking throughout pregnancy	0.04 (0.04)	0.282
Maternal age (y)	0.04×10^{-1} (0.03×10^{-1})	0.231
First trimester maternal BMI (kg/m^2)	0.07×10^{-1} (0.03×10^{-1})	<0.05
Total copper intake (mg)	0.06×10^{-3} (0.10×10^{-3})	0.563

[1]Sample size. Missing data in any of the independent variables in 18 pregnancies. SE, standard error. GW, gestational weeks. BMI, body mass index. Multiple linear regression model: dependent variable placenta copper concentration (ng/g ww). Adjusted R^2=9.8, F=4.1, p_{model}<0.001.

Models limited to gestational age, birth weight and either trace element total intake or intake from foods or intake from supplements yielded similar results (data not shown).

4. One carbon metabolism components as predictors of placenta trace element concentrations

The possible effects of elements of 1C metabolism during early and late pregnancy on placenta trace element concentrations (dependent variables) was studied taking into account plasma folate status at early pregnancy, and plasma tHcy, betaine, choline and cobalamin at ≤12 GW and at labour (independent variables). Multiple linear regression models were used and none was significant for zinc (adjusted R^2 from 0.4 to 2.8), selenium (adjusted R^2 from 1.4 to 3.7) and iron (adjusted $R^2 = 0$) (data not shown). Plasma cobalamin at ≤12 GW and plasma tHcy at labour were negative and positive predictors, respectively, of placenta copper concentration (**Table 30**). None of the other 1C metabolism analytes or folate categories affected placenta copper.

Table 30. Plasma cobalamin and tHcy as predictors of placenta copper concentrations.

	Models			
Independent variables	**Adjusted R^2**	**F [n]**	**B coefficient (SE)**	**p value**
Plasma cobalamin at ≤12 GW (pmol/L)[1]	8.9	3.0 [200]**	-0.259×10^{-3} (0.126×10^{-3})	<0.05
Plasma cobalamin at ≤12 GW (pmol/L)[2]	9.1	3.0 [200]**	-0.270×10^{-3} (0.125×10^{-3})	<0.05
Plasma tHcy at labour (µmol/L)	9.8	4.0 [192]***	0.016 (0.008)	<0.05
Plasma tHcy at labour (µmol/L)[3]	9.2	3.2 [192]**	0.017 (0.008)	<0.05

Multiple linear regression analyses: dependent variable placenta copper concentration (ng/g ww). All models were adjusted for birth weight, gestational age at time of delivery, pregnancy smoking patterns, previous pregnancies, total copper intake, [1]low or high early pregnancy plasma folate status (plasma folate tertile based categories) and plasma betaine at ≤12 GW, or [2]low or high early pregnancy plasma folate status and plasma choline at ≤12 GW, or [3]low or high early pregnancy plasma folate status. n, sample size varies due to missing data in any of the independent and adjusting variables. SE, standard error. GW, gestational weeks. **p_{model}<0.01, ***p_{model}<0.001.

5. Placenta trace element concentrations and adverse pregnancy outcome

5.1. Placenta trace element concentrations in pregnancies with preterm deliveries

Trace element concentrations from placentas of control or preterm deliveries were compared and no differences were observed (**Table 31**).

Table 31. Preterm deliveries and placenta trace element concentrations.

	Controls [181][1]	**Preterm [8]**
Zinc (ng/g ww)	6681 (6533, 6831)[2]	7000 (6493, 7547)
Copper (ng/g ww)	541 (525, 558)	537 (482, 599)
Selenium (ng/g ww)	76.1 (70.4, 82.3)	109.3 (73.9, 161.7)
Iron (µg/g ww)	61.2 (57.3, 65.5)	50.4 (32.1, 79.1)

[1]Sample size. [2]Trace element concentration geometric mean (95% confidence interval). Mann-Whitney U test for independent samples.

5.2. Placenta trace element concentrations in intrauterine growth restriction-affected pregnancies

Trace element concentrations from placentas of control or IUGR neonates were compared (**Table 32**). The percentage of IUGR neonates in the sample was 11.0% (95% CI: 7.2, 16.4%). Placenta copper and selenium concentrations were higher in IUGR neonates.

Table 32. IUGR-affected pregnancies and placenta trace element concentrations.

	Controls [162][1]	**IUGR [20]**
Zinc (ng/g ww)	6656 (6508, 6807)[2]	6926 (6389, 7509)
Copper (ng/g ww)	528 (514, 542)	611*** (541, 689)
Selenium (ng/g ww)	76.1 (70.1, 82.7)	102.3* (80.3, 130.4)
Iron (µg/g ww)	59.4 (55.2, 64.0)	63.9 (55.6, 73.3)

[1]Sample size. [2]Trace element concentration geometric mean (95% confidence interval). T-test: *p<0.05, ***p<0.001 IUGR vs control neonate placenta.

The effect of placental copper and selenium concentrations on IUGR risk were studied using multiple logistic regression analysis (**Table 33**). Placenta copper in the highest tertile (≥574.3ng/g ww) compared to the other tertiles was associated with an increase in probability of IUGR.

Table 33. Risk of IUGR according to placenta copper and selenium concentrations[1].

	R^2 [n]	**IUGR risk**
Copper	22.3 [176]**	4.1 (1.4, 12.6)[2]
Selenium	16.5 [176]	1.8 (0.66, 5.3)

[1]Multiple logistic regression. Models for placenta copper and selenium adjusted for smoking patterns at first trimester and throughout pregnancy, maternal age and maternal body mass index at first trimester. [2]Odds ratios (95% confidence interval) were estimated for placenta copper or selenium concentrations in the highest tertiles versus the other tertiles. n, sample size. p_{model}**<0.01.

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

PLACENTA TRACE ELEMENT STUDY DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

Placenta trace element study discussion

1. Zinc

The median placenta zinc concentration was 6809ng/g ww. Medians reported in other studies using ICP-MS are higher: 11579ng/g ww in Bangladesh (Kippler et al. 2010), 12630ng/g ww in China (Liu et al. 2013), 8017ng/g ww in Japan (Sakamoto et al. 2013), and 10461ng/g ww in Sweden (Osman et al. 2000). As the concentrations of the other addressed trace elements in the RTBC were also lower or slightly lower than in other studies using the same techniques, a comment on this can be made. In our study, placenta samples whose shortest dimension was 0.5-1cm, were thoroughly washed with deionised water to remove residual blood. Some of the other studies do not specify whether they cleaned the samples (Liu et al. 2013) or whether washing with water or saline was included in the process (Osman et al. 2000; Kippler et al. 2010). The median placenta zinc, copper and selenium concentrations, most similar to ours, were reported by Sakamoto *et al*. They washed their samples five times with saline solution (Sakamoto et al. 2013). Paper, which can potentially contaminate the samples, was used for blotting the specimens in two studies (Osman et al. 2000; Sakamoto et al. 2013), unlike in ours. Furthermore, the chorionic plate was discarded in some of these studies (Osman et al. 2000; Kippler et al. 2010; Sakamoto et al. 2013), unlike the RTBC samples that included the chorionic plate in addition to the chorionic villi. Studies comparing foetal side specimens including chorionic plate with maternal side specimens including *decidua basalis* have found differences in zinc, copper and iron concentrations (Manci and Blackburn 1987; Ronco et al. 2005; De Moraes et al. 2011), however similar studies comparing the chorionic villi with the chorionic plate are lacking. There is no consensus on placenta specimens collection and handling (Iyengar and Rapp 2001b), making comparisons among studies hard.

Placenta zinc concentration was positively correlated with those of copper and selenium. The association with copper has been previously reported in some (Ward et al. 1987; Zadorozhnaja et al. 2000; Odland et al. 2001), but not other studies (Tsuchiya

et al. 1984; Osman et al. 2000; Kantola et al. 2000; Ozdemir et al. 2009; Serafim et al. 2012). Some reports have described a positive correlation between placenta zinc and selenium (Osman et al. 2000; Punshon et al. 2016) while another reported no correlation (Odland et al. 2001). We assume that the positive correlations we observed are not due to the water content of the placentas because iron was not correlated with zinc, copper or selenium. It can be hypothesised that these three elements accumulate in placenta, specially bound to proteins, to avoid oxidative stress and/or metal toxicity. Superoxide radicals can be converted to hydrogen peroxide by superoxide dismutase 1 which contains zinc and copper; and hydrogen peroxide to water by the selenoproteins glutathione peroxidases (Mistry and Williams 2011). Another enzyme catalysing the former reaction is worth noting, the superoxide dismutase 3, which is extracellular and contains copper and possibly zinc (Marklund 1982; Marklund 1984). Peroxiredoxins in the reduced state can also convert hydrogen peroxide to water, needing to be reduced afterwards by thioredoxins. After the regeneration of peroxiredoxins, thioredoxins also need to be reduced in a reaction involving NADPH and the selenoproteins thioredoxin reductases (Lu and Holmgren 2014). The copper chaperone ATOX1 has antioxidant properties resembling those of superoxide dismutases, although the mechanism is unknown (Hung et al. 1998; Hatori and Lutsenko 2013). Unlike *in vitro* results, metallothionein's antioxidant role *in vivo* remains controversial (Chiaverini and De Ley 2010). Superoxide dismutase 1, glutathione peroxidases (Wang and Walsh 1996), superoxide dismutase 3 (Boggess et al. 1998), thioredoxin reductases (Oblong et al. 1993), the copper chaperone ATOX1 (Gambling et al. 2004), and metallothioneins (Waalkes et al. 1984) expression and/or activity have been detected in placenta. Although with controversy in superoxide dismutases, the aforementioned proteins have been reported to be upregulated by oxidative stress conditions (Shull et al. 1991; Lu et al. 1993; Stralin and Marklund 1994; Dalton et al. 1994; Lin and Culotta 1995; Sun et al. 1999; Park and Rho 2002; Moon et al. 2005). In support of this hypothesis, the two most abundant selenoproteins in placenta are glutathione peroxidases (Sonet et al. 2016).

Metallothioneins bind trace elements that are essential (e.g. copper, zinc) or toxic (e.g. cadmium) *in vivo* (Stillman 1995). Metallothionein can detoxify cadmium directly by the binding of the toxic metal to apometallothionein or replacing zinc atoms (Pinter et al. 2015). Experiments suggest zinc bound to metallothioneins is labile and serves to be effectively donated to maturing metalloproteins whereas cadmium is immobilised (Irvine et al. 2016). However placental zinc retention has been suggested under cadmium exposure. Zinc, copper, cadmium, nickel, lead and arsenic have been reported to upregulate metallothioneins via the metal-transcription factor 1 (Heuchel et al. 1994; He and Ma 2009). Higher concentrations of zinc and copper due to higher metallothionein levels for metal detoxification seems plausible. Selenium has also been implied in metal detoxification, especially of mercury. It has been proposed that methylmercury toxicity (and its associated oxidative stress) is mainly due to the replacement of selenocysteines in selenoproteins, most of which are antioxidant (Arnold et al. 1986). Methylmercury detoxification by selenium seems to rely on organic selenium for methylmercury excretion, inactivation or removal from some organs (Li et al. 2012; Yamashita et al. 2013b; Yamashita et al. 2013a; Li et al. 2014b; Wang et al. 2017), and more selenium and seleno amino acids to compensate the selenium deficiency of proteins (Ralston et al. 2008; Li et al. 2014b). Redistribution of selenium in blood and tissues in response to methylmercury has been reported in fishes (Huang et al. 2014). Selenium is also involved in arsenic and cadmium detoxification, especially through the reduction of reactive oxygen species generated by these toxic metals (Zwolak and Zaporowska 2012). Proteins with roles in oxidative stress and containing selenium have also been reported to be upregulated by zinc, copper or cadmium via the metal-transcription factor 1: selenoprotein H in human amniotic cells (Stoytcheva et al. 2010) and selenoprotein W in rat glial and myoblast cells (Amantana et al. 2002). Although the protein expression of these two selenoproteins has not been studied in placenta, their mRNA expression has been reported (Gu et al. 2000; Dikiy et al. 2007; Gilman et al. 2015). Therefore, we suggest that the positive correlations between zinc, copper and selenium may be due to the

retention of these elements in the placenta as a defensive mechanism against oxidative stress and metal toxicity. We consider that the weak correlations that we observed and the controversy in the bibliography regarding this topic, can be explained as these two adverse conditions can vary in intensity and in the reactive oxygen species and metals involved, leading to differences in the defence mechanisms related with zinc, copper and selenium.

Plasma cotinine and smoking patterns during pregnancy were not associated with placenta zinc concentrations. Our results are in agreement with some studies (Zadorozhnaja et al. 2000; Kantola et al. 2000; Pereg et al. 2001; Sorkun et al. 2007; Punshon et al. 2016), but other studies have reported higher zinc concentrations in the placentas of smokers (Kuhnert et al. 1987b; Ronco et al. 2005; Stasenko et al. 2010). All the studies reporting no association were based on self-reported smokers, but the studies finding an association classified smokers based on plasma thiocyanate (Kuhnert et al. 1987a), urine cotinine (Ronco et al. 2005), or placenta cadmium (Stasenko et al. 2010). We checked smoking status with plasma cotinine at ≤12, 24-27 GW and in the cord. Thiocyanate is considered a less specific marker of smoking than cotinine (Benowitz 1996), but our results differ from those in Ronco *et al*. using urine cotinine. We speculate the intensity of smoking between our study and the studies reporting associations might be different, however whether intensity of smoking affect placental zinc deposition has not been investigated to our knowledge. In addition, other differences in conditions that may affect placenta zinc concentrations through oxidative stress and heavy metal exposure might explain the results. For example, the hospitals of the studies reporting differences (Kuhnert et al. 1987a; Ronco et al. 2005; Stasenko et al. 2010) were in cities about five or eight-fold more populated than Reus or Tarragona. In addition, the chorionic plate was removed in two of the studies (Kuhnert et al. 1987a; Stasenko et al. 2010). The amount of residual blood in the samples is likely to be higher than in the RTBC because the entire placenta (Stasenko et al. 2010), half of it (Ronco et al. 2005), or several cotyledons (Kuhnert et al. 1987a) were the parts of the organ that were washed. Smoking leading to increased zinc content of the placental

blood supply but not in the tissue itself is a possibility. However, studies have reported no effect of smoking on maternal blood zinc concentration (Vir et al. 1981; Kuhnert et al. 1987b; Kantola et al. 2000). Moreover, there is controversy about whether maternal smoking reduces the zinc concentration in RBC of the foetus (Kuhnert et al. 1987b; Kuhnert et al. 1988a) or not (Aydogan et al. 2013). It should also be considered that reported maternal whole blood zinc concentrations range from below two orders of magnitude to the same order as placenta concentrations (Tsuchiya et al. 1984; Schramel et al. 1988; Al-Saleh et al. 2004; Ghneim et al. 2016). The same is true for cord whole blood (Tsuchiya et al. 1984; Schramel et al. 1988; Al-Saleh et al. 2004; Kippler et al. 2010). The reasons for the association between placenta zinc and smoking found in other studies using smoking markers, but not found in the RTBC are unclear.

Zinc was negatively correlated with birth weight and gestational age at delivery, but most studies have not found an association with birth weight (Kantola et al. 2000; Díaz et al. 2002; Odland et al. 2004; Ozdemir et al. 2009) or gestational age (Ward et al. 1987; Custódio et al. 2003; Reddy et al. 2014). Placenta zinc concentration was higher in those of female neonates than in male neonates, but other studies have found the contrary (Punshon et al. 2016) or no association (Osman et al. 2000; Phuapradit et al. 2000). The associations with gestational age and sex can contribute to that with birth weight. When adjusting for possible confounders no association with birth weight or gestational age was observed. Placenta zinc was not associated with IUGR, and this is in agreement with previous studies (Malhotra et al. 1990; Osada et al. 2002; Zadrozna et al. 2009). We speculate that the possible effect of placenta zinc on birth weight is influenced by its association with neonate sex. Given that in human placentas X chromosome inactivation is random and the expression behaviour of the inactive X chromosome can be heterogeneous (Moreira de Mello et al. 2010), if a gene affecting zinc metabolism or its regulation is located in the X chromosome, the effects of genetic variations would be more visible in males. A GWAS studying the main genetic factors associated with the variance in RBC zinc, copper and selenium found only in the case of zinc, a polymorphism in a gene in the chromosome X, i.e. *NBDY* g.56811695C>T. The

allele T was associated with higher RBC zinc (Evans et al. 2013). The *NBDY* gene codes for a protein that acts as an inhibitor of RNA degradation (D'Lima et al. 2016), but its direct link with zinc metabolism is unknown. The allele frequencies in the RTBC and in Evans *et al*.'s study can help test if the differences in the results of placenta zinc and sex could be due to this polymorphism.

We observed a negative correlation between maternal age and placenta zinc, but this association was not observed in multiple linear regression models taking into account birth weight, gestational age, smoking patterns, maternal BMI and zinc intake. There is controversy about whether an association between maternal age and placenta zinc exists and the sense of it (*Introduction* point *5.1*). Unknown factors may affect the relationship between maternal age and placenta zinc in the different populations that have been studied.

Total zinc intake was not associated with placenta zinc concentration. No human study has assessed this to our knowledge, and this is also true for the intake and placenta concentrations of the other addressed trace elements. Zinc supplementation was not associated with maternal blood zinc concentrations in humans (Caulfield et al. 2008), and in animal studies lower placenta zinc concentration was only observed in situations of deficient zinc intake (McKenzie et al. 1975; Fosmire et al. 1977; Masters et al. 1983a). About 50% of the RTBC participants did not meet the zinc RDA, based on the food frequency questionnaires and supplement intake. Only 6.4% in the first trimester and 16.5% in the rest of pregnancy actually took prenatal supplements containing zinc. Without zinc status measurements of these participants we cannot assess whether their zinc status was low. Zinc intakes not meeting the zinc RDA in pregnancy (11mg/d) seem common in populations with low use of micronutrients supplements. Mean total zinc intake ranged 9.8-11mg/d throughout pregnancy in a Spanish study using 3-day weighed food records (Carbone et al. 1992) or a Greek study using food frequency questionnaires (Petrakos et al. 2006). In pregnant women from the NHANES III, median

dietary zinc intake was 9.0mg/d, and 23.0mg/d with the addition of supplement intake (Briefel et al. 2000).

None of the analysed 1C metabolism components was associated with placenta zinc. In human, animal and *in* vitro studies there is controversy about whether concomitant or chronic folic acid affects zinc absorption and status, with slightly more evidence supporting no effect than a negative effect. According to the literature, a link between choline (as acetylcholine) and placenta zinc could exist. 1C metabolism could affect the regulation of zinc metabolism genes via methylation reactions, and indeed a foetal programming effect on liver and kidney zinc concentrations has been reported in rats (Król et al. 2011). Zinc status could also affect 1C metabolism because zinc is a component of BHMT (Millian and Garrow 1998), the main intestinal glutamate hydrolase folate hydrolase 1 (Halsted et al. 1998), DNA methyltransferases (Chuang et al. 1996) and possibly MTR (Matthews and Goulding 1997). The lower polyglutamate than monoglutamate folate absorption in humans after a zinc depletion intervention (Tamura et al. 1978), suggests an effect on folate hydrolase 1. Similar results were obtained in rats (Tamura et al. 1987; Canton et al. 1989), where the other (non-zinc-containing) glutamate hydrolase gamma glutamyl hydrolase is responsible for removal of glutamates from folates in the intestine. Higher glutamate hydrolase activity was reported in an *in vitro* study with pig pancreatic juice when higher concentrations of zinc were present in the medium (Bhandari et al. 1990). Studies in rats link zinc deficiency with increases in hepatic homocysteine (Duerre and Wallwork 1986) and decreases in plasma/serum homocysteine (Hong et al. 2000; Jing et al. 2015). Lower (Tamura et al. 1987; Hong et al. 2000) or non different (Jing et al. 2015) plasma/serum folate in zinc deficient compared to pair fed control rats have been reported. Our results show no association between placenta zinc concentration and 1C metabolism. Zinc deficiency risk in Spain is low (IZiNCG 2004), and in the RTBC a big proportion of folate intake is as folic acid supplements, where folate is monoglutamate. Thus, not observing and association between plasma folate and placenta zinc, potentially mediated by the effect of zinc deficiency on folate absorption, was expected. Moreover,

placenta zinc concentration may not be a marker of global zinc status, because it may be affected by other elements such as oxidative stress and metal toxicity.

2. Copper

The median placenta copper concentration was 534ng/g ww. Medians reported in other studies using ICP-MS are higher: 930ng/g ww in Bangladesh (Kippler et al. 2010), 690ng/g ww in China (Liu et al. 2013), 652ng/g ww in Japan (Sakamoto et al. 2013), and 953ng/g ww in Sweden (Osman et al. 2000). As previously mentioned in the case of placenta zinc, variations in reported placenta trace element concentrations between different studies may occur due to differences in sample collection and processing.

Placenta copper and selenium were positively correlated. As zinc was also associated in the same way with these elements, we speculate that the positive association among these trace elements can be due to all of them being involved in defence against oxidative stress and metal toxicity.

In addition to a positive correlation between placenta copper and plasma cotinine at 24-27 GW, we observed higher placenta copper concentration in first trimester smokers compared to non smokers. This result is contrary to previous studies where smoking status was assessed as self reported by the participants (Zadorozhnaja et al. 2000; Kantola et al. 2000), but also taking into account urine cotinine at delivery (Ronco et al. 2005) or placenta cadmium (Stasenko et al. 2010). Placenta cadmium has not been defined yet as a marker of smoking during pregnancy. Some studies reported no associations between placenta cadmium and smoking (Fagher et al. 1993; Zadorozhnaja et al. 2000; Odland et al. 2001; Terrones-Saldivar et al. 2008). However, there are more studies supporting a positive association between placenta cadmium and maternal smoking status (Roels et al. 1978; van Hattum et al. 1981; Kuhnert et al. 1987b; Lagerkvist et al. 1996; Bush et al. 2000; Osman et al. 2000; Kantola et al. 2000; Pereg et al. 2001; Falcón et al. 2002; Ronco et al. 2005; Sorkun et al. 2007). The reasons for the controversy are unclear, but differences among the studies of other sources of

cadmium exposure such as diet and contamination might be involved. As exposed previously, residual blood was unlikely to affect the placental trace element determinations in the RTBC placentas due to the cleaning step in the sample preparation procedure. This was possibly not the case in the studies reporting no effect of smoking on placenta copper because these studies either do not specify whether they cleaned the samples (Zadorozhnaja et al. 2000; Kantola et al. 2000) or washed bigger parts of the organ compared to the RTBC samples (Ronco et al. 2005; Stasenko et al. 2010). A possibility is that smoking increases the concentration of placental copper but decreases that in the circulating blood, and thus these studies did not observe differences between smokers and non smokers. Studies in women have not reported such smoking effect in blood copper, but absence of effect. Whole blood copper was not different between non pregnant smokers and non pregnant non smokers (Benes et al. 2005). No effect of smoking in maternal serum (Mochizuki et al. 1984; Kantola et al. 2000) and cord RBC (Aydogan et al. 2013) have been reported in pregnancy studies. We speculate whether the smoking effect on placenta copper was diluted due to the probably higher residual blood in the studies not reporting an effect (Zadorozhnaja et al. 2000; Kantola et al. 2000; Ronco et al. 2005; Stasenko et al. 2010). We deem sample size is not the reason for the absence of effect in these studies, because in two of them sample size was very close to ours (Zadorozhnaja et al. 2000; Stasenko et al. 2010). However, the positive association between smokers at least in the first trimester and placenta copper was not observed in smokers during only the first trimester or throughout pregnancy. Moreover, no effect of smoking patterns on placenta copper was observed in multiple linear regression models taking into account birth weight, gestational age, maternal age, maternal BMI and copper intake. Therefore, this finding must be regarded cautiously.

Placenta copper was negatively associated with birth weight in the RTBC and was associated with higher risk of IUGR. As commented in *Introduction* point *5.2*, there are conflicting findings in the literature regarding the association between placental copper and birth weight. Lower (Zadrozna et al. 2009) or similar (Osada et al. 2002) copper

concentrations have been found in placentas of IUGR compared to control neonates. In these two studies the sample size was small (≤51), the participants were self reported non-smokers and removal of residual blood was not specified. In addition in Osada *et al's*. study, the chorionic plate was discarded and not included in the analysis. However, when considering also the studies not assessing IUGR, but birth weight, we could not identify a pattern in the differences between them and our study that might explain the discrepancies. As we adjusted for other variables that can affect birth weight, we consider our approach more rigorous and propose that copper may be retained in the placenta in response to adverse conditions that can limit foetal growth such as oxidative stress or metal toxicity. Unknown factors that may vary in the different populations in the literature may affect the relationship between these two conditions and placenta copper.

Gestational age at delivery was positively associated with placenta copper after adjusting for different confounders. Higher placenta copper has been reported previously when comparing first trimester to term placentas (Kantola et al. 2000), but no differences comparing more advanced gestations, including preterm deliveries. In the analysis without adjustment we did not find an association, which is in line with the bibliography. We did not find differences according to preterm delivery either, but the number of preterm cases was reduced.

BMI and placenta copper were positively associated after adjusting for birth weight, gestational age, smoking patterns, maternal age and copper intake. There have been no previous reports on this to the best of our knowledge. Studies assessing BMI or categories of it and blood copper in non pregnant adults reported controvert results. BMI in adults and plasma/serum copper have been reported to be positively associated (Tungtrongchitr et al. 2003; Abiaka et al. 2003; Ghayour-Mobarhan et al. 2009; Dabbaghmanesh et al. 2011) or not associated (Chen et al. 1997; Rotter et al. 2015; Torkanlou et al. 2016). BMI in adults was positively associated with whole blood copper (Benes et al. 2005), but not associated with RBC copper (Rükgauer et al. 2001;

Ozata et al. 2002). In child studies, BMI and plasma/serum copper have been positively (Lima et al. 2006; Azab et al. 2014), negatively associated (Blazewicz et al. 2013) or not associated (Arvanitidou et al. 2007). Child BMI has been negatively associated with whole blood copper (Blazewicz et al. 2013) and not associated with RBC copper (Lima et al. 2006). Maternal whole blood copper concentrations were negatively associated with BMI at an unspecified time of pregnancy in a study in rural India (Mahanta et al. 2012). In summary, most studies in adults report positive associations between BMI and blood copper in line with the association that we observed in RTBC placentas. BMI and copper status might be related through oxidative stress. Plasma malondialdehyde (a marker of oxidative stress) and BMI were positively correlated in a case control study of obesity in adults (Olusi 2002). Higher RBC superoxide dismutase 1 activity was found in obese compared to control children (Erdeve et al. 2004), but the contrary was reported in adults (Olusi 2002). None of the aforementioned studies assessing BMI and blood copper have found differences in RBC copper according to BMI. We speculate whether higher levels of antioxidant copper proteins in plasma (such as superoxide dismutase 3) or placenta occur with increasing BMI, but such hypothesis has not been tested to the best of our knowledge in humans. In leptin mutant obese mouse model higher superoxide dismutase 3 protein levels were observed in plasma, compared to the wild type strain (Nakao et al. 2000).

Unlike previous reports in animal studies assessing diets ranging from copper deficient to with twice the copper requirements (Masters et al. 1983a; Ebesh et al. 1999; Andersen et al. 2007; Marques et al. 2016), total copper intake was not associated with placental copper concentration in our study. Copper intake may not have varied sufficiently among the participants to affect placenta copper concentrations. Moreover, adverse conditions such as oxidative stress and metal toxicity may be more important determinants of placenta copper concentrations and mask the effect of the intake on placenta copper. We found lower copper concentrations in the placentas of users of multivitamin supplements containing trace elements during the second and third trimester. About 78% of these users at this period took supplements containing only

iron (and in the ferrous state) out of the four trace elements. An interaction between iron and copper could exist. However, placenta iron and copper were not correlated, the uptake mechanisms in the intestine and placenta are different for both elements, and placenta copper was not affected by total copper intake. The ferroxidase enzymes, i.e. hephaestin in the enterocyte basolateral membrane, ceruloplasmin in blood plasma and liver, and zyklopen in the placenta; are copper containing enzymes necessary for non haem iron transport (*Introduction* point *5.2*). We hypothesise that the higher iron intake in these users might lead to copper redirection from the placenta to the blood, liver and intestine, with the exception of the copper for the zyklopen enzyme that will be retained in the placenta. The proportion of copper released from the placenta to the bloodstream, liver and intestine would be higher than that retained by zyklopen. In a pregnant rat study with diets from iron deficient to low (iron as 7.5-50mg/Kg diet), iron status as hepatic iron was negatively associated with placenta copper. However, iron status was also negatively associated with hepatic and serum copper, and serum ceruloplasmin and zyklopen activities (Gambling et al. 2004). A possibility not studied to the best of our knowledge is whether iron status is positively associated with intestine copper and hephaestin. Also, studies using adequate and high iron intake could shed light in the interaction of iron and copper.

Plasma cobalamin at ≤12 GW was a negative predictor of placenta copper and plasma tHcy at labour was a positive predictor. It can be considered that these two 1C metabolism analytes were associated with placenta copper in the same sense, because cobalamin is necessary for folate dependent homocysteine remethylation, and tHcy can accumulate under impaired 1C metabolism conditions. However the fact that different components of 1C metabolism associate with placenta copper at different stages of pregnancy complicates the interpretation of this result. In homocysteine remethylation betaine acquires more importance as a methyl donor toward late pregnancy, and the folate dependent pathway is the responsible of the remethylation at early pregnancy. Plasma tHcy would be a more appropriate marker of 1C metabolism in late pregnancy

due to the importance of both pathways at this pregnancy stage; and cobalamin a marker of the main pathway at early pregnancy, i.e. folate dependent remethylation.

There has been relatively little research to date on associations between folate and copper status. A possible release of copper from tissue stores to blood shortly after a pharmacological dose of folic acid was reported in mice (Hamed et al. 2009), and lower renal copper concentrations were reported in the offspring of rat dams fed folic acid supplemented diets (Król et al. 2011). In the cases of a possible association between 1C metabolism and copper, copper could be the effector. The enzyme adenosylhomocysteinase, which is present in human placentas (Hershfield et al. 1985), catalyses the reversible reaction adenosine + homocysteine ⇄ SAH, that due to the efficient removal of homocysteine and adenosine in the organism is in the hydrolytic direction (Cantoni 1985; Prigge and Chiang 2001). Human recombinant placental adenosylhomocysteinase has been reported to bind copper (Bethin et al. 1995b). The relation of copper to nicotinamide adenine dinucleotide (NAD^+) can regulate adenosylhomocysteinase activity according to *in vitro* studies. Copper acts as an inhibitor reducing the affinity of adenosylhomocysteinase for its NAD^+ cofactor, whereas higher concentrations on NAD^+ can prevent this inhibition. This has been proposed as a physiological adaptation to regulate adenosylhomocysteinase activity according to the redox status (Li et al. 2007). If the reaction catalysed by adenosylhomocysteinase is impaired by copper, tHcy would decrease and SAH would increase (with a subsequent inhibition of methyltransferases). However, adding to the scenario studies assessing copper status complicates the understanding of the relationship between copper and adenosylhomocysteinase. Downregulation of hepatic adenosylhomocysteinase protein levels have been reported in mice fed copper deficient diet compared to control diets (Bethin et al. 1995a). On the contrary, in a mouse model of Wilson disease, a disease where copper accumulates in liver and that can be due to mutations in the *ATP7B* copper pump; hepatic adenosylhomocysteinase protein levels were lower than in wild type mouse (Bethin et al. 1995a; Medici et al. 2013). In Medici *et al*.'s study, in the Wilson disease compared to wild type mouse higher hepatic SAH

and global DNA hypomethylation were observed. In this study DNA hypomethylation was rescued with chelators of copper or with betaine (Medici et al. 2013). Despite the controversy about the effect of copper on adenosylhomocysteinase, there is evidence of a positive association between copper and homocysteine. In uterine cervical cancer cell (HeLa) experiments, there was more release of homocysteine to the medium in cells with 64ng/ml copper in the medium than in cells with control medium (Hultberg et al. 1997). In a cervical dysplasia case control study, a positive correlation between plasma copper and tHcy was found in the whole sample, in the cases, and with borderline significance in controls (Thomson et al. 2000). Similar results were obtained in a peripheral vascular disease case control study (Mansoor et al. 2000). We hypothesised higher placenta copper concentrations increase adenosylhomocysteinase expression in this organ, leading to higher plasma tHcy concentrations at labour. The copper concentration in the placenta would affect the contribution of this organ to 1C metabolism at labour, and that would be the reason why plasma tHcy at ≤12 GW is not a predictor of placenta copper, but tHcy at labour is. As for plasma cobalamin at ≤12 GW, we are not sure of a possible cause for the negative association between it and placental copper. No previous studies have assessed this, but the fact that cobalamin and not folate status are associated with placenta copper may indicated that the other cobalamin pathway, methylmalonyl-CoA mutase may be involved.

3. Selenium

The median placenta selenium was 82.2ng/g ww. Medians reported in other studies using ICP-MS are higher: 245.6ng/g ww in Bangladesh (Kippler et al. 2010), 189.5ng/g ww in Sweden (Osman et al. 2000), 229.5ng/g ww in China (Liu et al. 2013) and 173.3ng/g ww in Japan (Sakamoto et al. 2013). As previously mentioned in the case of placenta zinc and copper, variations in reported placenta trace element concentrations between different studies may occur due to differences in sample collection and processing.

Smokers during the first trimester or throughout pregnancy had higher placenta selenium concentrations. Another study with a sample from rural Finland reported the same finding (Kantola et al. 2004), but most studies has found no association (Osman et al. 2000; Kantola et al. 2004; Punshon et al. 2016). Of these studies, the smoking status was assessed as self reported by the participants (Kantola et al. 2004; Punshon et al. 2016), except in one study where in a subsample urine cotinine was also taken into account (Osman et al. 2000). These studies probably had higher content of residual blood as the removal procedure was not specified (Kantola et al. 2004; Punshon et al. 2016), or whether washing with water or saline was included in the process (Osman et al. 2000). All the RTBC smokers were categorised as such taking into account plasma cotinine. We proposed that oxidative stress and metal toxicity, such as from cadmium, due to smoking, are associated with a placental selenium retention in smokers, especially in the aforementioned seleno proteins. The effect of smoking on maternal and cord blood selenium ranges from no effect to lower concentrations (*Introduction* point *5.3*), thus the likely higher residual blood in some of the previous studies may mask increases in placenta selenium concentration. However, the association between smoking and placenta selenium in the RTBC must be taken cautiously as it was not observed after adjusting for confounders such as birth weight, gestational age, maternal age, maternal BMI and selenium intake; or was plasma cotinine correlated with placenta selenium.

Placenta selenium was negatively correlated with birth weight, and higher concentrations were found in placentas of IUGR than control neonates. Most studies assessing birth weight have found no association, including a study from Saudi Arabia of 250 placentas with self reported non active smokers, and with the removal of residual blood with deionised water washes (Al-Saleh et al. 2015). Maternal whole blood selenium was not associated with birth weight, but cord selenium was positively associated with birth weight in non smokers (Kantola et al. 2004). As maternal and cord whole blood selenium concentrations (Schramel et al. 1988; Kosanovic et al. 2002; Kantola et al. 2004; Al-Saleh et al. 2004) are of the same order of magnitude as those of

the placenta; a possible dilution of the effect of smoking could have occurred in all these studies except Al-Saleh *et al*. There is controversy about whether the concentrations of selenium in placentas of IUGR neonates are lower (Klapec et al. 2008) or higher (Osada et al. 2002; Zadrozna et al. 2009) than in controls. Our study is the first assessing IUGR in a sample that includes active smokers. Nevertheless, we deem these results could be chance findings, as no association was observed for birth weight, and IUGR risk when taking into account possible confounders (gestational age, smoking patterns, maternal age, maternal BMI and selenium intake). Other studies adjusting for confounders has found this absence of association with IUGR or birth weight (Al-Saleh et al. 2015).

Total selenium intake was not associated with placenta selenium. This is in disagreement with the only study to our knowledge addressing this, which found higher concentrations in placentas of cattle fed with selenium supplemented diets (de Toledo and Perry 1985). Selenium intake affects the concentrations in plasma. In a clinical trial in UK elderly volunteers, after six month of 100μg/d or more of organic selenium supplementation, higher plasma selenium concentrations were observed (Bekaert et al. 2008). In a rat study with deficient to low selenium diets (2 to 119μg/Kg diet, 150μg/Kg diet is the required selenium for maintenance/growth in rats), plasma selenium was higher with higher selenium content in the diets (National Research Council (US) - Subcommittee on Laboratory Animal Nutrition 1995; Uthus et al. 2002). We suggest the same reasons as for zinc and copper for the absence of association in the RTBC, including non extreme intakes of selenium, and placenta selenium concentrations being more dependent on other conditions (oxidative stress and metal toxicity).

None of the analysed 1C metabolism components was associated with placenta selenium. Methyl groups derived from cobalamin dependent tHcy remethylation, but not from choline, seem to be involved in selenium detoxification (*Introduction* point *5.3*). The absence of selenium intakes above the UL plus total selenium intake not being

a predictor of placenta selenium in the RTBC, suggest that selenium detoxification is not a relevant contributor to the selenium content of this organ. Folic acid supplementation impairs selenomethionine intestinal absorption in rats (Nogales et al. 2011), and selenomethionine is the major form of dietary selenium (Institute of Medicine 2000; Burk and Hill 2015). In Nogales *et al*. the dams in which selenium intestinal absorption was measured in their pups, were fed diets containing folic acid as 2 or 8mg/Kg diet from gestation to lactation, that is 200% or 800% the requirements (National Research Council (US) - Subcommittee on Laboratory Animal Nutrition 1995). However, in the RTBC the median total folate intake was 638 (95% CI: 517, 839) µg/d (Sole-Navais 2016), and the Spanish recommended dietary intake in pregnancy is 500µg/d (Federación Española de Sociedades de Nutrición Alimentación y Dietética 2010). In addition to the lack of association between selenium concentrations in the placenta and selenium total intake, the possible effect reported in rats is likely non observable in our study due to folate intakes being less extreme.

In the aforementioned rat study, folic acid supplementation of 8mg/Kg diet induced changes in the selenium status of several compartments in the offspring (Ojeda et al. 2010; Nogales et al. 2011). The dietary selenium content in this experiment was 0.5mg/Kg diet, that is 125% the requirements of reproducing rats (National Research Council (US) - Subcommittee on Laboratory Animal Nutrition 1995), and in the RTBC about 100µg/d of selenium, that is 167% of the RDA (Institute of Medicine - Panel on Dietary Antioxidant and Related Compounds 2000). Thus, unlike folate, the intake of selenium may be similar in both studies. In the RTBC, at moderate folic acid intakes, no changes in the selenium status of the placenta were observed according to plasma folate.

Negative associations have been found between serum/plasma/whole blood selenium and plasma/serum tHcy in observational studies (Bates et al. 2002; González et al. 2004; Klapcinska et al. 2005; Bélanger et al. 2006). However, selenium deficient rats and mice have lower plasma tHcy concentrations (Uthus et al. 2002; Uthus and Ross

2007), and clinical trials of selenium supplementation failed to reduce plasma tHcy (Venn et al. 2003; Bekaert et al. 2008). In a genome-wide meta analysis, genetic polymorphisms located close to genes of the BHMT or transulphuration pathway were the major determinants of toenail and blood selenium concentrations (Cornelis et al. 2015). The BHMT and transulphuration pathways are involved in the generation of glycine and cysteine, respectively, which are necessary for the synthesis of the antioxidant glutathione. The higher tHcy with lower selenium concentrations found in observational studies might be due lower necessity of the antioxidants glutathione and seleno proteins. With high oxidative stress the opposite would occur, that is, selenium would be retained in seleno proteins and tHcy found at lower levels because of the synthesis of glutathione. However in animal studies, the induced selenium deficiency brings lower seleno protein activities as reported (Uthus et al. 2002; Uthus and Ross 2007). The lower tHcy concentrations could reflect an effort to compensate this by higher glutathione synthesis. Indeed higher plasma glutathione concentrations have been found in selenium deficient rats, but not mice (Uthus and Ross 2007). This speculative balance between 1C metabolism mediated glutathione synthesis and selenium retention in seleno proteins in the placenta seems to be looser in pregnancy according to our results, and the lack of BHMT in this organ could contribute to this.

4. Iron

Median placenta iron was 62.9µg/g ww. A study of populations from Northern Norway and Russia using ICP-OES had a median placenta iron of 103.39µg/g ww (Odland et al. 2004). However, the specimens of Odland *et al*. did not include the chorionic plate and were not cleaned of residual blood (Odland et al. 2001). Reported iron concentrations in whole blood range from the same order of magnitude to two orders below the concentrations of the placenta (Tsuchiya et al. 1984; Al-Saleh et al. 2004). Some authors attribute the higher placenta iron concentrations in older studies to improper blood removal (Iyengar and Rapp 2001a). Our lower iron concentrations may be due to the specimen being more thoroughly cleaned of residual blood.

To the best of our knowledge there are no studies assessing socioeconomic status and placenta iron concentrations, so the finding of lower concentrations in participants with low compared to high socioeconomic level cannot be compared with previous studies. No differences in total and supplement iron intake existed between both socioeconomic levels (data not shown). The high socioeconomic level group had characteristics indicative of a healthier pregnancy: lower proportion of smokers throughout pregnancy, lower maternal BMI and higher proportion of preconception folic acid supplement users (data not shown). However, in the RTBC, smoking and BMI were not associated with placenta iron concentrations, and as will be commented, iron intake and 1C metabolism were not either. There is controversy about whether smoking has a negative or no effect on the placenta iron concentrations (Ronco et al. 2005; Stasenko et al. 2010), and the effect of BMI is unknown. The causes for the lower iron concentration in the lower socioeconomic status of the RTBC are unclear, but this group had a relatively small sample size (n=17).

In English pregnant women, the use of iron supplements during pregnancy was not associated with placenta iron (Ward et al. 1987). We found borderline higher iron concentrations in placentas of users of trace element supplements (about 78% containing only iron) during the second and third trimester, but total iron intake was not associated with placenta iron concentrations. The intake effect, if any, on placenta concentrations seems to be small.

None of the analysed 1C metabolism components was associated with placenta iron. About 60% of the total iron intake in the RTBC came from supplements. The iron forms contained in these supplements are ferrous compound, none of which is haem iron. Folic acid simultaneous to non haem iron does not independently affect iron absorption (Buchanan 1971; Campbell et al. 1994), but folate deficient rabbits had higher non haem iron absorption compared to normal folate rabbits (Celada et al. 1979). Our results cannot shed light on this because placenta iron was not affected by total iron intake or intake from supplements when adjusting (data not shown). Other

observational studies have reported no differences in the iron concentrations in amniotic fluid and maternal plasma according to folic acid supplement use (Weekes et al. 1992; Tamura et al. 1994), and our findings in placenta are in line with them.

5. Strengths and limitations

Strengths of this study regarding its design and blood sample collection and handling have been described in *Genetic study discussion* point *5*. Placentas were not collected for the whole RTBC sample (*Material and methods* point *1.2.2*), thus the design for the placenta trace element sub study can be considered a nested case control study (Gordis 2008). This makes the study not completely appropriate for comparisons with studies assessing general populations.

The use of food frequency questionnaires to estimate the habitual intake of trace elements from food is imprecise for total intake estimations. Future analyses should include trace element determinations in blood to enable the study of interactions between the maternal, cord and placenta compartments, and with 1C metabolism, which has been measured in blood.

Although many placenta studies have used single-spot samples as we did, we consider this a limitation given that the trace elements are not uniformly distributed along the length and width of this organ. It is recommended to use multiple spots samples homogenised in a pooled sample (Iyengar and Rapp 2001a), however this was not possible in the RTBC as the collected specimens from other parts of the organ are aimed to other research applications. On the contrary, the special care to ensure all the digested blocks of placenta tissue had similar proportions of the foetal surface and the inner parts of the organ is a strength of this study, together with the thorough removal of residual blood. Due to this last point, the effects on the trace element concentrations of the tissue and not of the circulating blood were observed.

Oxidative stress and metal toxicity were common features of many hypotheses arising from these results. While measurements of oxidative stress and of trace element-

containing proteins involved in it are not available in the RTBC; toxic trace element concentrations were determined in the placentas. Due to the high degree of imputations because of a high proportion of placentas below the lower limits of detection, and some cases of implausible recovery percentages (*Material and methods* point *3.2* **Table 10**), these other trace elements were not taken into account in the quantitative analyses of this thesis. Further analyses using categorical variables for some of these toxic trace elements could help to test the hypotheses speculated here.

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

GENERAL COMMENT

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

General comment

Pregnancy is a physiological state in which 1C metabolism is extremely important, as numerous studies have proven. Progress in knowledge in this field is of potential benefit not only to the immediate health of the mother and her neonate, but possibly to the ageing process of the neonate by foetal programming events. In addition, given that the placenta is responsible for the flux of nutrients and waste products in the foeto-maternal unit, and in some cases act as a barrier or accumulator of toxins, the processes occurring in this organ are expected to affect pregnancy outcome as well. However little work has been done regarding the study of placental trace elements such as zinc, copper, selenium and iron and pregnancy outcomes. Regarding how 1C metabolism interacts with these trace elements in the placenta, no research exists.

This thesis consists of two parts. The first part focused on the possible interactions of folate status on the effects of polymorphisms of the 1C metabolic network that are most frequent in the RTBC (*MTHFR* c.665C>T, *BHMT* c.716G>A, *SLC19A1* c.80G>A, and *MTRR* c.66A>G). Several studies in non pregnant populations or *in vitro* had pointed to folate and related metabolites as modulators of the effect of these polymorphisms or of the pathways where these enzymes and transporter are located; but very few had been conducted in pregnancy, and virtually none in a longitudinal pregnancy study. The second part studied how maternal lifestyle factors and 1C metabolism during pregnancy may affect the aforementioned four trace elements in the placenta and whether placental concentrations of these are associated with adverse pregnancy outcomes.

The literature searches performed for both parts of the thesis revealed numerous gaps in knowledge in the related fields often due to discrepancies in the available evidence to date. This highlights the need to assess less conventional factors that are often overlooked or not investigated. In line with this, in the genetic chapter we have reported that folate status can modulate the effect of the *BHMT* c.716G>A polymorphism on DMG and DMG/betaine ratio at early pregnancy (also with a recent

publication, **Appendices**). Lower betaine to DMG conversion was found in heterozygotes compared to normal homozygotes at early pregnancy and only when folate status was high. At late pregnancy lower conversion was found in variant homozygotes compared to normal homozygotes, and this was not modulated by folate status. In the trace element chapter, the models did not predict the variability in the concentrations of zinc, selenium and iron in the placenta, and in the case of copper very poorly; and we pointed to oxidative stress and/or metal toxicity as the fields where determinant factors may belong. Nevertheless, according to our results tHcy could be a promising factor to take into account in the study of placenta copper status.

CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

Conclusions

Genetic study

- Objective: To report the genotype frequency of the MTHFR c.665C>T, BHMT c.716G>A, SLC19A1 c.80G>A, and MTRR c.66A>G polymorphisms in the RTBC.

The frequency of heterozygotes and variant homozygotes were: 49% and 17% for *MTHFR* c.665C>T, 41% and 11% for *BHMT* c.716G>A, 45% and 27% for *SLC19A1* c.80G>A, and 45% and 26% for *MTRR* c.66A>G polymorphisms.

- Objective: To investigate the effect of the MTHFR c.665C>T, BHMT c.716G>A, SLC19A1 c.80G>A, and MTRR c.66A>G maternal polymorphisms on related 1C metabolism analytes, in the RTBC.

MTHFR c.665 variant homozygotes had lower RBC folate and higher plasma tHcy concentrations throughout pregnancy compared to normal homozygotes.

BHMT c.716 variant allele carriers had lower plasma DMG concentrations throughout pregnancy compared to normal homozygotes.

Plasma and RBC folate and tHcy did not vary during pregnancy with *SLC19A1* c.80G>A genotype.

Early pregnancy plasma tHcy was higher in *MTRR* c.66 variant homozygotes compared to normal homozygotes.

- Objective: To investigate whether folate status modulates the effect of the MTHFR c.665C>T, BHMT c.716G>A, SLC19A1 c.80G>A, and MTRR c.66A>G maternal polymorphisms on 1C metabolism analytes other than plasma and RBC folate, in the RTBC.

The tHcy enhancing effect of *MTHFR* c.665 variant homozygote genotype was found in the lowest RBC folate tertile at ≤12 GW and lowest plasma folate tertiles at mid pregnancy.

BHMT c.716 heterozygotes compared to normal homozygotes had lower DMG at early pregnancy when folate status was high.

Plasma folate status did not modify the relationship between the *SLC19A1* c.80G>A polymorphism and plasma tHcy during pregnancy.

MTRR c.66 variant homozygotes compared to normal homozygotes had higher tHcy at early pregnancy in the mid plasma folate tertiles.

- Objective: To assess whether the MTHFR c.665C>T, BHMT c.716G>A, SLC19A1 c.80G>A, and MTRR c.66A>G maternal and offspring polymorphisms are associated with adverse pregnancy outcome (as IUGR and miscarriage), in the RTBC.

There was higher proportion of IUGR cases with neonate *MTHFR* c.665 normal allele increase.

The maternal *SLC19A1* c.80 variant homozygote genotype could be associated with miscarriage as this genotype had borderline higher proportion of miscarriage cases than the other genotypes.

Placenta trace element study

- Objective: To report placenta zinc, copper, selenium and iron concentrations in the RTBC.

The median concentrations in the placenta were 6809, 534 and 82.2ng/g of wet weight for zinc, copper and selenium, respectively. The median for iron was 62.9µg/g of wet weight.

- Objective: To study the associations between environmental factors and placenta zinc, copper, selenium and iron concentrations and pregnancy outcome, in the RTBC.

Placenta zinc, copper and selenium were positively correlated.

Neonate female sex was positively associated with placenta zinc.

Birth weight and use of trace element supplements at mid-late pregnancy were negatively associated with placenta copper. Elevated placenta copper was associated with IUGR risk.

High socioeconomic status was positively associated with placenta iron.

Intakes of each trace element were not associated with their respective concentrations in placenta.

- Objective: To investigate the associations of 1C metabolism analytes throughout pregnancy and placenta zinc, copper, selenium and iron concentrations, in the RTBC.

THcy at labour was positively associated with placenta copper. Plasma cobalamin at ≤12 GW was negatively associated with placenta copper.

1C metabolism components were not associated with placenta zinc, selenium and iron.

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

BIBLIOGRAPHY

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

Bibliography

Abiaka C, Olusi S, Al-Awadhi A (2003) Reference Ranges of Copper and Zinc and the Prevalence of Their Deficiencies in an Arab Population Aged 15-80 Years. Biol Trace Elem Res 91:33–43. doi: 10.1385/BTER:91:1:33

Acevedo CG, Rojas S, Ramirez M, Bravo I (1995) Transport and metabolism of adenosine in the perfused human placenta. Placenta 16:611–22.

Al-Saleh E, Al-Harmi J, Nandakumaran M, et al (2009) Effect of methotrexate administration on status of some essential trace elements and antioxidant enzymes in pregnant rats in late gestation. Gynecol Endocrinol 25:816–22. doi: 10.3109/09513590903056811

Al-Saleh E, Nandakumaran M, Al-Shammari M, et al (2004) Assessment of maternal-fetal status of some essential trace elements in pregnant women in late gestation: relationship with birth weight and placental weight. J Matern Fetal Neonatal Med 16:9–14. doi: 10.1080/14767050412331283157

Al-Saleh I, Al-Rouqi R, Obsum CA, et al (2015) Interaction between cadmium (Cd), selenium (Se) and oxidative stress biomarkers in healthy mothers and its impact on birth anthropometric measures. Int J Hyg Environ Health 218:66–90. doi: 10.1016/j.ijheh.2014.08.001

Alfthan G, Tapani K, Nissinen K, et al (2004) The effect of low doses of betaine on plasma homocysteine in healthy volunteers. Br J Nutr 92:665. doi: 10.1079/BJN20041253

Allen LH (2009) How common is vitamin B-12 deficiency? Am J Clin Nutr 89:693S–6S. doi: 10.3945/ajcn.2008.26947A

Allen RH (2000) Cecil Textbook of Medicine. In: Goldman L, Bennett JC (eds) 21st edn. Saunders,

Allen RH, Seetharam B, Podell E, Alpers DH (1978) Effect of proteolytic enzymes on the binding of cobalamin to R protein and intrinsic factor. In vitro evidence that a failure to partially degrade R protein is responsible for cobalamin malabsorption in pancreatic insufficiency. J Clin Invest 61:47–54. doi: 10.1172/JCI108924

Allen RH, Stabler SP, Lindenbaum J (1993a) Serum betaine, N,N-dimethylglycine and N-methylglycine levels in patients with cobalamin and folate deficiency and related inborn errors of metabolism. Metabolism 42:1448–1460.

Allen RH, Stabler SP, Savage DG, Lindenbaum J (1993b) Metabolic abnormalities in cobalamin (vitamin B12) and folate deficiency. FASEB J 7:1344–53.

Álvarez-Dardet C, Alonso J, Domingo A, Regidor E (1995) La medición de la clase social en Ciencias de la Salud. [Measurement of social class in health sciences.]. Barcelona

Amantana A, Vorachek WR, Butler JA, et al (2002) Effect of copper, zinc and cadmium on the promoter of selenoprotein W in glial and myoblast cells. J Inorg Biochem 91:356–362. doi: 10.1016/S0162-0134(02)00453-1

Amorim MR, Moura CM, Gomes AD, et al (2013) Betaine–homocysteine methyltransferase 742G>A polymorphism and risk of down syndrome offspring in a Brazilian population. Mol Biol Rep 40:4685–4689. doi: 10.1007/s11033-013-2563-x

Ananth C V., Elsasser D, Kinzler WL, et al (2007) Polymorphisms in methionine synthase reductase and betaine-homocysteine S-methyltransferase genes: Risk of placental abruption. Mol Genet Metab 91:104–110. doi: 10.1016/j.ymgme.2007.02.004

Anas M-KI, Hammer M-A, Lever M, et al (2007) The organic osmolytes betaine and proline are transported by a shared system in early preimplantation mouse embryos. J Cell Physiol 210:266–77. doi: 10.1002/jcp.20872

Anas M-KI, Lee MB, Zhou C, et al (2008) SIT1 is a betaine/proline transporter that is activated in mouse eggs after fertilization and functions until the 2-cell stage. Development 135:4123–4130. doi: 10.1242/dev.026575

Andersen HS, Gambling L, Holtrop G, McArdle HJ (2007) Effect of dietary copper deficiency on iron metabolism in the pregnant rat. Br J Nutr 97:239. doi: 10.1017/S0007114507239960

Andreini C, Banci L, Bertini I, Rosato A (2006) Zinc through the three domains of life. J Proteome Res 5:3173–8. doi: 10.1021/pr0603699

Andrews E, Grant A, Stephenson B (1963) Weight responses of sheep to cobalt and selenium in relation to vitamin B12 and selenium concentrations in liver and kidney. New Zeal J Agric Res 7:17–27.

ANSES (2013) French food composition table - Table Ciqual. https://pro.anses.fr/tableciqual/. Accessed 1 Jan 2016

Appling DR (1991) Compartmentation of folate-mediated one-carbon metabolism in eukaryotes. FASEB J 5:2645–2651. doi: 10.1146/annurev.nutr.012809.104810

Araki A, Sako Y (1987) Determination of free and total homocysteine in human plasma by high-performance liquid chromatography with fluorescence detection. J Chromatogr 422:43–52.

Arnaud J, Favier A, Herrmann MA, Pilorget JJ (1992) [Effect of folic acid and folinic acid on zinc

absorption]. Ann Nutr Metab 36:157–61.

Arnold A, Tan K, Rabenstein D (1986) Nuclear magnetic resonance studies of the solution chemistry of metal complexes. 23. Complexation of methylmercury by selenohydryl-containing amino acids and related molecules. Inorg Chem 25:2433–7.

Arvanitidou V, Voskaki I, Tripsianis G, et al (2007) Serum copper and zinc concentrations in healthy children aged 3-14 years in Greece. Biol Trace Elem Res 115:1–12. doi: 10.1385/BTER:115:1:1

Atamer Y, Kocyigit Y, Yokus B, et al (2005) Lipid peroxidation, antioxidant defense, status of trace metals and leptin levels in preeclampsia. Eur J Obs Gynecol Reprod Biol 119:60–66. doi: S0301-2115(04)00356-2 [pii]\r10.1016/j.ejogrb.2004.06.033

Aydogan U, Durmaz E, Ercan CM, et al (2013) Effects of smoking during pregnancy on DNA damage and ROS level consequences in maternal and newborns' blood. Arh Hig Rada Toksikol 64:35–46.

Azab SFA, Saleh SH, Elsaeed WF, et al (2014) Serum trace elements in obese Egyptian children: a case-control study. Ital J Pediatr 40:20. doi: 10.1186/1824-7288-40-20

Bae J, Shin SJ, Cha SH, et al (2007) Prevalent genotypes of methylenetetrahydrofolate reductase (MTHFR C677T and A1298C) in spontaneously aborted embryos. Fertil Steril 87:351–355. doi: 10.1016/j.fertnstert.2006.06.027

Baggott JE, Tamura T (2007) Iron-dependent formation of homocysteine from methionine and other thioethers. Eur J Clin Nutr 61:1359–63. doi: 10.1038/sj.ejcn.1602665

Baggott JE, Tamura T (2015) Homocysteine, iron and cardiovascular disease: a hypothesis. Nutrients 7:1108–18. doi: 10.3390/nu7021108

Bailey LB (1998) Dietary reference intakes for folate: the debut of dietary folate equivalents. Nutr Rev 56:294–299. doi: 10.1111/j.1753-4887.1998.tb01662.x

Bailey SW, Ayling JE (2009) The extremely slow and variable activity of dihydrofolate reductase in human liver and its implications for high folic acid intake. Proc Natl Acad Sci U S A 106:15424–9. doi: 10.1073/pnas.0902072106

Ball EW, Giles C (1964) Folic acid and vitamin B12 levels in pregnancy and their relation to megaloblastic anaemia. J Clin Pathol 17:165–174. doi: 10.1136/jcp.17.2.165

Banci L, Bertini I, Cantini F, et al (2009) Structure and dynamics of copper-free SOD: The protein before binding copper. Protein Sci 11:2479–2492. doi: 10.1110/ps.0210802

Banerjee R (1998) Vitamin B12 and B12-Proteins. In: Krautler B, Arigoni D, Golding B (eds).

Wiley-VCH, pp 189–197

Barbato JC, Catanescu O, Murray K, et al (2007) Targeting of metallothionein by L-homocysteine: a novel mechanism for disruption of zinc and redox homeostasis. Arterioscler Thromb Vasc Biol 27:49–54. doi: 10.1161/01.ATV.0000251536.49581.8a

Barbosa PR, Stabler SP, Machado ALK, et al (2008a) Association between decreased vitamin levels and MTHFR, MTR and MTRR gene polymorphisms as determinants for elevated total homocysteine concentrations in pregnant women. Eur J Clin Nutr 62:1010–21. doi: 10.1038/sj.ejcn.1602810

Barbosa PR, Stabler SP, Trentin R, et al (2008b) Evaluation of nutritional and genetic determinants of total homocysteine, methylmalonic acid and S-adenosylmethionine/S-adenosylhomocysteine values in Brazilian childbearing-age women. Clin Chim Acta 388:139–47.

Barnabé A, Aléssio ACM, Bittar LF, et al (2015) Folate, vitamin B12 and Homocysteine status in the post-folic acid fortification era in different subgroups of the Brazilian population attended to at a public health care center. Nutr J 14:19. doi: 10.1186/s12937-015-0006-3

Bartels PC, Helleman PW, Soons JB (1989) Investigation of red cell size-distribution histograms related to folate, vitamin B12 and iron state in the course of pregnancy. Scand J Clin Lab Invest 49:763–771. doi: 10.3109/00365518909091555

Bartholomew DW, Batshaw ML, Allen RH, et al (1988) Therapeutic approaches to cobalamin-C methylmalonic acidemia and homocystinuria. J Pediatr 112:32–9.

Baslund B, Gregers J, Nielsen CH (2008) Reduced folate carrier polymorphism determines methotrexate uptake by B cells and CD4+ T cells. Rheumatology (Oxford) 47:451–3. doi: 10.1093/rheumatology/ken073

Bates CJ, Thane CW, Prentice A, Delves HT (2002) Selenium status and its correlates in a British national diet and nutrition survey: people aged 65 years and over. J Trace Elem Med Biol 16:1–8.

Batey RG, Johnston R (1993) Effects of alcohol, carbon tetrachloride, and choline deficiency on iron metabolism in the rat. Alcohol Clin Exp Res 17:931–4.

Beedholm-Ebsen R, van de Wetering K, Hardlei T, et al (2010) Identification of multidrug resistance protein 1 (MRP1/ABCC1) as a molecular gate for cellular export of cobalamin. Blood 115:1632–9. doi: 10.1182/blood-2009-07-232587

Begley JA, Colligan PD, Chu RC, Hall CA (1993) Cobalamin metabolism in cultured human

chorionic villus cells. J Cell Physiol 156:43–7. doi: 10.1002/jcp.1041560107

Bekaert B, Cooper ML, Green FR, et al (2008) Effect of selenium status and supplementation with high-selenium yeast on plasma homocysteine and B vitamin concentrations in the UK elderly. Mol Nutr Food Res 52:1324–33. doi: 10.1002/mnfr.200700353

Bélanger MC, Dewailly É, Berthiaume L, et al (2006) Dietary contaminants and oxidative stress in Inuit of Nunavik. Metabolism 55:989–995. doi: 10.1016/j.metabol.2006.03.007

Benes B, Spevácková V, Smíd J, et al (2005) Effects of age, BMI, smoking and contraception on levels of Cu, Se and Zn in the blood of the population in the Czech Republic. Cent Eur J Public Health 13:202–7.

Benowitz NL (1996) Cotinine as a biomarker of environmental tobacco smoke exposure. Epidemiol Rev 18:188–204.

Bergen NE, Jaddoe VW, Timmermans S, et al (2012) Homocysteine and folate concentrations in early pregnancy and the risk of adverse pregnancy outcomes: the Generation R Study. BJOG 119:739–751. doi: 10.1111/j.1471-0528.2012.03321.x

Berry RJ, Li Z, Erickson JD, et al (1999) Prevention of neural-tube defects with folic acid in China. China-U.S. Collaborative Project for Neural Tube Defect Prevention. N Engl J Med 341:1485–90. doi: 10.1056/NEJM199911113412001

Bescond A, Augier T, Chareyre C, et al (1999) Influence of homocysteine on matrix metalloproteinase-2: activation and activity. Biochem Biophys Res Commun 263:498–503. doi: 10.1006/bbrc.1999.1391

Bethin KE, Cimato TR, Ettinger MJ (1995a) Copper binding to mouse liver S-adenosylhomocysteine hydrolase and the effects of copper on its levels. J Biol Chem 270:20703–20711. doi: 10.1074/jbc.270.35.20703

Bethin KE, Petrovic N, Ettinger MJ (1995b) Identification of a major hepatic copper binding protein as S-adenosylhomocysteine hydrolase. J Biol Chem 270:20698–20702.

BeVital A/S (2016) Assessment of nutritional status by vitamin marker. http://www.bevital.no/. Accessed 1 Jan 2016

Bhandari SD, Gregory JF, Renuart DR, Merritt AM (1990) Properties of pteroylpolyglutamate hydrolase in pancreatic juice of the pig. J Nutr 120:467–75.

Bialecka M, Kurzawski M, Roszmann A, et al (2012) Association of COMT, MTHFR, and SLC19A1(RFC-1) polymorphisms with homocysteine blood levels and cognitive impairment in Parkinson's disease. Pharmacogenet Genomics 22:716–24. doi:

10.1097/FPC.0b013e32835693f7

Biggers JD, Lawitts JA, Lechene CP (1993) The protective action of betaine on the deleterious effects of NaCl on preimplantation mouse embryos in vitro. Mol Reprod Dev 34:380–90. doi: 10.1002/mrd.1080340406

Birn H, Zhai X, Holm J, et al (2005) Megalin binds and mediates cellular internalization of folate binding protein. FEBS J 272:4423–30. doi: 10.1111/j.1742-4658.2005.04857.x

Biselli JM, Zampieri BL, Goloni-Bertollo EM, et al (2012) Genetic polymorphisms modulate the folate metabolism of Brazilian individuals with Down syndrome. Mol Biol Rep 39:9277–9284. doi: 10.1007/s11033-012-1629-5

Bjørke-Monsen AL, Roth C, Magnus P, et al (2013) Maternal B vitamin status in pregnancy week 18 according to reported use of folic acid supplements. Mol Nutr Food Res 57:645–652. doi: 10.1002/mnfr.201200114

Blazewicz A, Klatka M, Astel A, et al (2013) Differences in trace metal concentrations (Co, Cu, Fe, Mn, Zn, Cd, And Ni) in whole blood, plasma, and urine of obese and nonobese children. Biol Trace Elem Res 155:190–200. doi: 10.1007/s12011-013-9783-8

Blount BC, Mack MM, Wehr CM, et al (1997) Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. Proc Natl Acad Sci U S A 94:3290–5.

Boas WV, Gonçalves RO, Costa OLN, Goncalves MS (2015) Metabolism and gene polymorphisms of the folate pathway in Brazilian women with history of recurrent abortion. Rev Bras Ginecol Obstet 37:71–6. doi: 10.1590/SO100-720320140005223

Boggess KA, Oury TD, Kay HH, Crapo JD (1998) Extracellular superoxide dismutase localization and activity within the human placenta. Placenta 19:417–22.

Bolann BJ, Rahil-Khazen R, Henriksen H, et al (2007) Evaluation of methods for trace-element determination with emphasis on their usability in the clinical routine laboratory. Scand J Clin Lab Invest 67:353–66. doi: 10.1080/00365510601095281

Borden LA, Smith KE, Gustafson EL, et al (1995) Cloning and expression of a betaine/GABA transporter from human brain. J Neurochem 64:977–84.

Bose N, Greenspan P, Momany C (2002) Expression of recombinant human betaine: homocysteine S-methyltransferase for x-ray crystallographic studies and further characterization of interaction with S-adenosylmethionine. Protein Expr Purif 25:73–80. doi: 10.1006/prep.2001.1611

Bothwell TH (2000) Iron requirements in pregnancy and strategies to meet them. Am J Clin Nutr 72:257S–264S.

Botto LD, Yang Q (2000) 5,10-Methylenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review. Am J Epidemiol 151:862–77.

Botto N, Andreassi MG, Manfredi S, et al (2003) Genetic polymorphisms in folate and homocysteine metabolism as risk factors for DNA damage. Eur J Hum Genet 11:671–678. doi: 10.1038/sj.ejhg.5201024

Bou-Resli MN, Dashti HM, Mathew TC, Al-Zaid NS (2001) Pre- and postnatal tissue selenium of the rat in the growing state. Biol Neonate 80:169–72. doi: 47138

Boyles AL, Billups A V., Deak KL, et al (2006) Neural tube defects and folate pathway genes: Family-based association tests of gene-gene and gene-environment interactions. Environ Health Perspect 114:1547–1552. doi: 10.1289/ehp.9166

Brattström L, Lindgren A, Israelsson B, et al (1994) Homocysteine and cysteine: determinants of plasma levels in middle-aged and elderly subjects. J Intern Med 236:633–41.

Breen JG, Eisenmann C, Horowitz S, Miller RK (1994) Cell-specific increases in metallothionein expression in the human placenta perfused with cadmium. Reprod Toxicol 8:297–306. doi: 10.1016/0890-6238(94)90045-0

Briefel RR, Bialostosky K, Kennedy-Stephenson J, et al (2000) Zinc intake of the U.S. population: findings from the third National Health and Nutrition Examination Survey, 1988-1994. J Nutr 130:1367S–73S.

Brilakis ES, Berger PB, Ballman K V, Rozen R (2003) Methylenetetrahydrofolate reductase (MTHFR) 677C>T and methionine synthase reductase (MTRR) 66A>G polymorphisms: association with serum homocysteine and angiographic coronary artery disease in the era of flour products fortified with folic acid. Atherosclerosis 168:315–22.

Brouns R, Ursem N, Lindemans J, et al (2008) Polymorphisms in genes related to folate and cobalamin metabolism and the associations with complex birth defects. Prenat Diagn 28:485–93. doi: 10.1002/pd.2006

Brouwer IA, Verhoef P, Urgert R (2000) Betaine supplementation and plasma homocysteine in healthy volunteers. Arch Intern Med 160:2546–7.

Brown CA, McKinney KQ, Kaufman JS, et al (2000) A common polymorphism in methionine synthase reductase increases risk of premature coronary artery disease. J Cardiovasc Risk 7:197–200. doi: 10.1177/204748730000700306

Bruinse HW, van der Berg H, Haspels AA (1985) Maternal serum folacin levels during and after normal pregnancy. Eur J Obs Gynecol Reprod Biol 20:153–158. doi: 10.1016/0028-2243(85)90014-0

Buchanan WM (1971) The significance of folic acid, tissue iron stores, and tissue viability in determining iron uptake from serum by thyroid tissue slices. J Clin Pathol 24:328–33.

Bueno O, Molloy AM, Fernandez-Ballart JD, et al (2016) Common polymorphisms that affect folate transport or metabolism modify the effect of the MTHFR 677C > T polymorphism on folate status. J Nutr 146:1–8. doi: 10.3945/jn.115.223685

Burk RF, Hill KE (2015) Regulation of Selenium Metabolism and Transport. Annu Rev Nutr 35:150514143029003. doi: 10.1146/annurev-nutr-071714-034250

Burns J, Paterson CR (1993) Effect of iron-folate supplementation on serum copper concentration in late pregnancy. Acta Obstet Gynecol Scand 72:616–618.

Burton GJ, Hempstock J, Jauniaux E (2001) Nutrition of the human fetus during the first trimester - A review. Placenta 22:

Burton GJ, Watson AL, Hempstock J, et al (2002) Uterine glands provide histiotrophic nutrition for the human fetus during the first trimester of pregnancy. J Clin Endocrinol Metab 87:2954–2959. doi: 10.1210/jc.87.6.2954

Bush PG, Mayhew TM, Abramovich DR, et al (2000) A quantitative study on the effects of maternal smoking on placental morphology and cadmium concentration. Placenta 21:247–256. doi: 10.1053/plac.1999.0470

Butali A, Little J, Chevrier C, et al (2013) Folic acid supplementation use and the MTHFR C677T polymorphism in orofacial clefts etiology: An individual participant data pooled-analysis. Birth Defects Res A Clin Mol Teratol 97:509–14. doi: 10.1002/bdra.23133

Butterworth CE, Hatch K, Cole P, et al (1988) Zinc concentration in plasma and erythrocytes of subjects receiving folic acid supplementation. Am J Clin Nutr 47:484–486.

Cai B, Zhang T, Zhong R, et al (2014) Genetic variant in MTRR, but not MTR, is associated with risk of congenital heart disease: An integrated meta-analysis. PLoS One. doi: 10.1371/journal.pone.0089609

Callejón G, Mayor-Olea A, Jiménez AJ, et al (2007) Genotypes of the C677T and A1298C polymorphisms of the MTHFR gene as a cause of human spontaneous embryo loss. Hum Reprod 22:3249–3254. doi: 10.1093/humrep/dem337

Campbell NR, Hasinoff BB, Singh M, Robertson S (1994) Ferrous sulphate does not directly affect

pteroylmonoglutamic acid absorption in rats. Br J Nutr 72:447–53.

Canton MC, Cotter BM, Cremin FM, Morrissey PA (1989) The effect of dietary zinc deficiency on pancreatic gamma-glutamyl hydrolase (EC 3.4.22.12) activity and on the absorption of pteroylpolyglutamate in rats. Br J Nutr 62:185–93.

Cantoni GL (1985) The role of S-adenosylhomocysteine in the biological utilization of S-adenosylmethionine. Prog Clin Biol Res 198:47–65.

Cao Y, Xu J, Zhang Z, et al (2013) Association study between methylenetetrahydrofolate reductase polymorphisms and unexplained recurrent pregnancy loss: A meta-analysis. Gene 514:105–111. doi: 10.1016/j.gene.2012.10.091

Carbone P, Sobreviela M, Jimenez D, et al (1992) Hair zinc and dietary zinc intake during pregnancy and puerperium. Eur J Obstet Gynecol Reprod Biol 47:103–108. doi: 10.1016/0028-2243(92)90038-Z

Castro C, Breksa AP, Salisbury E, Garrow TA (2002) Betaine-homocysteine S-methyltransferase (BHMT) transcription is inhibited by S-adenosylmethionine (ADOMET). In: Milstien S, Kapatos G, Levine RA, Shane B (eds) Chemistry and Biology of Pteridines and Folates. Bethesda, pp 549–556

Castro R, Rivera I, Ravasco P, et al (2003) 5,10-methylenetetrahydrofolate reductase 677C → T and 1298A → C mutations are genetic determinants of elevated homocysteine. QJM 96:297–303. doi: 10.1093/qjmed/hcg039

Caudill MA (2010) Folate in health and disease. In: Bailey LB (ed) 2nd edn. CRC Press, pp 449–465

Caulfield LE, Donangelo CM, Chen P, et al (2008) Red blood cell metallothionein as an indicator of zinc status during pregnancy. Nutrition 24:1081–1087. doi: 10.1016/j.nut.2008.05.011

Celada A, Rudolf H, Donath A (1979) Effect of experimental chronic alcohol ingestion and folic acid deficiency on iron absorption. Blood 54:906–15.

Cetin I, Berti C, Mandò C, Parisi F (2011) Placental iron transport and maternal absorption. Ann Nutr Metab 59:55–58. doi: 10.1159/000332133

Chambers JC, McGregor a, Jean-Marie J, et al (1999) Demonstration of rapid onset vascular endothelial dysfunction after hyperhomocysteinemia: an effect reversible with vitamin C therapy. Circulation 99:1156–1160. doi: 10.1161/01.CIR.99.9.1156

Chan J, Deng L, Mikael LG, et al (2010) Low dietary choline and low dietary riboflavin during pregnancy influence reproductive outcomes and heart development in mice. Am J Clin

Nutr 91:1035–1043. doi: 10.3945/ajcn.2009.28754

Chancy CD, Kekuda R, Huang W, et al (2000) Expression and differential polarization of the reduced-folate transporter-1 and the folate receptor ?? in mammalian retinal pigment epithelium. J Biol Chem 275:20676–20684. doi: 10.1074/jbc.M002328200

Chandler AL, Hobbs CA, Mosley BS, et al (2012) Neural tube defects and maternal intake of micronutrients related to one-carbon metabolism or antioxidant activity. Birth Defects Res Part A - Clin Mol Teratol 94:864–874. doi: 10.1002/bdra.23068

Chango A, Boisson F, Barbé F, et al (2000a) The effect of 677C-->T and 1298A-->C mutations on plasma homocysteine and 5,10-methylenetetrahydrofolate reductase activity in healthy subjects. Br J Nutr 83:593–6.

Chango A, Emery-Fillon N, de Courcy GP, et al (2000b) A polymorphism (80G->A) in the reduced folate carrier gene and its associations with folate status and homocysteinemia. Mol Genet Metab 70:310–315. doi: 10.1006/mgme.2000.3034

Chango A, Nour AA, Bousserouel S, et al (2009) Time course gene expression in the one-carbon metabolism network using HepG2 cell line grown in folate-deficient medium. J Nutr Biochem 20:312–320. doi: 10.1016/j.jnutbio.2008.04.004

Chango A, Potier De Courcy G, Boisson F, et al (2000c) 5,10-methylenetetrahydrofolate reductase common mutations, folate status and plasma homocysteine in healthy French adults of the Supplementation en Vitamines et Mineraux Antioxydants (SU.VI.MAX) cohort. Br J Nutr 84:891–6.

Chen C, Hedstrom O, Whanger PD (1993) Effect of vitamin B12 on performance and tissue selenium content in rats fed sub-toxic levels of selenite. Toxicology 85:101–115. doi: 10.1016/0300-483X(93)90035-Q

Chen CL, Whanger PD (1993) Effect of vitamin B12 status on selenium methylation and toxicity in rats: in vivo and in vitro studies. Toxicol Appl Pharmacol 118:65–72.

Chen H, Attieh ZK, Su T, et al (2004) Hephaestin is a ferroxidase that maintains partial activity in sex-linked anemia mice. Blood 103:3933–3939. doi: 10.1182/blood-2003-09-3139

Chen H, Attieh ZK, Syed B a, et al (2010) Identification of zyklopen, a new member of the vertebrate multicopper ferroxidase family, and characterization in rodents and human cells. J Nutr 140:1728–1735. doi: 10.3945/jn.109.117531

Chen H, Liu S, Ji L, et al (2016a) Folic Acid Supplementation Mitigates Alzheimer's Disease by Reducing Inflammation: A Randomized Controlled Trial. Mediators Inflamm. doi:

10.1155/2016/5912146

Chen H, Yang X, Lu M (2016b) Methylenetetrahydrofolate reductase gene polymorphisms and recurrent pregnancy loss in China: a systematic review and meta-analysis. Arch Gynecol Obstet 293:283–90. doi: 10.1007/s00404-015-3894-8

Chen L, Liu L, Hong K, et al (2012) Three genetic polymorphisms of homocysteine-metabolizing enzymes and risk of coronary heart disease: a meta-analysis based on 23 case-control studies. DNA Cell Biol 31:238–49. doi: 10.1089/dna.2011.1281

Chen L, Qi H, Korenberg J, et al (1996) Purification and properties of human cytosolic folylpoly-gamma-glutamate synthetase and organization, localization, and differential splicing of its gene. J Biol Chem 271:13077–13087. doi: 10.1074/jbc.271.22.13077

Chen M-DD, Lin PY, Sheu WH (1997) Zinc status in plasma of obese individuals during glucose administration. Biol Trace Elem Res 60:123–9. doi: 10.1007/BF02783315

Chen Y, Zhao S, Wang Y, et al (2014) Homocysteine reduces protein S-nitrosylation in endothelium. Int J Mol Med 34:1277–1285. doi: 10.3892/ijmm.2014.1920

Chern MK, Pietruszko R (1995) Human aldehyde dehydrogenase E3 isozyme is a betaine Aldehyde dehydrogenase. Biochem Biophys Res Commun 561–568. doi: 10.1006/bbrc.1995.2168

Chern MK, Pietruszko R (1999) Evidence for mitochondrial localization of betaine aldehyde dehydrogenase in rat liver: purification, characterization, and comparison with human cytoplasmic E3 isozyme. Biochem Cell Biol 77:179–187.

Chiaverini N, De Ley M (2010) Protective effect of metallothionein on oxidative stress-induced DNA damage. Free Radic Res 44:605–13. doi: 10.3109/10715761003692511

Chien PF, Smith K, Watt PW, et al (1993) Protein turnover in the human fetus studied at term using stable isotope tracer amino acids. Am J Physiol 265:E31-5.

Chiplonkar SA, Agte V V (2006) Predicting bioavailable zinc from lower phytate forms, folic Acid and their interactions with zinc in vegetarian meals. J Am Coll Nutr 25:26–33.

Chmurzynska A, Malinowska AM (2011) Homocysteine homeostasis in the rat is maintained by compensatory changes in cystathionine β-synthase, betaine-homocysteine methyltransferase, and phosphatidylethanolamine N-methyltransferase gene transcription occurring in response to maternal protein a. Nutr Res 31:572–577. doi: 10.1016/j.nutres.2011.07.001

Choi SW, Friso S, Ghandour H, et al (2004) Vitamin B-12 deficiency induces anomalies of base

substitution and methylation in the DNA of rat colonic epithelium. J Nutr 134:750–5.

Choumenkovitch SF, Selhub J, Wilson PWF, et al (2002) Folic acid intake from fortification in United States exceeds predictions. J Nutr 132:2792–2798.

Christensen B, Arbour L, Tran P, et al (1999) Genetic polymorphisms in methylenetetrahydrofolate reductase and methionine synthase, folate levels in red blood cells, and risk of neural tube defects. Am J Med Genet 84:151–157. doi: 10.1002/(SICI)1096-8628(19990521)84:2<151::AID-AJMG12>3.0.CO;2-T

Christensen EI, Birn H (2002) Megalin and cubilin: multifunctional endocytic receptors. Nat Rev Mol Cell Biol 3:256–66. doi: 10.1038/nrm778

Christensen KE, Mikael LG, Leung KY, et al (2015) High folic acid consumption leads to pseudo-MTHFR deficiency, altered lipid metabolism, and liver injury in mice. Am J Clin Nutr 101:646–658. doi: 10.3945/ajcn.114.086603

Christensen KE, Wu Q, Wang X, et al (2010) Steatosis in mice is associated with gender, folate intake, and expression of genes of one-carbon metabolism. J Nutr 140:1736–1741. doi: 10.3945/jn.110.124917

Christensen KE, Zada YF, Rohlicek C V., et al (2013) Risk of congenital heart defects is influenced by genetic variation in folate metabolism. Cardiol Young 23:89–98. doi: 10.1017/S1047951112000431

Chuang LS, Ng HH, Chia JN, Li BF (1996) Characterisation of independent DNA and multiple Zn-binding domains at the N terminus of human DNA-(cytosine-5) methyltransferase: modulating the property of a DNA-binding domain by contiguous Zn-binding motifs. J Mol Biol 257:935–48. doi: 10.1006/jmbi.1996.0213

Chwatko G, Jakubowski H (2005) The determination of homocysteine-thiolactone in human plasma. Anal Biochem 337:271–7. doi: 10.1016/j.ab.2004.11.035

Ciappio E, Mason JB (2010) Folate in health and disease. In: Bailey LB (ed) 2nd edn. CRC Press, pp 235–262

Cichowicz DJ, Shane B (1987) Mammalian folylpoly-gamma-glutamate synthetase. 2. Substrate specificity and kinetic properties. Biochemistry 26:513–521. doi: 10.1021/bi00376a025

Cikot RJ, Steegers-Theunissen RP, Thomas CM, et al (2001) Longitudinal vitamin and homocysteine levels in normal pregnancy. Br J Nutr 85:49–58. doi: S0007114501000083 [pii]

Clarke R, Halsey J, Lewington S, et al (2010) Effects of Lowering Homocysteine Levels With B

Vitamins on Cardiovascular Disease, Cancer, and Cause-Specific Mortality. Arch Intern Med 170:1622–1631. doi: 10.1001/archinternmed.2010.348

Clarke R, Lewington S, Landray M (2003) Homocysteine, renal function, and risk of cardiovascular disease. Kidney Int Suppl 63:S131-3. doi: 10.1046/j.1523-1755.63.s84.7.x

Clarke S, Banfield K (2001) Homocysteine in health and disease. In: Carmel R, Jacobsen DW (eds) 1st edn. Cambridge, pp 63–78

Clifford a. J, Chen K, McWade L, et al (2012) Gender and Single Nucleotide Polymorphisms in MTHFR, BHMT, SPTLC1, CRBP2, CETP, and SCARB1 Are Significant Predictors of Plasma Homocysteine Normalized by RBC Folate in Healthy Adults. J Nutr 142:1764–1771. doi: 10.3945/jn.112.160333

Coleman JE (1998) Zinc enzymes. Curr Opin Chem Biol 2:222–34.

Collin SM, Metcalfe C, Refsum H, et al (2010) Circulating folate, vitamin B12, homocysteine, vitamin B12 transport proteins, and risk of prostate cancer: a case-control study, systematic review, and meta-analysis. Cancer Epidemiol Biomarkers Prev 19:1632–42. doi: 10.1158/1055-9965.EPI-10-0180

Colomina JM, Cavallé-Busquets P, Fernàndez-Roig S, et al (2016) Maternal Folate Status and the BHMT c.716G>A Polymorphism Affect the Betaine Dimethylglycine Pathway during Pregnancy. Nutrients 8:621. doi: 10.3390/nu8100621

Coppedè F, Bosco P, Lorenzoni V, et al (2014) The MTRR 66A>G polymorphism and maternal risk of birth of a child with Down syndrome in Caucasian women: A case-control study and a meta-analysis. Mol Biol Rep 41:5571–5583. doi: 10.1007/s11033-014-3462-5

Cornelis MC, Fornage M, Foy M, et al (2015) Genome-wide association study of selenium concentrations. Hum Mol Genet 24:1469–1477. doi: 10.1093/hmg/ddu546

Cousins RJ (2010) Gastrointestinal factors influencing zinc absorption and homeostasis. Int J Vitam Nutr Res 80:243–248. doi: 10.1024/0300-9831/a000030

Creus M, Deulofeu R, Pe Ñ Arrubia J, et al (2013) Plasma homocysteine and vitamin B12 serum levels, red blood cell folate concentrations, C677T methylenetetrahydrofolate reductase gene mutation and risk of recurrent miscarriage: a case-control study in Spain. Clin Chem Lab Med 51:693–699. doi: 10.1515/cclm-2012-0452

Crosby WM, Metcoff J, Costiloe JP, et al (1977) Fetal malnutrition: an appraisal of correlated factors. Am J Obstet Gynecol 128:22–31.

Custódio PJ, Carvalho ML, Nunes F (2003) Trace elements determination by energy dispersive X-

ray fluorescence (EDXRF) in human placenta and membrane: A comparative study. In: Analytical and Bioanalytical Chemistry. pp 1101–1106

Czeizel AE, Dudás I (1992) Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. N Engl J Med 327:1832–5. doi: 10.1056/NEJM199212243272602

D'Andrea G, Di Giulio A, Salucci M, Oratore A (1995) Binding and iron delivering of ovotransferrin to cholesterol-depleted chick-embryo red blood cells. Cell Signal 7:67–74.

D'Lima NG, Ma J, Winkler L, et al (2016) A human microprotein that interacts with the mRNA decapping complex. Nat Chem Biol 1–11. doi: 10.1038/nchembio.2249

Dabbaghmanesh MH, Salehi NM, Siadatan J, Omrani GR (2011) Copper Concentration in a Healthy Urban Adult Population of Southern Iran. Biol Trace Elem Res 144:217–224. doi: 10.1007/s12011-011-9074-1

Dalton T, Palmiter RD, Andrews GK (1994) Transcriptional induction of the mouse metallothionein-I gene in hydrogen peroxide-treated Hepa cells involves a composite major late transcription factor/antioxidant response element and metal response promoter elements. Nucleic Acids Res 22:5016–23.

Daly SF, Molloy AM, Mills JL, et al (1999) The influence of 5,10 methylenetetrahydrofolate reductase genotypes on enzyme activity in placental tissue. Br J Obs Gynaecol 106:1214–1218.

Dancis J, Lee C, Mendoza S, Liebes L (1993) Nucleoside transport by perfused human placenta. Placenta 14:547–554. doi: 10.1016/S0143-4004(05)80207-3

Danishpajooh IO, Gudi T, Chen Y, et al (2001) Nitric oxide inhibits methionine synthase activity in vivo and disrupts carbon flow through the folate pathway. J Biol Chem 276:27296–303. doi: 10.1074/jbc.M104043200

Danzeisen R, Fosset C, Chariana Z, et al (2002) Placental ceruloplasmin homolog is regulated by iron and copper and is implicated in iron metabolism. Am J Physiol Cell Physiol 282:C472–C478. doi: 10.1152/ajpcell.00019.2001

Danzeisen R, Ponnambalam S, Lea RG, et al (2000) The effect of ceruloplasmin on iron release from placenta (BeWo) cells; evidence for an endogenous cu oxidase. Placenta 21:805–812. doi: 10.1053/plac.2000.0582

Davis SR, Quinlivan EP, Shelnutt KP, et al (2005) The methylenetetrahydrofolate reductase 677C->T polymorphism and dietary folate restriction affect plasma one-carbon

metabolites and red blood cell folate concentrations and distribution in women. J Nutr 135:1040–4.

Dawson EB, Croft HA, Clark RR, McGanity WJ (1969) Study of nine cation levels in term placentas. II. Changes associated with toxemia of pregnancy and fetal prematurity. Am J Obstet Gynecol 103:1144–7.

Dawson KM, Baltz JM (1997) Organic osmolytes and embryos: substrates of the Gly and beta transport systems protect mouse zygotes against the effects of raised osmolarity. Biol Reprod 56:1550–8.

de Benoist B (2008) Conclusions of a WHO Technical Consultation on folate and vitamin B12 deficiencies. Food Nutr Bull 29:S238-44.

de Lau LML, van Meurs JBJ, Uitterlinden AG, et al (2010) Genetic variation in homocysteine metabolism, cognition, and white matter lesions. Neurobiol Aging 31:2020–2022. doi: 10.1016/j.neurobiolaging.2008.10.004

De Moraes ML, De Faria Barbosa R, Santo RE, et al (2011) Distribution of calcium, iron, copper, and zinc in two portions of placenta of teenager and adult women. Biol Trace Elem Res 143:1271–1281. doi: 10.1007/s12011-011-8963-7

de Toledo LR, Perry TW (1985) Distribution of supplemental selenium in the serum, hair, colostrum, and fetus of parturient dairy cows. J Dairy Sci 68:3249–54. doi: 10.3168/jds.S0022-0302(85)81233-9

de Vogel S, Meyer K, Fredriksen Å, et al (2013) Serum folate and vitamin B12 concentrations in relation to prostate cancer risk--a Norwegian population-based nested case-control study of 3000 cases and 3000 controls within the JANUS cohort. Int J Epidemiol 42:201–10. doi: 10.1093/ije/dys199

de Weerd S, Steegers-Theunissen RPM, de Boo TM, et al (2003) Maternal periconceptional biochemical and hematological parameters, vitamin profiles and pregnancy outcome. Eur J Clin Nutr 57:1128–34. doi: 10.1038/sj.ejcn.1601654

Del Nero U, Rudge M, Novo N, et al (2002) Metodologia para Estudo do Volume e Densidade Absoluta da Placenta Humana de Termo Material e Métodos. Rev Bras Ginecol Obs 24:669–673.

Delgado-Reyes C V, Wallig M a, Garrow T a (2001) Immunohistochemical detection of betaine-homocysteine S-methyltransferase in human, pig, and rat liver and kidney. Arch Biochem Biophys 393:184–186. doi: 10.1006/abbi.2001.2474

Deng L, Elmore CL, Lawrance AK, et al (2008) Methionine synthase reductase deficiency results in adverse reproductive outcomes and congenital heart defects in mice. Mol Genet Metab 94:336–342. doi: 10.1016/j.ymgme.2008.03.004

Devlin AM, Clarke R, Birks J, et al (2006) Interactions among polymorphisms in folate-metabolizing genes and serum total homocysteine concentrations in a healthy elderly population. Am J Clin Nutr 83:708–713.

DeVos L, Chanson A, Liu Z, et al (2008) Associations between single nucleotide polymorphisms in folate uptake and metabolizing genes with blood folate, homocysteine, and DNA uracil concentrations. Am J Clin Nutr 88:1149–1158.

Diamond AM (2015) The subcellular location of selenoproteins and the impact on their function. Nutrients 7:3938–3948. doi: 10.3390/nu7053938

Di Simone N, Maggiano N, Caliandro D, et al (2003) Homocysteine induces trophoblast cell death with apoptotic features. Biol Reprod 69:1129–1134. doi: 10.1095/biolreprod.103.015800

Díaz E, Halhali A, Luna C, et al (2002) Newborn birth weight correlates with placental zinc, umbilical insulin-like growth factor I, and leptin levels in preeclampsia. Arch Med Res 33:40–47. doi: 10.1016/S0188-4409(01)00364-2

Dikiy A, Novoselov S V., Fomenko DE, et al (2007) SelT, SelW, SelH, and Rdx 12: Genomics and molecular insights into the functions of selenoproteins of a novel thioredoxin-like family. Biochemistry 46:6871–6882. doi: 10.1021/bi602462q

Direcció General de Salut Pública (Departament de Salut - Generalitat de Catalunya) (2005) Protocol de seguiment de l'embaràs a Catalunya [Catalonia pregnancy monitoring protocol].

Dirección General de Salud Pública (Ministerio de Sanidad y Consumo - Gobierno de España) (2001) Recomendaciones sobre suplementación con ácido fólico para la prevención de defectos del tubo neural [Folic acid supplementation recommendations for the prevention of neural tube defects].

Dirección General de Salud Pública (Ministerio de Sanidad y Consumo - Gobierno de España) (1998) Recomendaciones sobre suplementación con ácido fólico para la prevención de defectos del tubo neural [Folic acid supplementation recommendations for the prevention of neural tube defects].

Dobrzynski W, Szymanski W aw, Zachara B aw, et al (1998) Decreased selenium concentration in maternal and cord blood in preterm compared with term delivery. Analyst 123:93–97.

doi: 10.1039/a704884j

Dominguez-Salas P, Moore SE, Cole D, et al (2013) DNA methylation potential: dietary intake and blood concentrations of one-carbon metabolites and cofactors in rural African women. Am J Clin Nutr 97:1217–27. doi: 10.3945/ajcn.112.048462

Donangelo CM, King JC (2012) Maternal zinc intakes and homeostatic adjustments during pregnancy and lactation. Nutrients 4:782–798.

Drummond JT, Huang S, Blumenthal RM, Matthews RG (1993) Assignment of enzymatic function to specific protein regions of cobalamin-dependent methionine synthase from Escherichia coli. Biochemistry 32:9290–9295.

Duerre JA, Wallwork JC (1986) Methionine metabolism in isolated perfused livers from rats fed on zinc-deficient and restricted diets. Br J Nutr 56:395–405.

Ebbing M, Bønaa KH, Arnesen E, et al (2010) Combined analyses and extended follow-up of two randomized controlled homocysteine-lowering B-vitamin trials. J Intern Med 268:367–382. doi: 10.1111/j.1365-2796.2010.02259.x

Ebesh O, Barone A, Harper RG, Wapnir RA (1999) Combined effect of high-fat diet and copper deficiency during gestation on fetal copper status in the rat. Biol Trace Elem Res 67:139–150. doi: 10.1007/BF02784069

EFSA - CONTAM Panel (2009) Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on cadmium in food. EFSA J 980:1–139.

EFSA - NDA Panel (2016) Dietary Reference Values for choline. EFSA J 14:4484.

Ek J, Magnus EM (1981) Plasma and red blood cell folate during normal pregnancies. Acta Obstet Gynecol Scand 60:247–51.

Elmore CL, Wu X, Leclerc D, et al (2007) Metabolic derangement of methionine and folate metabolism in mice deficient in methionine synthase reductase. Mol Genet Metab 91:85–97. doi: 10.1016/j.ymgme.2007.02.001

Emmert JL, Garrow TA, Baker DH (1996) Hepatic betaine-homocysteine methyltransferase activity in the chicken is influenced by dietary intake of sulfur amino acids, choline and betaine. J Nutr 126:2050–2058.

Enciso M, Sarasa J, Xanthopoulou L, et al (2016) Polymorphisms in the MTHFR gene influence embryo viability and the incidence of aneuploidy. Hum Genet 135:555–68. doi: 10.1007/s00439-016-1652-z

Enders A (1965) A comparative study of the fine structure of the trophoblast in several

hemochorial placentas. Am J Anat 116:29–67. doi: 10.1002/aja.1001160103

Enders AC, Blankenship TN (1999) Comparative placental structure. Adv. Drug Deliv. Rev. 38:3–15.

Erdeve O, Siklar Z, Kocaturk PA, et al (2004) Antioxidant superoxide dismutase activity in obese children. Biol Trace Elem Res 98:219–28. doi: 10.1385/BTER:98:3:219

Erdogan MO, Yildiz SH, Solak M, et al (2010) C677T polymorphism of the methylenetetrahydrofolate reductase gene does not affect folic acid, vitamin B12, and homocysteine serum levels in Turkish children with neural tube defects. Genet Mol Res 9:1197–203. doi: 10.4238/vol9-2gmr816

Esfahani ST, Cogger EA, Caudill MA (2003) Heterogeneity in the prevalence of methylenetetrahydrofolate reductase gene polymorphisms in women of different ethnic groups. J Am Diet Assoc 103:200–207. doi: 10.1053/jada.2003.50030

Evans DF, Pye G, Bramley R, et al (1988) Measurement of gastrointestinal pH profiles in normal ambulant human subjects. Gut 29:1035–41. doi: 10.1136/gut.29.8.1035

Evans DM, Zhu G, Dy V, et al (2013) Genome-wide association study identifies loci affecting blood copper, selenium and zinc. Hum Mol Genet 22:3998–4006. doi: 10.1093/hmg/ddt239

Evans JC, Huddler DP, Jiracek J, et al (2002) Betaine-homocysteine methyltransferase: Zinc in a distorted barrel. Structure 10:1159–1171. doi: 10.1016/S0969-2126(02)00796-7

Facco F, You W, Grobman W (2009) Genetic thrombophilias and intrauterine growth restriction: a meta-analysis. Obstet Gynecol 113:1206–16. doi: 10.1097/AOG.0b013e3181a6e96a

Fagher U, Laudanski T, Schütz A, et al (1993) The relationship between cadmium and lead burdens and preterm labor. Int J Gynecol Obstet 40:109–114. doi: 10.1016/0020-7292(93)90368-7

Fairweather-Tait SJ, Bao Y, Broadley MR, et al (2011) Selenium in human health and disease. Antioxid Redox Signal 14:1337–1383. doi: 10.1089/ars.2010.3275

Falcón M, Viñas P, Osuna E, Luna A (2002) Environmental exposures to lead and cadmium measured in human placenta. Arch Environ Health 57:598–602. doi: 10.1080/00039890209602094

Farkas SA, Bottiger AK, Isaksson HS, et al (2013) Epigenetic alterations in folate transport genes in placental tissue from fetuses with neural tube defects and in leukocytes from subjects with hyperhomocysteinemia. Epigenetics 8:303–316. doi: 10.4161/epi.23988

Federación Española de Sociedades de Nutrición Alimentación y Dietética (2010) Ingestas Dietéticas de Referencia (IDR) para la Población Española, 2010. Act dietética 14:196–7.

Feix A, Winkelmayer WC, Eberle C, et al (2004) Methionine synthase reductase MTRR 66A > G has no effect on total homocysteine, folate, and Vitamin B12 concentrations in renal transplant patients. Atherosclerosis 174:43–8. doi: 10.1016/j.atherosclerosis.2003.12.036

Feng Q, Kalari K, Fridley BL, et al (2011) Betaine-homocysteine methyltransferase: Human liver genotype-phenotype correlation. Mol Genet Metab 102:126–133. doi: 10.1016/j.ymgme.2010.10.010

Ferguson PL, Flintoff WF (1999) Topological and functional analysis of the human reduced folate carrier by hemagglutinin epitope insertion. J Biol Chem 274:16269–16278. doi: 10.1074/jbc.274.23.16269

Fernandes-Costa F, Metz J (1979) Transplacental transport in the rabbit of vitamin B12 bound to human transcobalamin I, II and III. Br J Haematol 43:625–30.

Fernàndez-Roig S, Cavallé-Busquets P, Fernandez-Ballart JD, et al (2013) Low folate status enhances pregnancy changes in plasma betaine and dimethylglycine concentrations and the association between betaine and homocysteine. Am J Clin Nutr 97:1252–1259. doi: 10.3945/ajcn.112.054189

Finkelstein JD (1990) Methionine metabolism in mammals. J Nutr Biochem 1:228–237.

Finkelstein JD, Harris BJ, Kyle WE (1972) Methionine metabolism in mammals: Kinetic study of betaine-homocysteine methyltransferase. Arch Biochem Biophys 153:320–324. doi: 10.1016/0003-9861(72)90451-1

Finkelstein JD, Harris BJ, Martin JJ, Kyle WE (1982) Regulation of hepatic betaine-homocysteine methyltransferase by dietary methionine. Biochem Biophys Res Commun 108:344–348. doi: 10.1016/0006-291X(82)91872-1

Finkelstein JD, Kyle WE, Harris BJ (1974) Methionine metabolism in mammals: regulatory effects of S-adenosylhomocysteine. Arch Biochem Biophys 165:774–9.

Finkelstein JD, Martin JJ (1986) Methionine metabolism in mammals. Adaptation to methionine excess. J Biol Chem 261:1582–1587. doi: 10.1016/0955-2863(90)90070-2

Finkelstein JD, Martin JJ (1984a) Methionine metabolism in mammals. Distribution of homocysteine between competing pathways. J Biol Chem 259:9508–13.

Finkelstein JD, Martin JJ (1984b) Inactivation of betaine-homocysteine methyltransferase by adenosylmethionine and adenosylethionine. Biochem Biophys Res Commun 118:14–9.

Finkelstein JD, Martin JJ, Kyle WE, Harris BJ (1978) Methionine metabolism in mammals: Regulation of methylenetetrahydrofolate reductase content of rat tissues. Arch Biochem Biophys 191:153–160. doi: 10.1016/0003-9861(78)90077-2

Fisher MC, Zeisel SH, Mar MH, Sadler TW (2001) Inhibitors of choline uptake and metabolism cause developmental abnormalities in neurulating mouse embryos. Teratology 64:114–122. doi: 10.1002/tera.1053

Fisher MC, Zeisel SH, Mar MH, Sadler TW (2002) Perturbations in choline metabolism cause neural tube defects in mouse embryos in vitro. FASEB J 16:619–621. doi: 10.1096/fj.01-0564fje

Fiskerstrand T, Refsum H, Kvalheim G, Ueland PM (1993) Homocysteine and other thiols in plasma and urine: automated determination and sample stability. Clin Chem 39:263–71.

Födinger M, Dierkes J, Skoupy S, et al (2003) Effect of glutamate carboxypeptidase II and reduced folate carrier polymorphisms on folate and total homocysteine concentrations in dialysis patients. J Am Soc Nephrol 14:1314–9.

Food Fortification Initiative (2016) Country Profiles.

Forman JP, Rimm EB, Stampfer MJ, Curhan GC (2005) Folate Intake and the Risk of Incident Hypertension Among US Women. J Am Med Assoc 293:320–329.

Fosmire GJ, Greeley S, Sandstead HH (1977) Maternal and fetal response to various suboptimal levels of zinc intake during gestation in the rat. J Nutr 107:1543–50.

Foster M, Dilworth M, Woods D (1964) COBALAMIN AND THE SYNTHESIS OF METHIONINE BY ESCHERICHIA COLI. Nature 201:39–42.

Fowler B (2001) Homocysteine in health and disease. In: Carmel R, Jacobsen D (eds) 1st edn. Cambridge, pp 163–175

Fredriksen A, Meyer K, Ueland PM, et al (2007) Large-scale population-based metabolic phenotyping of thirteen genetic polymorphisms related to one-carbon metabolism. Hum Mutat 28:856–65. doi: 10.1002/humu.20522

Friedman PA, Shia MA, Wallace JK (1977) A saturable high affinity binding site for transcobalamin II-vitamin B12 complexes in human placental membrane preparations. J Clin Invest 59:51–8. doi: 10.1172/JCI108621

Friesen RW, Novak EM, Hasman D, Innis SM (2007) Relationship of dimethylglycine, choline, and betaine with oxoproline in plasma of pregnant women and their newborn infants. J Nutr 137:2641–2646. doi: 137/12/2641 [pii]

Froese DS, Wu X, Zhang J, et al (2008) Restricted role for methionine synthase reductase defined by subcellular localization. Mol Genet Metab 94:68–77. doi: 10.1016/j.ymgme.2007.11.019

Frosst P, Blom HJ, Milos R, et al (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 10:111–3. doi: 10.1038/ng0595-111

Fujii K, Galivan JH, Huennekens FM (1977) Activation of methionine synthase: further characterization of flavoprotein system. Arch Biochem Biophys 178:662–70.

Fuller NJ, Bates CJ, Evans PH, Lucas A (1992) High folate intakes related to zinc status in preterm infants. Eur J Pediatr 151:51–53. doi: 10.1007/BF02073892

Fuller NJ, Evans PH, Howlett M, Bates CJ (1988) The effects of dietary folate and zinc on the outcome of pregnancy and early growth in rats. Br J Nutr 59:251–9.

Furness DLF, Fenech MF, Khong YT, et al (2008) One-carbon metabolism enzyme polymorphisms and uteroplacental insufficiency. Am J Obstet Gynecol. doi: 10.1016/j.ajog.2008.06.020

Furness DLF, Yasin N, Dekker GA, et al (2012) Maternal red blood cell folate concentration at 10-12 weeks gestation and pregnancy outcome. J Matern Fetal Neonatal Med 25:1423–1427. doi: 10.3109/14767058.2011.636463

Galivan J, Ryan TJ, Chave K, et al (2000) Glutamyl hydrolase: pharmacological role and enzymatic characterization. Pharmacol Ther 85:207–15.

Gamble M V., Liu X, Ahsan H, et al (2005) Folate, homocysteine, and arsenic metabolism in arsenic-exposed individuals in Bangladesh. Environ Health Perspect 113:1683–1688. doi: 10.1289/ehp.8084

Gamble M V., Liu X, Ahsan H, et al (2006) Folate and arsenic metabolism: A double-blind, placebo-controlled folic acid-supplementation trial in Bangladesh. Am J Clin Nutr 84:1093–1101. doi: 10.1097/00001648-200611001-01422

Gamble M V, Liu X, Slavkovich V, et al (2007) Folic acid supplementation lowers blood arsenic. Am J Clin Nutr 86:1202–9.

Gambling L, Czopek A, Andersen HS, et al (2009) Fetal iron status regulates maternal iron metabolism during pregnancy in the rat. Am J Physiol Regul Integr Comp Physiol 296:R1063–R1070. doi: 10.1152/ajpregu.90793.2008

Gambling L, Danzeisen R, Gair S, et al (2001) Effect of iron deficiency on placental transfer of iron and expression of iron transport proteins in vivo and in vitro. Biochem J 356:883–

889. doi: 10.1042/0264-6021:3560883

Gambling L, Dunford S, McArdle HJ (2004) Iron deficiency in the pregnant rat has differential effects on maternal and fetal copper levels. J Nutr Biochem 15:366–72. doi: 10.1016/j.jnutbio.2003.12.009

Ganji V, Kafai MR (2006) Trends in serum folate, RBC folate, and circulating total homocysteine concentrations in the United States: analysis of data from National Health and Nutrition Examination Surveys, 1988-1994, 1999-2000, and 2001-2002. J Nutr 136:153–8.

Ganu R, Garrow T, Koutmos M, et al (2013) Splicing variants of the porcine betaine-homocysteine S-methyltransferase gene: Implications for mammalian metabolism. Gene 529:228–237. doi: 10.1016/j.gene.2013.07.103

Ganz AB, Shields K, Fomin VG, et al (2016) Genetic impairments in folate enzymes increase dependence on dietary choline for phosphatidylcholine production at the expense of betaine synthesis. FASEB J 30:3321–3333. doi: 10.1096/fj.201500138RR

García-Minguillán CJ, Fernandez-Ballart JD, Ceruelo S, et al (2014) Riboflavin status modifies the effects of methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) polymorphisms on homocysteine. Genes Nutr 9:435. doi: 10.1007/s12263-014-0435-1

Gardiner MR (1966) Chronic selenium toxicity studies in sheep. Aust Vet J 42:442–8.

Gaughan DJ, Kluijtmans LAJ, Barbaux S, et al (2001) The methionine synthase reductase (MTRR) A66G polymorphism is a novel genetic determinant of plasma homocysteine concentrations. Atherosclerosis 157:451–456. doi: 10.1016/S0021-9150(00)00739-5

Gaull GE, Von Berg W, Räihä NC, Sturman JA (1973) Development of methyltransferase activities of human fetal tissues. Pediatr Res 7:527–33. doi: 10.1203/00006450-197305000-00006

Gaunt TR, Rodríguez S, Day IN (2007) Cubic exact solutions for the estimation of pairwise haplotype frequencies: implications for linkage disequilibrium analyses and a web tool "CubeX". BMC Bioinformatics 8:428. doi: 10.1186/1471-2105-8-428

Gebel TW (2002) Arsenic methylation is a process of detoxification through accelerated excretion. Int J Hyg Environ Health 205:505–8. doi: 10.1078/1438-4639-00177

Geisel J, Zimbelmann I, Schorr H, et al (2001) Genetic defects as important factors for moderate hyperhomocysteinemia. Clin Chem Lab Med 39:698–704. doi: 10.1515/CCLM.2001.115

Geisser P (1990) In vitro studies on interactions of iron salts and complexes with food-stuffs and medicaments. Arzneimittelforschung 40:754–60.

Geissler C, Singh M (2011) Iron, meat and health. Nutrients 3:283–316.

Gelineau-van Waes J, Maddox JR, Smith LM, et al (2008) Microarray analysis of E9.5 reduced folate carrier (RFC1; Slc19a1) knockout embryos reveals altered expression of genes in the cubilin-megalin multiligand endocytic receptor complex. BMC Genomics 9:156. doi: 10.1186/1471-2164-9-156

Geller J, Kronn D, Jayabose S, Sandoval C (2002) Hereditary folate malabsorption: family report and review of the literature. Medicine (Baltimore) 81:51–68.

GeneCards (2016) Human gene database. http://www.genecards.org/.

Gentry-Nielsen MJ, Preheim LC, Lyman KN, et al (2001) Use of rat models to mimic alterations in iron homeostasis during human alcohol abuse and cirrhosis. Alcohol 23:71–81.

George L, Mills JL, Johansson A, et al (2002) Plasma folate levels and risk of spontaneous abortion. JAMA 288:1867–1873.

Ghayour-Mobarhan M, Shapouri-Moghaddam A, Azimi-Nezhad M, et al (2009) The relationship between established coronary risk factors and serum copper and zinc concentrations in a large Persian Cohort. J Trace Elem Med Biol 23:167–175. doi: 10.1016/j.jtemb.2009.03.006

Ghishan FK, Said HM, Wilson PC, et al (1986) Intestinal transport of zinc and folic acid: A mutual inhibitory effect. Am J Clin Nutr 43:258–262.

Ghneim HK, Al-Sheikh YA, Alshebly MM, Aboul-Soud MAM (2016) Superoxide dismutase activity and gene expression levels in Saudi women with recurrent miscarriage. Mol Med Rep 13:2606–2612. doi: 10.3892/mmr.2016.4807

Gibson RS, Hess SY, Hotz C, Brown KH (2008) Indicators of zinc status at the population level: a review of the evidence. Br J Nutr 99:S14–S23. doi: 10.1017/s0007114508006818

Giedroc DP, Chen X, Apuy JL (2001) Metal Response Element (MRE)-Binding Transcription Factor-1 (MTF-1): Structure, Function, and Regulation. Antioxid Redox Signal 3:577–596. doi: 10.1089/15230860152542943

Gilman CL, Soon R, Sauvage L, et al (2015) Umbilical cord blood and placental mercury, selenium and selenoprotein expression in relation to maternal fish consumption. J Trace Elem Med Biol 30:17–24. doi: 10.1016/j.jtemb.2015.01.006

Gimsing P, Nexø E, Hippe E (1983) Determination of cobalamins in biological material. II. The cobalamins in human plasma and erythrocytes after desalting on nonpolar adsorbent material, and separation by one-dimensional thin-layer chromatography. Anal Biochem

129:296–304.

Giugliani ER, Jorge SM, Gonçalves AL (1985) Serum vitamin B12 levels in parturients, in the intervillous space of the placenta and in full-term newborns and their interrelationships with folate levels. Am J Clin Nutr 41:330–5.

Giusti B, Sestini I, Saracini C, et al (2008) High-throughput multiplex single-nucleotide polymorphism (SNP) analysis in genes involved in methionine metabolism. Biochem Genet 46:406–23. doi: 10.1007/s10528-008-9159-5

Glanville T, Yates Z, Ovadia L, et al (2006) Fetal folate C677T methylenetetrahydrofolate reductase gene polymorphism and low birth weight. J Obstet Gynaecol 26:11–4. doi: 10.1080/01443610500363865

Gnana-Prakasam JP, Reddy SK, Veeranan-Karmegam R, et al (2011) Polarized distribution of heme transporters in retinal pigment epithelium and their regulation in the iron-overload disease hemochromatosis. Invest Ophthalmol Vis Sci 52:9279–86. doi: 10.1167/iovs.11-8264

Goel R, Misra PK (1982) Plasma copper in foetal malnutrition. Acta Paediatr Scand 71:421–3.

Gong D, Gu H, Zhang Y, et al (2012) Methylenetetrahydrofolate reductase C677T and reduced folate carrier 80 G>A polymorphisms are associated with an increased risk of conotruncal heart defects. Clin Chem Lab Med 50:1455–61. doi: 10.1515/cclm-2011-0759

González B, Pajares MA, Too HP, et al (2002) Crystallization and preliminary X-ray study of recombinant betaine-homocysteine S-methyltransferase from rat liver. Acta Crystallogr D Biol Crystallogr 58:1507–10.

González S, Huerta JM, Alvarez-Uría J, et al (2004) Serum selenium is associated with plasma homocysteine concentrations in elderly humans. J Nutr 134:1736–1740.

Gordis L (2008) Epidemiology. In: 4th edn. Saunders, pp 177–99

Gow AJ, Buerk DG, Ischiropoulos H (1997) A novel reaction mechanism for the formation of S-nitrosothiol in vivo. J Biol Chem 272:2841–5.

Gow AJ, Cobb F, Stamler JS (2001) Homocysteine in health and disease. In: Carmel R, Jacobsen DW (eds) 1st edn. Cambridge, pp 39–45

Goyer RA, Cherian MG (1992) Role of metallothionein in human placenta and rats exposed to cadmium. IARC Sci Publ 239–247.

Goyette P, Pai a, Milos R, et al (1998) Gene structure of human and mouse methylenetetrahydrofolate reductase (MTHFR). Mamm Genome 9:652–656. doi:

10.1007/s003359900838

Goyette P, Sumner JS, Milos R, et al (1994) Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification. Nat. Genet. 7:195–200.

Grant C, Lalor G, Fletcher H, et al (2010) Elements in Human Placentae in Jamaica. West Indian Med J 59:479–85.

Gray H (1918) Anatomy of the human body. In: 20th edn. Lea & Febiger, Philadelphia,

Green TJ, Skeaff CM, Whiting SJ, Gibson RS (2003) Effect of folic acid supplementation on plasma zinc concentrations of young women. Nutrition 19:522–523. doi: 10.1016/S0899-9007(02)01100-0

Greene NDE, Copp AJ (2009) Development of the vertebrate central nervous system: Formation of the neural tube. Prenat. Diagn. 29:303–311.

Griffin TJ, Smith LM (2000) Single-nucleotide polymorphism analysis by MALDI-TOF mass spectrometry. Trends Biotechnol 18:77–84.

Gu QP, Sun Y, Ream LW, Whanger PD (2000) Selenoprotein W accumulates primarily in primate skeletal muscle, heart, brain and tongue. Mol Cell Biochem 204:49–56.

Guerra-Shinohara EM, Paiva AA, Rondo PH, et al (2002) Relationship between total homocysteine and folate levels in pregnant women and their newborn babies according to maternal serum levels of vitamin B12. BJOG 109:784–791.

Guillén M, Corella D, Portolés O, et al (2001) Prevalence of the methylenetetrahydrofolate reductase 677C > T mutation in the Mediterranean Spanish population. Association with cardiovascular risk factors. Eur J Epidemiol 17:255–261. doi: 10.1023/A:1017978503416

Gulati S, Chen Z, Brody LC, et al (1997) Defects in auxiliary redox proteins lead to functional methionine synthase deficiency. J Biol Chem 272:19171–19175. doi: 10.1074/jbc.272.31.19171

Gulec S, Anderson GJ, Collins JF (2014) Mechanistic and regulatory aspects of intestinal iron absorption. Am J Physiol Gastrointest Liver Physiol 307:G397-409. doi: 10.1152/ajpgi.00348.2013

Gunshin H, Mackenzie B, Berger U V., et al (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 388:482–488. doi: 10.1038/41343

Gupta N, Gupta S, Dama M, et al (2011) Strong association of 677 C>T substitution in the MTHFR gene with male infertility - a study on an Indian population and a Meta-Analysis. PLoS One. doi: 10.1371/journal.pone.0022277

Gupta VJ, Wilcken DE (1978) The detection of cysteine-homocysteine mixed disulphide in plasma of normal fasting man. Eur J Clin Invest 8:205–7.

Gutiérrez-Torres DS, González-Horta C, Del Razo LM, et al (2015) Prenatal Exposure to Sodium Arsenite Alters Placental Glucose 1, 3, and 4 Transporters in Balb/c Mice. Biomed Res Int 2015:175025. doi: 10.1155/2015/175025

Guttormsen AB, Solheim E, Refsum H (2004) Variation in plasma cystathionine and its relation to changes in plasma concentrations of homocysteine and methionine in healthy subjects during a 24-h observation period. Am J Clin Nutr 79:76–9.

Haendeler J, Hoffmann J, Tischler V, et al (2002) Redox regulatory and anti-apoptotic functions of thioredoxin depend on S-nitrosylation at cysteine 69. Nat cell biolology 4:743–749. doi: 10.1038/ncb851

Hajjar KA, Mauri L, Jacovina AT, et al (1998) Tissue plasminogen activator binding to the annexin II tail domain. Direct modulation by homocysteine. J Biol Chem 273:9987–93.

Håkansson K, Carlsson M, Svensson LA, Liljas A (1992) Structure of native and apo carbonic anhydrase II and structure of some of its anion-ligand complexes. J Mol Biol 227:1192–204.

Hall M, Gamble M, Slavkovich V, et al (2007) Determinants of arsenic metabolism: Blood arsenic metabolites, plasma folate, cobalamin, and homocysteine concentrations in maternal-newborn pairs. Environ Health Perspect 115:1503–1509. doi: 10.1289/ehp.9906

Hall MN, Liu X, Slavkovich V, et al (2009a) Influence of cobalamin on arsenic metabolism in Bangladesh. Environ Health Perspect 117:1724–1729. doi: 10.1289/ehp.0900734

Hall MN, Liu X, Slavkovich V, et al (2009b) Folate, cobalamin, cysteine, homocysteine, and Arsenic metabolism among children in Bangladesh. Environ Health Perspect 117:825–831. doi: 10.1289/ehp.0800164

Halsted CH, Ling EH, Luthi-Carter R, et al (1998) Folylpoly-gamma-glutamate carboxypeptidase from pig jejunum: Molecular characterization and relation to glutamate carboxypeptidase II. J Biol Chem 273:20417–20424. doi: 10.1074/jbc.273.32.20417

Halsted CH, Villanueva J a, Devlin AM, et al (2002) Folate deficiency disturbs hepatic methionine metabolism and promotes liver injury in the ethanol-fed micropig. Proc Natl Acad Sci U S A 99:10072–10077. doi: 10.1073/pnas.112336399

Hambidge M, Hackshaw A, Wald N (1993) Neural tube defects and serum zinc. Br J Obstet Gynaecol 100:746–9.

Hamed E, Attia MS, Bassiouny K (2009) Synthesis, spectroscopic and thermal characterization of copper(II) and iron(III) complexes of folic acid and their absorption efficiency in the blood. Bioinorg Chem Appl 979680. doi: 10.1155/2009/979680

Hamer DH (1986) Metallothionein. Annu Rev Biochem 55:913–951. doi: 10.1146/annurev.bi.55.070186.004405

Hansen M, Samman S, Madsen LT, et al (2001) Folic acid enrichment of bread does not appear to affect zinc absorption in young women. Am J Clin Nutr 74:125–129.

Hao L, Ma J, Zhu J, et al (2007) High prevalence of hyperhomocysteinemia in Chinese adults is associated with low folate, vitamin B-12, and vitamin B-6 status. J Nutr 137:407–13.

Hardman B, Manuelpillai U, Wallace EM, et al (2006) Expression, Localisation and Hormone Regulation of the Human Copper Transporter hCTR1 in Placenta and Choriocarcinoma Jeg-3 Cells. Placenta 27:968–977. doi: 10.1016/j.placenta.2005.10.011

Hardman B, Manuelpillai U, Wallace EM, et al (2004) Expression and Localization of Menkes and Wilson Copper Transporting ATPases in Human Placenta. Placenta 25:512–517. doi: 10.1016/j.placenta.2003.11.013

Hardman B, Michalczyk A, Greenough M, et al (2007) Hormonal regulation of the Menkes and Wilson copper-transporting ATPases in human placental Jeg-3 cells. Biochem J 402:241–250. doi: 10.1042/BJ20061099

Harmon DL, Woodside J V, Yarnell JW, et al (1996) The common “thermolabile” variant of methylene tetrahydrofolate reductase is a major determinant of mild hyperhomocysteinaemia. QJM 89:571–7.

Harpel PC, Chang VT, Borth W (1992) Homocysteine and other sulfhydryl compounds enhance the binding of lipoprotein(a) to fibrin: a potential biochemical link between thrombosis, atherogenesis, and sulfhydryl compound metabolism. Proc Natl Acad Sci U S A 89:10193–7.

Hatori Y, Lutsenko S (2013) An Expanding Range of Functions for the Copper Chaperone/Antioxidant Protein Atox1. Antioxid Redox Signal 19:945–957. doi: 10.1089/ars.2012.5086

Hay WW (1991) The placenta: Not just a conduit for maternal fuels. In: Diabetes. pp 44–50

Hazra A, Kraft P, Lazarus R, et al (2009) Genome-wide significant predictors of metabolites in the one-carbon metabolism pathway. Hum Mol Genet 18:4677–4687. doi: 10.1093/hmg/ddp428

Hazra A, Wu K, Kraft P, et al (2007) Twenty-four non-synonymous polymorphisms in the one-carbon metabolic pathway and risk of colorectal adenoma in the Nurses' Health Study. Carcinogenesis 28:1510–1519. doi: 10.1093/carcin/bgm062

He X, Hong X, Zeng F, et al (2009) Zinc antagonizes homocysteine-induced fetal heart defects in rats. Cardiovasc Toxicol 9:151–159. doi: 10.1007/s12012-009-9048-0

He X, Ma Q (2009) Induction of metallothionein I by arsenic via metal-activated transcription factor 1: critical role of C-terminal cysteine residues in arsenic sensing. J Biol Chem 284:12609–21. doi: 10.1074/jbc.M901204200

Hegsted D, Kinney T, Cartaya J (1948) The effect of low protein and low choline diets on the absorption of iron and copper. Am J Pathol 24:722.

Heil SG, Lievers KJ, Boers GH, et al (2000) Betaine-homocysteine methyltransferase (BHMT): genomic sequencing and relevance to hyperhomocysteinemia and vascular disease in humans. Mol Genet Metab 71:511–519. doi: 10.1006/mgme.2000.3078

Helston RM, Phillips SR, McKay JA, et al (2007) Zinc transporters in the mouse placenta show a coordinated regulatory response to changes in dietary zinc intake. Placenta 28:437–44. doi: 10.1016/j.placenta.2006.07.002

Henderson GB, Zevely EM (1983) Structural requirements for anion substrates of the methotrexate transport system in L1210 cells. Arch Biochem Biophys 221:438–46.

Henderson GI, Perez T, Schenker S, et al (1995) Maternal-to-fetal transfer of 5-methyltetrahydrofolate by the perfused human placental cotyledon: evidence for a concentrative role by placental folate receptors in fetal folate delivery. J Lab Clin Med 126:184–203.

Hershfield MS, Aiyar VN, Premakumar R, Small WC (1985) S-Adenosylhomocysteine hydrolase from human placenta. Affinity purification and characterization. Biochem J 230:43–52.

Hess SY, Peerson JAM, King JC, Brown KH (2007) Use of serum zinc concentration as an indicator of population zinc status. Food Nutr Bull 28:S403-29.

Heuchel R, Radtke F, Georgiev O, et al (1994) The transcription factor MTF-1 is essential for basal and heavy metal-induced metallothionein gene expression. EMBO J 13:2870–5. doi: 10.1002/cssc.201100629

Hill DM, Johnson LJ, Burns PJ, et al (2002) Effects of temperature on stability of blood homocysteine in collection tubes containing 3-deazaadenosine. Clin Chem 48:2017–22.

Hirooka T, Galambos JT (1966) Selenium metabolism. I. Respiratory excretion. Biochim Biophys

Acta 130:313–20.

Hmadcha A, Bedoya FJ, Sobrino F, Pintado E (1999) Methylation-dependent gene silencing induced by interleukin 1beta via nitric oxide production. J Exp Med 190:1595–604.

Hobbs CA, Cleves MA, Karim MA, et al (2010) Maternal folate-related gene environment interactions and congenital heart defects. Obstet Gynecol 116:316–22. doi: 10.1097/AOG.0b013e3181e80979

Hoffman JL, McConnell KP (1987) Periodate-oxidized adenosine inhibits the formation of dimethylselenide and trimethylselenonium ion in mice treated with selenite. Arch Biochem Biophys 254:534–40.

Hogeveen M, den Heijer M, Semmekrot B a., et al (2013) Umbilical choline and related methylamines betaine and dimethylglycine in relation to birth weight. Pediatr Res 73:783–787. doi: 10.1038/pr.2013.54

Holm PI, Bleie Ø, Ueland PM, et al (2004) Betaine as a Determinant of Post-methionine load Total Plasma Homocysteine before and after B-Vitamin Supplementation. Arterioscler Thromb Vasc Biol 24:301–307. doi: 10.1161/01.ATV.0000114569.54976.31

Holm PI, Ueland PM, Kvalheim G, Lien EA (2003) Determination of choline, betaine, and dimethylglycine in plasma by a high-throughput method based on normal-phase chromatography-tandem mass spectrometry. Clin Chem 49:286–94.

Holm PI, Ueland PM, Vollset SE, et al (2005) Betaine and folate status as cooperative determinants of plasma homocysteine in humans. Arterioscler Thromb Vasc Biol 25:379–85. doi: 10.1161/01.ATV.0000151283.33976.e6

Holmes VA, Wallace JMW, Alexander HD, et al (2005) Homocysteine Is Lower in the Third Trimester of Pregnancy in Women with Enhanced Folate Status from Continued Folic Acid Supplementation. Clin Chem 51:629–634. doi: 10.1373/clinchem.2004.032698

Holmes M V, Newcombe P, Hubacek JA, et al (2011) Effect modification by population dietary folate on the association between MTHFR genotype, homocysteine, and stroke risk: a meta-analysis of genetic studies and randomised trials. Lancet 378:584–594. doi: 10.1016/S0140-6736(11)60872-6

Homocysteine Lowering Trialists' Collaboration. (2005) Dose-dependent effects of folic acid on blood concentrations of homocysteine: a meta-analysis of the randomized trials. Am J Clin Nutr 82:806–12.

Homocysteine Studies Collaboration (2002) Homocysteine and risk of ischemic heart disease

and stroke: a meta-analysis. JAMA 288:2015–22.

Hong KH, Keen CL, Mizuno Y, et al (2000) Effects of dietary zinc deficiency on homocysteine and folate metabolism in rats. J Nutr Biochem 11:165–9.

Horie A, Ishida K, Watanabe Y, et al (2014) Membrane transport mechanisms of choline in human intestinal epithelial LS180 cells. Biopharm Drug Dispos 35:532–542. doi: 10.1002/bdd.1917

Hou X, Chen X, Shi J (2015) Genetic polymorphism of MTHFR C677T and premature coronary artery disease susceptibility: A meta-analysis. Gene 565:39–44. doi: 10.1016/j.gene.2015.03.062

Huang SS-Y, Hung SSO, Chan HM (2014) Maintaining tissue selenium species distribution as a potential defense mechanism against methylmercury toxicity in juvenile white sturgeon (Acipenser transmontanus). Aquat Toxicol 156:88–95. doi: 10.1016/j.aquatox.2014.08.004

Huang T, Ren J, Huang J, Li D (2013) Association of homocysteine with type 2 diabetes: a meta-analysis implementing Mendelian randomization approach. BMC Genomics 14:867. doi: 10.1186/1471-2164-14-867

Huang Y, Khartulyari S, Morales ME, et al (2008) Quantification of key red blood cell folates from subjects with defined MTHFR 677C > T genotypes using stable isotope dilution liquid chromatography / mass spectrometry. 2403–2412. doi: 10.1002/rcm

Hubmacher D, Tiedemann K, Bartels R, et al (2005) Modification of the structure and function of fibrillin-1 by homocysteine suggests a potential pathogenetic mechanism in homocystinuria. J Biol Chem 280:34946–55. doi: 10.1074/jbc.M504748200

Hubner U, Alwan A, Jouma M, et al (2008) Low serum vitamin B12 is associated with recurrent pregnancy loss in Syrian women. Clin Chem Lab Med 46:1265–1269. doi: 10.1515/CCLM.2008.247

Hübner U, Schorr H, Eckert R, et al (2007) Stability of plasma homocysteine, S-adenosylmethionine, and S-adenosylhomocysteine in EDTA, acidic citrate, and Primavette collection tubes. Clin Chem 53:2217–8. doi: 10.1373/clinchem.2007.093930

Hultberg B, Andersson A, Isaksson A (1997) Copper ions differ from other thiol reactive metal ions in their effects on the concentration and redox status of thiols in HeLa cell cultures. Toxicology 117:89–97.

Hung IH, Casareno RL, Labesse G, et al (1998) HAH1 is a copper-binding protein with distinct

amino acid residues mediating copper homeostasis and antioxidant defense. J Biol Chem 273:1749–54.

Huxley T (1869) An introduction to the classification of animals. Churchill & sons, London, pp 87–114

Hyvonen-Dabek M, Nikkinen-Vilkki P, Dabek JT (1984) Selenium and other elements in human maternal and umbilical serum, as determined simultaneously by proton-induced X-ray emission. Clin Chem 30:529–533.

Imbard A, Smulders YM, Barto R, et al (2013) Plasma choline and betaine correlate with serum folate, plasma S-adenosyl-methionine and S-adenosyl-homocysteine in healthy volunteers. Clin Chem Lab Med 51:683–692. doi: 10.1515/cclm-2012-0302

Imperatore A, Florio P, Torres PB, et al (2006) Urocortin 2 and urocortin 3 are expressed by the human placenta, deciduas, and fetal membranes. Am J Obstet Gynecol 195:288–295. doi: 10.1016/j.ajog.2005.12.048

Infante-Rivard C, Rivard G-E, Yotov W V, et al (2002) Absence of association of thrombophilia polymorphisms with intrauterine growth restriction. N Engl J Med 347:19–25. doi: 10.1056/NEJM200207043470105

Institute of Medicine (2016a) Pubchem. https://pubchem.ncbi.nlm.nih.gov/compound/.

Institute of Medicine (2000) Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline. National Academies Press, Washington, D.C.

Institute of Medicine (2016b) Gene. http://www.ncbi.nlm.nih.gov/gene/.

Institute of Medicine (2016c) Nucleotide. http://www.ncbi.nlm.nih.gov/nuccore/.

Institute of Medicine - Panel on Dietary Antioxidant and Related Compounds (2000) Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids, 1st edn. Washington (DC)

Institute of Medicine - Panel on Micronutrients (2001) Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc, 1st edn. National Academies Press., Washington (DC)

Irvine GW, Pinter TBJ, Stillman MJ (2016) Defining the metal binding pathways of human metallothionein 1a: balancing zinc availability and cadmium seclusion. Metallomics 8:71–81. doi: 10.1039/C5MT00225G

Isotalo PA, Wells GA, Donnelly JG (2000) Neonatal and fetal methylenetetrahydrofolate

reductase genetic polymorphisms: an examination of C677T and A1298C mutations. Am J Hum Genet 67:986–90. doi: 10.1086/303082

IUPAC-IUB (1975) The Nomenclature of Corrinoids. http://www.chem.qmul.ac.uk/iupac/misc/B12.html.

Iyengar G, Rapp A (2001a) Human placenta as a “dual” biomarker for monitoring fetal and maternal environment with special reference to potentially toxic trace elements. Part 2: essential minor, trace and other (non-essential) elements in human placenta. Sci Total Environ 280:207–219.

Iyengar G, Rapp A (2001b) Human placenta as a “dual” biomarker for monitoring fetal and maternal environment with special reference to potentially toxic trace elements Part 1: Physiology, function and sampling of placenta for elemental characterisation. Sci Total Environ 280:195–206.

IZiNCG (2004) Chapter 4: Research needs.

Jacobsen DW (2000) Hyperhomocysteinemia and oxidative stress: time for a reality check? Arterioscler Thromb Vasc Biol 20:1182–4.

Jacobsen DW (2001) Homocysteine in health and disease. In: Carmel R, Jacobsen DW (eds) 1st edn. Cambridge, pp 9–20

Jacques PF, Bostom AG, Selhub J, et al (2003) Effects of polymorphisms of methionine synthase and methionine synthase reductase on total plasma homocysteine in the NHLBI Family Heart Study. Atherosclerosis 166:49–55.

Jacques PF, Bostom AG, Williams RR, et al (1996) Relation Between Folate Status, a Common Mutation in Methylenetetrahydrofolate Reductase, and Plasma Homocysteine Concentrations. Circulation 93:7–9. doi: 10.1161/01.CIR.93.1.7

Jadavji NM, Bahous RH, Deng L, et al (2014) Mouse model for deficiency of methionine synthase reductase exhibits short-term memory impairment and disturbances in brain choline metabolism. Biochem J 461:205–12. doi: 10.1042/BJ20131568

Jakubowski H (1997) Metabolism of homocysteine thiolactone in human cell cultures. Possible mechanism for pathological consequences of elevated homocysteine levels. J Biol Chem 272:1935–42.

Jakubowski H (2001) Translational accuracy of aminoacyl-tRNA synthetases: implications for atherosclerosis. J Nutr 131:2983S–7S.

Jakubowski H (1999) Protein homocysteinylation: possible mechanism underlying pathological

consequences of elevated homocysteine levels. FASEB J 13:2277–83.

Jakubowski H (2004) Molecular basis of homocysteine toxicity in humans. Cell Mol Life Sci 61:470–87. doi: 10.1007/s00018-003-3204-7

Jakubowski H (2006) Pathophysiological Consequences of Homocysteine Excess. J Nutr 136:16365–16405.

Jarabak J, Bachur N (1971) A soluble dihydrofolate reductase from human placenta: purification and properties. Arch Biochem Biophys 142:417–25.

Jardine MJ, Kang A, Zoungas S, et al (2012) The effect of folic acid based homocysteine lowering on cardiovascular events in people with kidney disease: systematic review and meta-analysis. BMJ 344:e3533.

Jean-Claude F, Ireland-Ripert J, Toque C, et al (1995) Répertoire général des aliments : Tables de composition, XXVII. Paris

Jiang X, Yan J, Caudill MA (2013) Handbook of vitamins. In: Zempleni J, Suttie JW, Gregory III JF, Stover PJ (eds) 5th edn. CRC Press, pp 491–513

Jing M, Rech L, Wu Y, et al (2015) Effects of zinc deficiency and zinc supplementation on homocysteine levels and related enzyme expression in rats. J Trace Elem Med Biol 30:77–82. doi: 10.1016/j.jtemb.2014.10.013

Joannides R, Haefeli WE, Linder L, et al (1995) Nitric Oxide Is Responsible for Flow-Dependent Dilatation of Human Peripheral Conduit Arteries In Vivo. Circulation 91:1314–1319. doi: 10.1161/01.CIR.91.5.1314

Joncquel-Chevalier Curt M, Voicu PM, Fontaine M, et al (2015) Creatine biosynthesis and transport in health and disease. Biochimie 119:146–165.

Kalin S, Rimm E (2010) Folate in health and disease. In: Bailey LB (ed) 2nd edn. CRC Press, pp 263–290

Kalinowski J, Chavez ER (1991) Tissue composition and trace mineral content of the dam and litter under low dietary zinc intake during gestation and lactation of first-litter gilts. J Trace Elem Electrolytes Health Dis 5:35–46.

Kamath A V, Darling IM, Morris ME (2003) Choline uptake in human intestinal Caco-2 cells is carrier-mediated. J Nutr 133:2607–2611.

Kang SS, Wong PWK, Zhou J, Cook HY (1986) Total homocyst(e)ine in plasma and amniotic fluid of pregnant women. Metabolism 35:889–891. doi: 10.1016/0026-0495(86)90049-1

Kantola M, Purkunen R, Kröger P, et al (2004) Selenium in pregnancy: Is selenium an active

defective ion against environmental chemical stress? Environ Res 96:51–61. doi: 10.1016/j.envres.2004.03.003

Kantola M, Purkunen R, Kröger P, et al (2000) Accumulation of cadmium, zinc, and copper in maternal blood and developmental placental tissue: differences between Finland, Estonia, and St. Petersburg. Environ Res 83:54–66. doi: 10.1006/enrs.1999.4043

Kauwell GP, Bailey LB, Gregory JF, et al (1995) Zinc status is not adversely affected by folic acid supplementation and zinc intake does not impair folate utilization in human subjects. J Nutr 125:66–72.

Keating E, Gonçalves P, Campos I, et al (2009) Folic acid uptake by the human syncytiotrophoblast: Interference by pharmacotherapy, drugs of abuse and pathological conditions. Reprod Toxicol 28:511–520. doi: 10.1016/j.reprotox.2009.07.001

Keating JN, Wada L, Stokstad EL, King JC (1987) Folic acid: Effect on zinc absorption in humans and in the rat. Am J Clin Nutr 46:835–9.

Keaveney EM, Price RK, Hamill LL, et al (2015) Postprandial plasma betaine and other methyl donor-related responses after consumption of minimally processed wheat bran or wheat aleurone, or wheat aleurone incorporated into bread. Br J Nutr 113:445–53. doi: 10.1017/S0007114514003778

Keefer RC, Tuma DJ, Barak AJ (1973) Relationship of hepatic trace metals to intestinal transport in choline-deficient rats. Am J Clin Nutr 26:409–14.

Kelleher BP, Broin SD (1991) Microbiological assay for vitamin B12 performed in 96-well microtitre plates. J Clin Pathol 44:592–5.

Kempson SA, Zhou Y, Danbolt NC (2014) The betaine/GABA transporter and betaine: roles in brain, kidney, and liver. Front Physiol 5:159. doi: 10.3389/fphys.2014.00159

Khandanpour N, Willis G, Meyer FJ, et al (2009) Peripheral arterial disease and methylenetetrahydrofolate reductase (MTHFR) C677T mutations: A case-control study and meta-analysis. J Vasc Surg 49:711–718. doi: 10.1016/j.jvs.2008.10.004

Kim EE, Wyckoff HW (1991) Reaction mechanism of alkaline phosphatase based on crystal structures. Two-metal ion catalysis. J Mol Biol 218:449–464. doi: 10.1016/0022-2836(91)90724-K

Kim KN, Kim YJ, Chang N (2004) Effects of the interaction between the C677T 5,10-methylenetetrahydrofolate reductase polymorphism and serum B vitamins on homocysteine levels in pregnant women. Eur J Clin Nutr 58:10–6. doi:

10.1038/sj.ejcn.1601729

Kim SY, Park SY, Choi JW, et al (2011) Association between MTHFR 1298A>C polymorphism and spontaneous abortion with fetal chromosomal aneuploidy. Am J Reprod Immunol 66:252–258. doi: 10.1111/j.1600-0897.2011.00996.x

Kimura T, Kambe T (2016) The Functions of Metallothionein and ZIP and ZnT Transporters: An Overview and Perspective. Int J Mol Sci 17:336. doi: 10.3390/ijms17030336

King JC (2011) Zinc: an essential but elusive nutrient. Am J Clin Nutr 94:679S–84S. doi: 10.3945/ajcn.110.005744

Kippler M, Hoque AMW, Raqib R, et al (2010) Accumulation of cadmium in human placenta interacts with the transport of micronutrients to the fetus. Toxicol Lett 192:162–168. doi: 10.1016/j.toxlet.2009.10.018

Klapcinska B, Poprzecki S, Danch A, et al (2005) Selenium levels in blood of upper Silesian population: evidence of suboptimal selenium status in a significant percentage of the population. Biol Trace Elem Res 108:1–15. doi: 10.1385/BTER:108:1-3:001

Klapec T, Ćavar S, Kasač Z, et al (2008) Selenium in placenta predicts birth weight in normal but not intrauterine growth restriction pregnancy. J Trace Elem Med Biol 22:54–58. doi: 10.1016/j.jtemb.2007.10.004

Klotz K, Weistenhöfer W, Drexter H (2013) Cadmium: from toxicity to essentiality. In: Sigel A, Sigel H, Sigel R (eds) 1st edn. Springer Netherlands, pp 85–98

Kluijtmans LAJ, Young IS, Boreham CA, et al (2003) Genetic and nutritional factors contributing to hyperhomocysteinemia in young adults. Blood 101:2483–2488. doi: 10.1182/blood.V101.7.2483

Knock E, Deng L, Wu Q, et al (2008) Strain differences in mice highlight the role of DNA damage in neoplasia induced by low dietary folate. J Nutr 138:653–658.

Knock E, Deng L, Wu Q, et al (2006) Low dietary folate initiates intestinal tumours in mice, with altered expression of G2-M checkpoint regulators polo-like kinase 1 and cell Division cycle 25c. Cancer Res 66:10349–10356. doi: 10.1158/0008-5472.CAN-06-2477

Koc H, Mar M-H, Ranasinghe A, et al (2002) Quantitation of choline and its metabolites in tissues and foods by liquid chromatography/electrospray ionization-isotope dilution mass spectrometry. Anal Chem 74:4734–40.

Koebnick C, Heins UA, Dagnelie PC, et al (2002) Longitudinal concentrations of vitamin B(12) and vitamin B(12)-binding proteins during uncomplicated pregnancy. Clin Chem 48:928–

33.

Kolhouse JF, Utley C, Allen RH (1980) Isolation and characterization of methylmalonyl-CoA mutase from human placenta. J Biol Chem 255:2708–2712.

Koorts a M, Viljoen M (2007) Ferritin and ferritin isoforms I: Structure-function relationships, synthesis, degradation and secretion. Arch Physiol Biochem 113:30–54. doi: 10.1080/13813450701318583

Kordas K, Ettinger AS, Lamadrid-Figueroa H, et al (2009) Methylenetetrahydrofolate reductase (MTHFR) C677T, A1298C and G1793A genotypes, and the relationship between maternal folate intake, tibia lead and infant size at birth. Br J Nutr 102:907–14. doi: 10.1017/S0007114509318280

Kosanovic M, Jokanovic M, Jevremovic M, et al (2002) Maternal and fetal cadmium and selenium status in normotensive and hypertensive pregnancy. Biol Trace Elem Res 89:97–103. doi: 10.1385/bter:89:2:97

Kowalczuk S, Bröer A, Munzinger M, et al (2005) Molecular cloning of the mouse IMINO system: an Na+- and Cl--dependent proline transporter. Biochem J 386:417–22. doi: 10.1042/BJ20050100

Krachler M, Rossipal E, Micetic-Turk D (1999) Trace element transfer from the mother to the newborn--investigations on triplets of colostrum, maternal and umbilical cord sera. Eur J Clin Nutr 53:486–494. doi: 10.1038/sj.ejcn.1600781

Kraus F, Redline R, Gersell D, et al (2004) Placental pathology. In: Armed Forces Institute of Pathology (ed) 1st edn. Washington (DC), pp 1–22

Krebs N, Hambidge K, Hagerman R, et al (1989) Nutrient availability: chemical and biological aspects. In: Southgate D, Johnson I, Fenwick G (eds). Royal Society of Chemistry, Cambridge, pp 226–229

Krikke GG, Grooten IJ, Vrijkotte TGM, et al (2016) Vitamin B12 and folate status in early pregnancy and cardiometabolic risk factors in the offspring at age 5-6 years: Findings from the ABCD multi-ethnic birth cohort. BJOG An Int J Obstet Gynaecol 123:384–392. doi: 10.1111/1471-0528.13574

Król E, Krejpcio Z, Chmurzynska A (2011) Folic acid and protein content in maternal diet and postnatal high-fat feeding affect the tissue levels of iron, zinc, and copper in the rat. Biol Trace Elem Res 144:885–93. doi: 10.1007/s12011-011-9048-3

Kuhnert BR, Kuhnert PM, Debanne S, Williams TG (1987a) The relationship between cadmium,

zinc, and birth weight in pregnant women who smoke. Am J Obstet Gynecol 157:1247–51.

Kuhnert BR, Kuhnert PM, Lazebnik N, Erhard P (1988a) The effect of maternal smoking on the relationship between maternal and fetal zinc status and infant birth weight. J Am Coll Nutr 7:309–16.

Kuhnert BR, Kuhnert PM, Zarlingo TJ (1988b) Associations between placental cadmium and zinc and age and parity in pregnant women who smoke. Obstet Gynecol 71:67–70.

Kuhnert PM, Kuhnert BR, Erhard P, et al (1987b) The effect of smoking on placental and fetal zinc status. Am J Obstet Gynecol 157:1241–1246.

Kumudini N, Uma A, Naushad SM, et al (2014) Association of seven functional polymorphisms of one-carbon metabolic pathway with total plasma homocysteine levels and susceptibility to Parkinson's disease among South Indians. Neurosci Lett 568:1–5. doi: 10.1016/j.neulet.2014.03.044

Kutzbach C, Stokstad ELR (1971) Mammalian methylenetetrahydrofolate reductase Partial purification, properties, and inhibition by S-adenosylmethionine. Biochim Biophys Acta 250:459–477. doi: 10.1016/0005-2744(71)90247-6

Laanpere M, Altmae S, Kaart T, et al (2011) Folate-metabolizing gene variants and pregnancy outcome of IVF. Reprod Biomed Online 22:603–614. doi: 10.1016/j.rbmo.2011.03.002

Laftah AH, Latunde-Dada GO, Fakih S, et al (2009) Haem and folate transport by proton-coupled folate transporter/haem carrier protein 1 (SLC46A1). Br J Nutr 101:1150–6. doi: 10.1017/S0007114508066762

Lagerkvist BJ, Sandberg S, Frech W, et al (1996) Is placenta a good indicator of cadmium and lead exposure? Arch Environ Health 51:389–394.

Lai S-C, Nakayama Y, Sequeira JM, et al (2013) The transcobalamin receptor knockout mouse: a model for vitamin B12 deficiency in the central nervous system. FASEB J 27:2468–75. doi: 10.1096/fj.12-219055

Lambert BD, Titgemeyer EC, Stokka GL, et al (2002) Methionine supply to growing steers affects hepatic activities of methionine synthase and betaine-homocysteine methyltransferase, but not cystathionine synthase. J Nutr 132:2004–2009.

Landon M (1975) Folate metabolism in pregnancy. Clin Obstet Gynaecol 2:413–430.

Landon M (1972) Placental y-glutamyl carboxipeptidase. Int J Biochem 3:387–388.

Landon MJ, Eyre DH, Hytten FE (1975) Transfer of folate to the fetus. Br J Obstet Gynaecol 82:12–19.

Laraqui A, Allami A, Carri?? A, et al (2007) Relation between plasma homocysteine, gene polymorphisms of homocysteine metabolism-related enzymes, and angiographically proven coronary artery disease. Eur J Intern Med 18:474–483. doi: 10.1016/j.ejim.2007.02.020

Lassi ZS, Salam RA, Haider BA, Bhutta ZA (2013) Folic acid supplementation during pregnancy for maternal health and pregnancy outcomes. Cochrane database Syst Rev 3:CD006896. doi: 10.1002/14651858.CD006896.pub2

Latunde-Dada GO, Takeuchi K, Simpson RJ, McKie AT (2006) Haem carrier protein 1 (HCP1): Expression and functional studies in cultured cells. FEBS Lett 580:6865–70. doi: 10.1016/j.febslet.2006.11.048

Lazo JS, Kondo Y, Dellapiazza D, et al (1995) Enhanced sensitivity to oxidative stress in cultured embryonic cells from transgenic mice deficient in metallothionein I and II genes. J Biol Chem 270:5506–10.

Le Blanc S, Garrick MD, Arredondo M (2012) Heme carrier protein 1 transports heme and is involved in heme-Fe metabolism. AJP Cell Physiol 302:C1780–C1785. doi: 10.1152/ajpcell.00080.2012

Le Kim D, Betzing H (1976) Intestinal absorption of polyunsaturated phosphatidylcholine in the rat. Hoppe Seylers Z Physiol Chem 357:1321–31.

Leclerc D, Odievre M, Wu Q, et al (1999) Molecular cloning, expression and physical mapping of the human methionine synthase reductase gene. Gene 240:75–88. doi: S037811199900431X [pii]

Leclerc D, Wilson A, Dumas R, et al (1998) Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria. Proc Natl Acad Sci U S A 95:3059–64.

Lee HH, Prasad AS, Brewer GJ, Owyang C (1989) Zinc absorption in human small intestine. Am J Physiol 256:G87-91.

Lee JE, Jacques PF, Dougherty L, et al (2010) Are dietary choline and betaine intakes determinants of total homocysteine concentration? Am J Clin Nutr 91:1303–1310. doi: 10.3945/ajcn.2009.28456

Lee MB, Kooistra M, Zhang B, et al (2012) Betaine-homocysteine methyltransferase is active in the mouse blastocyst and promotes inner cell mass development. J Biol Chem 287:33094–33103. doi: 10.1074/jbc.M112.365478

Lever M, George PM, Dellow WJ, et al (2005) Homocysteine, glycine betaine, and N,N-dimethylglycine in patients attending a lipid clinic. Metabolism 54:1–14. doi: 10.1016/j.metabol.2004.07.007

Li F, Feng Q, Lee C, et al (2008) Human betaine-homocysteine methyltransferase (BHMT) and BHMT2: Common gene sequence variation and functional characterization. Mol Genet Metab 94:326–335. doi: 10.1016/j.ymgme.2008.03.013

Li M, Li Y, Chen J, et al (2007) Copper ions inhibit S-adenosylhomocysteine hydrolase by causing dissociation of NAD+ cofactor. Biochemistry 46:11451–8. doi: 10.1021/bi700395d

Li S, Zhi L, Liu Y, et al (2016) Effect of in ovo feeding of folic acid on the folate metabolism, immune function and epigenetic modification of immune effector molecules of broiler. Br J Nutr 115:411–21. doi: 10.1017/S0007114515004511

Li WX, Dai SX, Zheng JJ, et al (2015) Homocysteine Metabolism Gene Polymorphisms (MTHFR C677T, MTHFR A1298C, MTR A2756G and MTRR A66G) Jointly Elevate the Risk of Folate Deficiency. Nutrients 7:6670–87. doi: 10.3390/nu7085303

Li X, Sun Q, Li X, et al (2014a) Dietary betaine supplementation to gestational sows enhances hippocampal IGF2 expression in newborn piglets with modified DNA methylation of the differentially methylated regions. Eur J Nutr. doi: 10.1007/s00394-014-0799-4

Li X, Yin D, Yin J, et al (2014b) Dietary selenium protect against redox-mediated immune suppression induced by methylmercury exposure. Food Chem Toxicol 72:169–177. doi: 10.1016/j.fct.2014.07.023

Li Y-F, Dong Z, Chen C, et al (2012) Organic selenium supplementation increases mercury excretion and decreases oxidative damage in long-term mercury-exposed residents from Wanshan, China. Environ Sci Technol 46:11313–8. doi: 10.1021/es302241v

Liang S, Zhou Y, Wang H, et al (2014) The effect of multiple single nucleotide polymorphisms in the folic acid pathway genes on homocysteine metabolism. Biomed Res Int. doi: 10.1155/2014/560183

Liaugaudas G, Jacques P, Selhub J, et al (2001) Renal insufficiency, vitamin B(12) status, and population attributable risk for mild hyperhomocysteinemia among coronary artery disease patients in the era of folic acid-fortified cereal grain flour. Arter Thromb Vasc Biol 21:849–51.

Lima SCVC, Arrais RF, Sales CH, et al (2006) Assessment of copper and lipid profile in obese children and adolescents. Biol Trace Elem Res 114:19–29. doi: 10.1385/BTER:114:1:19

Lin SJ, Culotta VC (1995) The ATX1 gene of Saccharomyces cerevisiae encodes a small metal homeostasis factor that protects cells against reactive oxygen toxicity. Proc Natl Acad Sci U S A 92:3784–8.

Linnell JC, Hoffbrand A V., Hussein HAA, et al (1974) Tissue distribution of coenzyme and other forms of vitamin B12 in control subjects and patients with pernicious anaemia. Clin. Sci. Mol. Med. 46:163–172.

Liu J, Jin L, Zhang L, et al (2013) Placental concentrations of manganese and the risk of fetal neural tube defects. J Trace Elem Med Biol 27:322–325. doi: 10.1016/j.jtemb.2013.04.001

Liu J, Qi J, Yu X, et al (2014) Investigations of single nucleotide polymorphisms in folate pathway genes in Chinese families with neural tube defects. J Neurol Sci 337:61–66. doi: 10.1016/j.jns.2013.11.017

Liu M, Ge Y, Cabelof DC, et al (2005) Structure and regulation of the murine reduced folate carrier gene: Identification of four noncoding exons and promoters and regulation by dietary folates. J Biol Chem 280:5588–5597. doi: 10.1074/jbc.M412662200

Liuzzi JP, Aydemir F, Nam H, et al (2006) Zip14 (Slc39a14) mediates non transferrin bound iron uptake into cells. Proc Natl Acad Sci 103:13612–13617.

Llanos MN, Ronco AM (2009) Fetal growth restriction is related to placental levels of cadmium, lead and arsenic but not with antioxidant activities. Reprod Toxicol 27:88–92. doi: 10.1016/j.reprotox.2008.11.057

Locke AE, Dooley KJ, Tinker SW, et al (2010) Variation in folate pathway genes contributes to risk of congenital heart defects among individuals with Down syndrome. Genet Epidemiol 34:613–623. doi: 10.1002/gepi.20518

Lopreato FR, Stabler SP, Carvalho FR, et al (2008) Relationships between gene polymorphisms of folate-related proteins and vitamins and metabolites in pregnant women and neonates. Clin Chim Acta 398:134–139. doi: 10.1016/j.cca.2008.09.004

Lorenzo Alonso MJ, Bermejo Barrera A, Cocho de Juan JA, et al (2005) Selenium levels in related biological samples: Human placenta, maternal and umbilical cord blood, hair and nails. J Trace Elem Med Biol 19:49–54. doi: 10.1016/j.jtemb.2005.07.006

Lu D, Maulik N, Moraru II, et al (1993) Molecular adaptation of vascular endothelial cells to oxidative stress. Am J Physiol 264:C715-22.

Lu J, Holmgren A (2014) The thioredoxin antioxidant system. Free Radic Biol Med 66:75–87. doi: 10.1016/j.freeradbiomed.2013.07.036

Luck AN, Mason AB (2012) Transferrin-Mediated Cellular Iron Delivery. Curr Top Membr 69:3–35. doi: 10.1016/B978-0-12-394390-3.00001-X

Lucock M, Daskalakis I, Hinkins M, Yates Z (2001) An examination of polymorphic genes and folate metabolism in mothers affected by a spina bifida pregnancy. Mol Genet Metab 73:322–32. doi: 10.1006/mgme.2001.3205

Lucock M, Yates Z, Boyd L, et al (2013) Vitamin C-related nutrient-nutrient and nutrient-gene interactions that modify folate status. Eur J Nutr 52:569–582. doi: 10.1007/s00394-012-0359-8

Ludwig ML, Matthews RG (1997) Structure-based perspectives on B12-dependent enzymes. Annu Rev Biochem 66:269–313. doi: 10.1146/annurev.biochem.66.1.269

Luo L, Chen Y, Wang L, et al (2015) Polymorphisms of Genes Involved in the Folate Metabolic Pathway Impact the Occurrence of Unexplained Recurrent Pregnancy Loss. Reprod Sci 22:845–51. doi: 10.1177/1933719114565033

Luo YL, Cheng YL, Ye P, et al (2012) Association between MTHFR polymorphisms and orofacial clefts risk: a meta-analysis. Birth Defects Res A Clin Mol Teratol 94:237–44. doi: 10.1002/bdra.23005

Lussier-Cacan S, Xhignesse M, Piolot A, et al (1996) Plasma total homocysteine in healthy subjects: Sex-specific relation with biological traits. Am J Clin Nutr 64:587–593.

Lynch SR, Cook JD (1980) Interaction of vitamin C and iron. Ann N Y Acad Sci 355:32–44. doi: 10.1111/j.1749-6632.1980.tb21325.x

Mahanta LB, Roy TD, Dutta RG, Devi A (2012) Nutritional Status and the Impact of Socioeconomic Factors on Pregnant Women in Kamrup District of Assam. Ecol Food Nutr 51:463–480. doi: 10.1080/03670244.2012.705701

Majors AK, Sengupta S, Willard B, et al (2002) Homocysteine binds to human plasma fibronectin and inhibits its interaction with fibrin. Arterioscler Thromb Vasc Biol 22:1354–9.

Malhotra a, Fairweather-Tait SJ, Wharton P a, Gee H (1990) Placental zinc in normal and intra-uterine growth-retarded pregnancies. Br J Nutr 63:613–21. doi: 10.1079/BJN19900147

Manci EA, Blackburn WR (1987) Regional variations in the levels of zinc, iron, copper, and calcium in the term human placenta. Placenta 8:497–502. doi: 10.1016/0143-4004(87)90078-6

Mando C, Tabano S, Colapietro P, et al (2011) Transferrin receptor gene and protein expression and localization in human IUGR and normal term placentas. Placenta 32:44–50. doi:

10.1016/j.placenta.2010.10.009

Mansoor MA, Bergmark C, Haswell SJ, et al (2000) Correlation between plasma total homocysteine and copper in patients with peripheral vascular disease. Clin Chem 46:385–91.

Mansoori N, Tripathi M, Alam R, et al (2014) Serum folic acid and RFC A80G polymorphism in Alzheimer's disease and vascular dementia. Am J Alzheimers Dis Other Demen 29:38–44. doi: 10.1177/1533317513505131

Mao S, Xiang W, Huang S, Zhang A (2014) Association between homocysteine status and the risk of nephropathy in type 2 diabetes mellitus. Clin Chim Acta 431:206–210. doi: 10.1016/j.cca.2014.02.007

Marchant JS, Subramanian VS, Parker I, Said HM (2002) Intracellular trafficking and membrane targeting mechanisms of the human reduced folate carrier in mammalian epithelial cells. J Biol Chem 277:33325–33333. doi: 10.1074/jbc.M205955200

Marini NJ, Gin J, Ziegle J, et al (2008) The prevalence of folate-remedial MTHFR enzyme variants in humans. Proc Natl Acad Sci U S A 105:8055–8060. doi: 10.1073/pnas.0802813105

Marklund SL (1984) Extracellular superoxide dismutase in human tissues and human cell lines. J Clin Invest 74:1398–403. doi: 10.1172/JCI111550

Marklund SL (1982) Human copper-containing superoxide dismutase of high molecular weight. Proc Natl Acad Sci U S A 79:7634–7638. doi: 10.1073/pnas.79.24.7634

Marques RS, Cooke RF, Rodrigues MC, et al (2016) Effects of organic or inorganic cobalt, copper, manganese, and zinc supplementation to late-gestating beef cows on productive and physiological responses of the offspring. J Anim Sci 94:1215. doi: 10.2527/jas.2015-0036

Martí-Carvajal AJ, Solà I, Lathyris D, et al (2013) Homocysteine-lowering interventions for preventing cardiovascular events. Cochrane database Syst Rev 1:CD006612. doi: 10.1002/14651858.CD006612.pub3

Martínez-Frías M-L, Pérez B, Desviat LR, et al (2006) Maternal polymorphisms 677C-T and 1298A-C of MTHFR, and 66A-G MTRR genes: is there any relationship between polymorphisms of the folate pathway, maternal homocysteine levels, and the risk for having a child with Down syndrome? Am J Med Genet A 140:987–97. doi: 10.1002/ajmg.a.31203

Mask G, Lane H (1993) Selected measures of selenium status in full-term and preterm neonates, their mothers and nonpregnant women. Nutr Res 13:901–911.

Masters DG, Keen CL, Lönnerdal B, Hurley LS (1983a) Comparative aspects of dietary copper and zinc deficiencies in pregnant rats. J Nutr 113:1448–51.

Masters DG, Keen CL, Lönnerdal B, Hurley LS (1983b) Zinc deficiency teratogenicity: the protective role of maternal tissue catabolism. J Nutr 113:905–12.

Matsuo Y, Greenberg D (1959) A crystalline enzyme that cleaves homoserine and cystathionine. IV. Mechanism of action, reversibility, and substrate specificity. J Biol Chem 234:516–9.

Matté C, Mackedanz V, Stefanello F, et al (2009) Chronic hyperhomocysteinemia alters antioxidant defenses and increases DNA damage in brain and blood of rats: protective effect of folic acid. Neurochem Int 54:7–13.

Matte JJ, Guay F, Le Floc'h N, Girard CL (2010) Bioavailability of dietary cyanocobalamin (vitamin B12) in growing pigs. J Anim Sci 88:3936–44. doi: 10.2527/jas.2010-2979

Matthews RG, Goulding CW (1997) Enzyme-catalyzed methyl transfers to thiols: the role of zinc. Curr Opin Chem Biol 1:332–9.

Matthews RG, Vanoni MA, Hainfeld JF, Wall J (1984) Methylenetetrahydrofolate reductase. Evidence for spatially distinct subunit domains obtained by scanning transmission electron microscopy and limited proteolysis. J Biol Chem 259:11647–50.

McArdle HJ, Tysoe J (1988) Effect of nicotine on transferrin binding and iron uptake by cultured rat placenta. J Cell Physiol 134:509–513. doi: 10.1002/jcp.1041340326

McBreairty LE, McGowan R a, Brunton J a, Bertolo RF (2013) Partitioning of [methyl-3H]methionine to methylated products and protein is altered during high methyl demand conditions in young Yucatan miniature pigs. J Nutr 143:804–9. doi: 10.3945/jn.112.172593

McDonald SD, Perkins SL, Jodouin CA, Walker MC (2002) Folate levels in pregnant women who smoke: an important gene/environment interaction. Am J Obstet Gynecol 187:620–5.

McKeever MP, Weir DG, Molloy A, Scott JM (1991) Betaine-homocysteine methyltransferase: organ distribution in man, pig and rat and subcellular distribution in the rat. Clin Sci (Lond) 81:551–6.

McKenzie JM, Fosmire GJ, Sandstead HH (1975) Zinc deficiency during the latter third of pregnancy: effects on fetal rat brain, liver, and placenta. J Nutr 105:1466–1475.

McKie AT, Marciani P, Rolfs A, et al (2000) A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. Mol Cell 5:299–309.

McMaster D, Ewing A, Erwin C, et al (1985) The influence of dietary vitamins on zinc uptake

from the lumen of the perfused rat gut. Nutr Res 267–270.

McNulty B, McNulty H, Marshall B, et al (2013) Impact of continuing folic acid after the first trimester of pregnancy: findings of a randomized trial of Folic Acid Supplementation in the Second and Third Trimesters. Am J Clin Nutr 98:92–8. doi: 10.3945/ajcn.112.057489

McNulty H, Strain JJ, Pentieva K, Ward M (2012) C1 metabolism and CVD outcomes in older adults. Proc Nutr Soc 71:213–221. doi: 10.1017/S0029665111003387

Meadows NJ, Grainger SL, Ruse W, et al (1983) Oral iron and the bioavailability of zinc. Br Med J (Clin Res Ed) 287:1013–4.

Medici V, Shibata NM, Kharbanda KK, et al (2013) Wilson's disease: Changes in methionine metabolism and inflammation affect global DNA methylation in early liver disease. Hepatology 57:555–565. doi: 10.1002/hep.26047

Melse-Boonstra A, Holm PI, Ueland PM, et al (2005) Betaine concentration as a determinant of fasting total homocysteine concentrations and the effect of folic acid supplementation on betaine concentrations. Am J Clin Nutr 81:1378–1382.

Mendiburo MJ, Le Blanc S, Espinoza A, et al (2011) Transepithelial heme-iron transport: Effect of heme oxygenase overexpression. Eur J Nutr 50:363–371. doi: 10.1007/s00394-010-0144-5

Meyer K, Fredriksen Å, Ueland PM (2004) High-Level Multiplex Genotyping of Polymorphisms Involved in Folate or Homocysteine Metabolism by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. Clin Chem 50:391–402. doi: 10.1373/clinchem.2003.026799

Meyer LA, Durley AP, Prohaska JR, Harris ZL (2001) Copper transport and metabolism are normal in aceruloplasminemic mice. J Biol Chem 276:36857–36861. doi: 10.1074/jbc.M105361200\nM105361200 [pii]

Michel V, Yuan Z, Ramsubir S, Bakovic M (2006) Choline transport for phospholipid synthesis. Exp Biol Med 231:490–504. doi: 231/5/490 [pii]

Michelakos T, Kousoulis AA, Katsiardanis K, et al (2013) Serum Folate and B12 Levels in Association With Cognitive Impairment Among Seniors: Results From the VELESTINO Study in Greece and Meta-Analysis. J Aging Health 25:589–616. doi: 10.1177/0898264313482488

Midttun Ø, Hustad S, Ueland PM (2009) Quantitative profiling of biomarkers related to B-vitamin status, tryptophan metabolism and inflammation in human plasma by liquid

chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 23:1371–9. doi: 10.1002/rcm.4013

Midttun Ø, Kvalheim G, Ueland PM (2013) High-throughput, low-volume, multianalyte quantification of plasma metabolites related to one-carbon metabolism using HPLC-MS/MS. Anal Bioanal Chem 405:2009–17. doi: 10.1007/s00216-012-6602-6

Midttun O, Townsend MK, Nygård O, et al (2014) Most blood biomarkers related to vitamin status, one-carbon metabolism, and the kynurenine pathway show adequate preanalytical stability and within-person reproducibility to allow assessment of exposure or nutritional status in healthy women and cardiovas. J Nutr 144:784–90. doi: 10.3945/jn.113.189738

Miller J, McLachan a D, Klug a (1985) Repetitive zinc-binding doains in the protein transcription factor IIA from Xenopus oocytes. EMBO J 4:1609–1614.

Millian NS, Garrow T a (1998) Human betaine-homocysteine methyltransferase is a zinc metalloenzyme. Arch Biochem Biophys 356:93–98. doi: 10.1006/abbi.1998.0757

Mills JL, Carter TC, Kay DM, et al (2012) Folate and vitamin B12-related genes and risk for omphalocele. Hum Genet 131:739–746. doi: 10.1007/s00439-011-1117-3

Mills JL, Fan R, Brody LC, et al (2014) Maternal choline concentrations during pregnancy and choline-related genetic variants as risk factors for neural tube defects. Am J Clin Nutr 100:1069–1074. doi: 10.3945/ajcn.113.079319

Milne D (1989) Effects of folic acid supplements on zinc-65 absorption in humans and in the rat. J Trace Elem Exp Med 2:297–304.

Milne D, Lukaski H, Johnson P (1990) Effect of folic acid supplements on zinc balance and metabolism in men fed diets adequate in zinc. J Trace Elem Exp Med 3:319–326.

Milne DB, Canfield WK, Mahalko JR, Sandstead HH (1984) Effect of oral folic acid supplements on zinc, copper, and iron absorption and excretion. Am J Clin Nutr 39:535–9.

Milton NG (2008) Homocysteine inhibits hydrogen peroxide breakdown by catalase. Open Enzym Inhib J 1:34–41.

Misiak B, Łaczmański Ł, Słoka N, et al (2016) Metabolic dysregulation in first-episode schizophrenia patients with respect to genetic variation in one-carbon metabolism. Psychiatry Res 238:60–67.

Mislanova C, Martsenyuk O, Huppertz B, Obolenskaya M (2011) Placental markers of folate-related metabolism in preeclampsia. Reproduction 142:467–476. doi: 10.1530/REP-10-0484

Mistry HD, Williams PJ (2011) The importance of antioxidant micronutrients in pregnancy. Oxid. Med. Cell. Longev.

Mitchell LE, Long J, Garbarini J, et al (2010) Variants of folate metabolism genes and risk of left-sided cardiac defects. Birth Defects Res Part A - Clin Mol Teratol 88:48–53. doi: 10.1002/bdra.20622

Miyauchi S, Srinivas SR, Fei Y-J, et al (2006) Functional characteristics of NaS2, a placenta-specific Na+-coupled transporter for sulfate and oxyanions of the micronutrients selenium and chromium. Placenta 27:550–9. doi: 10.1016/j.placenta.2005.05.004

Mochizuki M, Maruo T, Masuko K, Ohtsu T (1984) Effects of smoking on fetoplacental-maternal system during pregnancy. Am J Obstet Gynecol 149:413–420. doi: 10.1016/0002-9378(84)90156-X

Moestrup SK, Birn H, Fischer PB, et al (1996) Megalin-mediated endocytosis of transcobalamin-vitamin-B12 complexes suggests a role of the receptor in vitamin-B12 homeostasis. Proc Natl Acad Sci U S A 93:8612–7.

Moestrup SK, Verroust PJ (2001) Megalin- and cubilin-mediated endocytosis of protein-bound vitamins, lipids, and hormones in polarized epithelia. Annu Rev Annu Rev Nutr 21:133–17407. doi: 10.1146/annurev.nutr.21.1.407

Mohtaram S, Sheikhha MH, Honarvar N, et al (2016) An Association Study of the SLC19A1 Gene Polymorphisms/Haplotypes with Idiopathic Recurrent Pregnancy Loss in an Iranian Population. Genet Test Mol Biomarkers 20:235–40. doi: 10.1089/gtmb.2015.0230

Molloy AM (2010) Folate in health and disease. In: Bailey LB (ed) 2nd edn. CRC Press, pp 381–408

Molloy AM (2012) Genetic aspects of folate metabolism. Subcell Biochem 56:105–30. doi: 10.1007/978-94-007-2199-9_7

Molloy AM, Daly S, Mills JL, et al (1997) Thermolabile variant of 5,10-methylenetetrahydrofolate reductase associated with low red-cell folates: Implications for folate intake recommendations. Lancet 349:1591–1593. doi: 10.1016/S0140-6736(96)12049-3

Molloy AM, Mills JL, Cox C, et al (2005) Choline and homocysteine interrelations in umbilical cord and maternal plasma at delivery. Am J Clin Nutr 82:836–842.

Molloy AM, Mills JL, Kirke PN, et al (1998) Whole-blood folate values in subjects with different methylenetetrahydrofolate reductase genotypes: differences between the radioassay and microbiological assays. Clin Chem 44:186–8.

Molloy AM, Mills JL, McPartlin J, et al (2002) Maternal and fetal plasma homocysteine concentrations at birth: The influence of folate, vitamin B-12, and the 5,10-methylenetetrahydrofolate reductase 677C -> T variant. Am J Obstet Gynecol 186:499–503.

Molloy AM, Scott JM (1997) Microbiological assay for serum, plasma, and red cell folate using cryopreserved, microtiter plate method. Methods Enzymol 281:43–53.

Montjean D, Benkhalifa M, Dessolle L, et al (2011) Polymorphisms in MTHFR and MTRR genes associated with blood plasma homocysteine concentration and sperm counts. Fertil Steril 95:635–640. doi: 10.1016/j.fertnstert.2010.08.054

Moon S, Fernando MR, Lou MF (2005) Induction of thioltransferase and thioredoxin/thioredoxin reductase systems in cultured porcine lenses under oxidative stress. Invest Ophthalmol Vis Sci 46:3783–9. doi: 10.1167/iovs.05-0237

Moreira de Mello JC, de Araújo ESS, Stabellini R, et al (2010) Random X inactivation and extensive mosaicism in human placenta revealed by analysis of allele-specific gene expression along the X chromosome. PLoS One 5:e10947. doi: 10.1371/journal.pone.0010947

Morin I, Devlin AM, Leclerc D, et al (2003a) Evaluation of genetic variants in the reduced folate carrier and in glutamate carboxypeptidase II for spina bifida risk. Mol Genet Metab 79:197–200.

Morin I, Platt R, Weisberg I, et al (2003b) Common variant in betaine-homocysteine methyltransferase (BHMT) and risk for spina bifida. Am J Med Genet A 119A:172–176. doi: 10.1002/ajmg.a.20115

Mostowska A, Myka M, Lianeri M, et al (2011) Folate and choline metabolism gene variants and development of uterine cervical carcinoma. Clin Biochem 44:596–600. doi: 10.1016/j.clinbiochem.2011.02.007

MRC Vitamin Study Research Group (1991) Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. Lancet 338:131–7.

Mudd SH, Brosnan JT, Brosnan ME, et al (2007) Methyl balance and transmethylation fluxes in humans. Am. J. Clin. Nutr. 85:19–25.

Mudd SH, Cerone R, Schiaffino MC, et al (2001) Glycine N-methyltransferase deficiency: A novel inborn error causing persistent isolated hypermethioninaemia. J Inherit Metab Dis 24:448–464. doi: 10.1023/A:1010577512912

Mudd SH, Finkelstein JD, Refsum H, et al (2000) Homocysteine and its disulfide derivatives: a suggested consensus terminology. Arterioscler Thromb Vasc Biol 20:1704–6.

Mukherjee MD, Sandstead HH, Ratnaparkhi M V, et al (1984) Maternal zinc, iron, folic acid, and protein nutriture and outcome of human pregnancy. Am J Clin Nutr 40:496–507.

Murphy-Chutorian DR, Wexman MP, Grieco AJ, et al (1985) Methionine intolerance: a possible risk factor for coronary artery disease. J Am Coll Cardiol 6:725–30.

Murphy MM, Molloy AM, Ueland PM, et al (2007) Longitudinal study of the effect of pregnancy on maternal and fetal cobalamin status in healthy women and their offspring. J Nutr 137:1863–7.

Murphy MM, Scott JM, Arija V, et al (2004) Maternal homocysteine before conception and throughout pregnancy predicts fetal homocysteine and birth weight. Clin Chem 50:1406–12. doi: 10.1373/clinchem.2004.032904

Murphy MM, Scott JM, McPartlin JM, Fernandez-Ballart JD (2002) The pregnancy-related decrease in fasting plasma homocysteine is not explained by folic acid supplementation, hemodilution, or a decrease in albumin in a longitudinal study. Am J Clin Nutr 76:614–9.

Murri M, Luque-Ramírez M, Insenser M, et al (2013) Circulating markers of oxidative stress and polycystic ovary syndrome (PCOS): a systematic review and meta-analysis. Hum Reprod Update 19:268–88. doi: 10.1093/humupd/dms059

Nagele P, Meissner K, Francis A, et al (2011) Genetic and environmental determinants of plasma total homocysteine levels: impact of population-wide folate fortification. Pharmacogenet Genomics 21:426–31. doi: 10.1097/FPC.0b013e32834741ff

Nakai Y, Inoue K, Abe N, et al (2007) Functional characterization of human proton-coupled folate transporter/heme carrier protein 1 heterologously expressed in mammalian cells as a folate transporter. J Pharmacol Exp Ther 322:469–76. doi: 10.1124/jpet.107.122606

Nakao C, Ookawara T, Sato Y, et al (2000) Extracellular superoxide dismutase in tissues from obese (ob/ob) mice. Free Radic Res 33:229–41.

Nandakumaran M, Dashti HM, Al-Saleh E, Al-Zaid NS (2003) Transport kinetics of zinc, copper, selenium, and iron in perfused human placental lobule in vitro. Mol Cell Biochem 252:91–96. doi: 10.1023/A:1025565720489

National Institutes of Health (2016a) MedlinePlus. https://www.nlm.nih.gov/medlineplus/.

National Institutes of Health (2016b) National Institute of Neurological Disorders and Stroke list of disorders. http://www.ninds.nih.gov/.

National Research Council (US) - Subcommittee on Laboratory Animal Nutrition (1995) Nutrient Requirements of Laboratory Animals, Fourth Revised Edition, 1995. In: Poos M, Overton J, Blackwell D (eds) 4th edn. National Academies Press, Washington (DC), pp 11–58

Nelen WLDM, Blom HJ, Steegers EAP, et al (2000) Hyperhomocysteinemia and recurrent early pregnancy loss: A meta-analysis. Fertil Steril 74:1196–1199. doi: 10.1016/S0015-0282(00)01595-8

Nexo E (1977) Characterization of the cobalamins attached to transcobalamin I and transcobalamin II in human plasma. Scand J Haematol 18:358–60.

Nexo E (1998) Vitamin B12 and B12-Proteins. In: Krautler B, Arigoni D, Golding BT (eds). Wiley-VCH, pp 461–475

Nexo E, Hollenberg MD (1980) Characterization of the particulate and soluble acceptor for transcobalamin II from human placenta and rabbit liver. Biochim Biophys Acta 628:190–200. doi: 10.1016/0304-4165(80)90366-9

Nickel A, Kottra G, Schmidt G, et al (2009) Characteristics of transport of selenoamino acids by epithelial amino acid transporters. Chem Biol Interact 177:234–241. doi: 10.1016/j.cbi.2008.09.008

Nielsen MJ, Rasmussen MR, Andersen CBF, et al (2012) Vitamin B12 transport from food to the body's cells—a sophisticated, multistep pathway. Nat Rev Gastroenterol Hepatol 9:345–354. doi: 10.1038/nrgastro.2012.76

Nishi A, Numata S, Tajima A, et al (2014) Meta-analyses of blood homocysteine levels for gender and genetic association studies of the MTHFR C677T polymorphism in schizophrenia. Schizophr Bull 40:1154–1163. doi: 10.1093/schbul/sbt154

Nishimura T, Sai Y, Fujii J, et al (2010) Roles of TauT and system A in cytoprotection of rat syncytiotrophoblast cell line exposed to hypertonic stress. Placenta 31:1003–1009. doi: 10.1016/j.placenta.2010.08.003

Nishio E, Watanabe Y (1997) Homocysteine as a modulator of platelet-derived growth factor action in vascular smooth muscle cells: a possible role for hydrogen peroxide. Br J Pharmacol 122:269–74. doi: 10.1038/sj.bjp.0701391

Niu X, Chen X, Xiao Y, et al (2014) The differences in homocysteine level between obstructive sleep apnea patients and controls: A meta-analysis. PLoS One. doi: 10.1371/journal.pone.0095794

Nogales F, Ojeda ML, Delgado MJ, et al (2011) Effects of antioxidant supplementation on

duodenal Se-Met absorption in ethanol-exposed rat offspring in vivo. J Reprod Dev 57:708–14.

Nose Y, Kim B-E, Thiele DJ (2006) Ctr1 drives intestinal copper absorption and is essential for growth, iron metabolism, and neonatal cardiac function. Cell Metab 4:235–244. doi: 10.1016/j.cmet.2006.08.009

Noy V, Baracaldo CM, Forero Y, et al (2002) [Stability and effect of ingestion on the levels of folate in plasma]. Biomédica Rev del Inst Nac Salud 22:46–50.

O'Brien KO, Zavaleta N, Caulfield LE, et al (2000) Prenatal iron supplements impair zinc absorption in pregnant Peruvian women . J Nutr 130:2251–2255.

O'Broin S, Kelleher B (1992) Microbiological assay on microtitre plates of folate in serum and red cells. J Clin Pathol 45:344–7.

O'Leary VB, Mills JL, Pangilinan F, et al (2005) Analysis of methionine synthase reductase polymorphisms for neural tube defects risk association. Mol Genet Metab 85:220–227. doi: 10.1016/j.ymgme.2005.02.003

Obeid R, Fedosov SN, Nexo E (2015) Cobalamin coenzyme forms are not likely to be superior to cyano- and hydroxyl-cobalamin in prevention or treatment of cobalamin deficiency. Mol Nutr Food Res 59:1364–72. doi: 10.1002/mnfr.201500019

Obeid R, Munz W, Jäger M, et al (2005) Biochemical indexes of the B vitamins in cord serum are predicted by maternal B vitamin status. Am J Clin Nutr 82:133–139. doi: 82/1/133 [pii]

Oblong JE, Gasdaska PY, Sherrill K, Powis G (1993) Purification of human thioredoxin reductase: properties and characterization by absorption and circular dichroism spectroscopy. Biochemistry 32:7271–7.

Odland JØ, Nieboer E, Romanova N, et al (2001) Factor analysis of essential and toxic elements in human placentas from deliveries in arctic and subarctic areas of Russia and Norway. J Environ Monit 3:177–84.

Odland JO, Nieboer E, Romanova N, Thomassen Y (2004) Elements in placenta and pregnancy outcome in arctic and subarctic areas. Int J Circumpolar Health 63:169–187. doi: 10.3402/ijch.v63i2.17703

Ogueh O, Clough A, Hancock M, Johnson MR (2011) A longitudinal study of the control of renal and uterine hemodynamic changes of pregnancy. Hypertens Pregnancy 30:243–259. doi: 10.3109/10641955.2010.484079

Ohuchi S, Matsumoto Y, Morita T, Sugiyama K (2009) High casein diet decreases plasma

homocysteine concentration in rats. J Nutr Sci Vitaminol (Tokyo) 55:22–30. doi: 10.3177/jnsv.55.22

Ojeda ML, Jotty K, Nogales F, et al (2010) Selenium or selenium plus folic acid intake improves the detrimental effects of ethanol on pups' selenium balance. Food Chem Toxicol 48:3486–91. doi: 10.1016/j.fct.2010.09.028

Olson GE, Winfrey VP, Hill KE, Burk RF (2008) Megalin mediates selenoprotein p uptake by kidney proximal tubule epithelial cells. J Biol Chem 283:6854–6860. doi: 10.1074/jbc.M709945200

Olson O (1986) Selenium toxicity in animals with emphasis on man. Int J Toxicol 5:45–70.

Olteanu H, Banerjee R (2001) Human methionine synthase reductase, a soluble P-450 reductase-like dual flavoprotein, is sufficient for NADPH-dependent methionine synthase activation. J Biol Chem 276:35558–63. doi: 10.1074/jbc.M103707200

Olteanu H, Munson T, Banerjee R (2002) Differences in the efficiency of reductive activation of methionine synthase and exogenous electron acceptors between the common polymorphic variants of human methionine synthase reductase. Biochemistry 41:13378–85.

Olteanu H, Wolthers KR, Munro AW, et al (2004) Kinetic and Thermodynamic Characterization of the Common Polymorphic Variants of Human Methionine Synthase Reductase. Biochemistry 43:1988–1997. doi: 10.1021/bi035910i

Olthof MR, van Vliet T, Boelsma E, Verhoef P (2003) Low dose betaine supplementation leads to immediate and long term lowering of plasma homocysteine in healthy men and women. J Nutr 133:4135–4138.

Olusi SO (2002) Obesity is an independent risk factor for plasma lipid peroxidation and depletion of erythrocyte cytoprotectic enzymes in humans. Int J Obes Relat Metab Disord 26:1159–64. doi: 10.1038/sj.ijo.0802066

Ong CN, Chia SE, Foo SC, et al (1993) Concentrations of heavy metals in maternal and umbilical cord blood. Biometals 6:61–6.

Orozco LD, Morselli M, Rubbi L, et al (2015) Epigenome-wide association of liver methylation patterns and complex metabolic traits in mice. Cell Metab 21:905–917. doi: 10.1016/j.cmet.2015.04.025

Osada H, Watanabe Y, Nishimura Y, et al (2002) Profile of trace element concentrations in the feto-placental unit in relation to fetal growth. Acta Obstet Gynecol Scand 81:931–7.

Osaki S, Johnson DA, Frieden E (1966) The possible significance of the ferrous oxidase activity of ceruloplasmin in normal human serum. J Biol Chem 241:2746–2751.

Osman K, Åkesson A, Berglund M, et al (2000) Toxic and essential elements in placentas of Swedish women. Clin Biochem 33:131–138. doi: 10.1016/S0009-9120(00)00052-7

Ou X, Yang H, Ramani K, et al (2007) Inhibition of human betaine-homocysteine methyltransferase expression by S-adenosylmethionine and methylthioadenosine. Biochem J 401:87–96. doi: 10.1042/BJ20061119

Outten CE, O'Halloran T V (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292:2488–2492. doi: 10.1126/science.1060331

Overbye A, Fengsrud M, Seglen PO (2007) Proteomic analysis of membrane-associated proteins from rat liver autophagosomes. Autophagy 3:300–322. doi: 3910 [pii]

Overmoyer BA, McLaren CE, Brittenham GM (1987) Uniformity of liver density and nonheme (storage) iron distribution. Arch Pathol Lab Med 111:549–554.

Oyen J, Svingen GF, Gjesdal CG, et al (2015) Plasma dimethylglycine, nicotine exposure and risk of low bone mineral density and hip fracture: the Hordaland Health Study. Osteoporos Int 26:1573–1583. doi: 10.1007/s00198-015-3030-4

Ozata M, Mergen M, Oktenli C, et al (2002) Increased oxidative stress and hypozincemia in male obesity. Clin Biochem 35:627–631. doi: 10.1016/S0009-9120(02)00363-6

Ozdemir U, Gulturk S, Aker A, et al (2007) Correlation between birth weight, leptin, zinc and copper levels in maternal and cord blood. J Physiol Biochem 63:121–8.

Ozdemir Y, Börekci B, Levet A, Kurudirek M (2009) Assessment of trace element concentration distribution in human placenta by wavelength dispersive X-ray fluorescence: Effect of neonate weight and maternal age. Appl Radiat Isot 67:1790–1795. doi: 10.1016/j.apradiso.2009.06.007

Padmanabhan N, Jia D, Geary-Joo C, et al (2013) Mutation in folate metabolism causes epigenetic instability and transgenerational effects on development. Cell 155:81–93. doi: 10.1016/j.cell.2013.09.002

Pagán K, Hou J, Goldenberg RL, et al (2001) Effect of smoking on serum concentrations of total homocysteine and B vitamins in mid-pregnancy. Clin Chim Acta 306:103–109. doi: 10.1016/S0009-8981(01)00402-8

Pan Y, Zhang W, Ma J, et al (2012) Infants' MTHFR polymorphisms and nonsyndromic orofacial clefts susceptibility: a meta-analysis based on 17 case-control studies. Am J Med Genet A

158A:2162–9. doi: 10.1002/ajmg.a.35503

Paoli P, Sbrana F, Tiribilli B, et al (2010) Protein N-homocysteinylation induces the formation of toxic amyloid-like protofibrils. J Mol Biol 400:889–907. doi: 10.1016/j.jmb.2010.05.039

Paré G, Chasman DI, Parker AN, et al (2009) Novel associations of CPS1, MUT, NOX4, and DPEP1 with plasma Homocysteine in a healthy population a genome-wide evaluation of 13 974 participants in the women's genome health study. Circ Cardiovasc Genet 2:142–150. doi: 10.1161/CIRCGENETICS.108.829804

Park EI, Garrow T (1999) Interaction between dietary methionine and methyl donor intake on rat liver betaine-homocysteine methyltransferase gene expression and organization of the human gene. J Biol Chem 274:7816–7824. doi: 10.1074/jbc.274.12.7816

Park EI, Renduchintala MS, Garrow TA (1997) Diet-induced changes in hepatic betaine-homocysteine methyltransferase activity are mediated by changes in the steady-state level of its mRNA. J Nutr Biochem 8:541–545. doi: 10.1016/S0955-2863(97)00101-0

Park EY, Rho HM (2002) The transcriptional activation of the human copper/zinc superoxide dismutase gene by 2,3,7,8-tetrachlorodibenzo-p-dioxin through two different regulator sites, the antioxidant responsive element and xenobiotic responsive element. Mol Cell Biochem 240:47–55.

Parle-McDermott A, Mills JL, Molloy AM, et al (2006) The MTHFR 1298CC and 677TT genotypes have opposite associations with red cell folate levels. Mol Genet Metab 88:290–294. doi: 10.1016/j.ymgme.2006.02.011

Parle-McDermott A, Pangilinan F, Mills JL, et al (2005) A polymorphism in the MTHFD1 gene increases a mother's risk of having an unexplained second trimester pregnancy loss. Mol Hum Reprod 11:477–480. doi: 10.1093/molehr/gah204

Parthasarathy S, Subbaiah P V, Ganguly J (1974) The mechanism of intestinal absorption of phosphatidylcholine in rats. Biochem J 140:503–8.

Pavía J, Muñoz M, Jiménez E, et al (1997) Pharmacological characterization and distribution of muscarinic receptors in human placental syncytiotrophoblast brush-border and basal plasma membranes. Eur J Pharmacol 320:209–14.

Pavlíková M, Sokolová J, Janosíková B, et al (2012) Rare allelic variants determine folate status in an unsupplemented European population. J Nutr 142:1403–9. doi: 10.3945/jn.112.160549

Pearce LL, Gandley RE, Han W, et al (2000) Role of metallothionein in nitric oxide signaling as

revealed by a green fluorescent fusion protein. Proc Natl Acad Sci U S A 97:477–82. doi: 10.1073/pnas.97.1.477

Pei L, Liu J, Zhang Y, et al (2009) Association of reduced folate carrier gene polymorphism and maternal folic acid use with neural tube defects. Am J Med Genet Part B Neuropsychiatr Genet 150:874–878. doi: 10.1002/ajmg.b.30911

Pei L, Zhu H, Zhu J, et al (2006) Genetic Variation of Infant Reduced Folate Carrier (A80G) and Risk of Orofacial Defects and Congenital Heart Defects in China. Ann Epidemiol 16:352–356. doi: 10.1016/j.annepidem.2005.02.014

Pereg D, Lagueux J, Dewailly É, et al (2001) Cigarette smoking during pregnancy: comparison of biomarkers for inclusion in epidemiological studies. Biomarkers 6:161–73. doi: 10.1080/13547500014750001

Perez-D'Gregorio RE, Miller RK (1998) Transport and endogenous release of vitamin B12 in the dually perfused human placenta. J Pediatr 132:S35-42.

Pérez-Mato I, Castro C, Ruiz FA, et al (1999) Methionine adenosyltransferase S-nitrosylation is regulated by the basic and acidic amino acids surrounding the target thiol. J Biol Chem 274:17075–9.

Perna AF, Satta E, Acanfora F, et al (2006) Increased plasma protein homocysteinylation in hemodialysis patients. Kidney Int 69:869–76. doi: 10.1038/sj.ki.5000070

Perrone RD, Madias NE, Levey AS (1992) Serum creatinine as an index of renal function: new insights into old concepts. Clin Chem 38:1933–53.

Peters-Regehr T, Bode JG, Kubitz R, Häussinger D (1999) Organic osmolyte transport in quiescent and activated rat hepatic stellate cells (Ito cells). Hepatology 29:173–180. doi: 10.1002/hep.510290111

Peters BA, Hall MN, Liu X, et al (2015) Folic acid and creatine as therapeutic approaches to lower blood arsenic: A randomized controlled trial. Environ Health Perspect 123:1294–1301. doi: 10.1289/ehp.1409396

Petrakos G, Panagopoulos P, Koutras I, et al (2006) A comparison of the dietary and total intake of micronutrients in a group of pregnant Greek women with the Dietary Reference Intakes. Eur J Obstet Gynecol Reprod Biol 127:166–171. doi: 10.1016/j.ejogrb.2005.07.034

Pfeiffer C, Fazili Z, Zhang M (2010) Folate in health and disease. In: Bailey LB (ed) 2nd edn. CRC Press, pp 517–574

Pfeiffer CM, Osterloh JD, Kennedy-Stephenson J, et al (2008) Trends in circulating

concentrations of total homocysteine among US adolescents and adults: findings from the 1991-1994 and 1999-2004 National Health and Nutrition Examination Surveys. Clin Chem 54:801–13. doi: 10.1373/clinchem.2007.100214

Phuapradit W, Thuvasethakul P, Sassanarakkit S, et al (2000) Nutrients and Hormones in Heat-Dried Human Placenta. J Med Assoc Thail 83:690–694.

Pickell L, Brown K, Li D, et al (2011) High intake of folic acid disrupts embryonic development in mice. Birth Defects Res Part A - Clin Mol Teratol 91:8–19. doi: 10.1002/bdra.20754

Pickell L, Li D, Brown K, et al (2009) Methylenetetrahydrofolate reductase deficiency and low dietary folate increase embryonic delay and placental abnormalities in mice. Birth Defects Res Part A - Clin Mol Teratol 85:531–541. doi: 10.1002/bdra.20575

Pietruszko R, Chern M-K (2001) Betaine aldehyde dehydrogenase from rat liver mitochondrial matrix. Chem Biol Interact 130–132:193–199. doi: 10.1016/S0009-2797(00)00277-5

Pinter TBJ, Irvine GW, Stillman MJ (2015) Domain Selection in Metallothionein 1A: Affinity-Controlled Mechanisms of Zinc Binding and Cadmium Exchange. Biochemistry 54:5006–5016. doi: 10.1021/acs.biochem.5b00452

Plumptre L, Masih SP, Ly A, et al (2015) High concentrations of folate and unmetabolized folic acid in a cohort of pregnant Canadian women and umbilical cord blood. Am J Clin Nutr 102:848–857. doi: 10.3945/ajcn.115.110783

Poirier LA, Wise CK, Delongchamp RR, Sinha R (2001) Blood determinations of S-adenosylmethionine, S-adenosylhomocysteine, and homocysteine: Correlations with diet. Cancer Epidemiol Biomarkers Prev 10:649–655. doi: S0026-0495(01)68451-8 [pii] 10.1053/meta.2001.25655

Prasodjo A, Pfeiffer CM, Fazili Z, et al (2014) Serum cotinine and whole blood folate concentrations in pregnancy. Ann Epidemiol. doi: 10.1016/j.annepidem.2014.04.004

Preynat A, Lapierre H, Thivierge MC, et al (2010) Effects of supplementary folic acid and vitamin B(12) on hepatic metabolism of dairy cows according to methionine supply. J Dairy Sci 93:2130–42. doi: 10.3168/jds.2009-2796

Prigge ST, Chiang PK (2001) Homocysteine in health and disease. In: Carmel R, Jacobsen DW (eds) 1st edn. Cambridge, pp 79–91

Pullakhandam R, Nair KM, Pamini H, Punjal R (2011) Bioavailability of iron and zinc from multiple micronutrient fortified beverage premixes in Caco-2 cell model. J Food Sci 76:H38-42. doi: 10.1111/j.1750-3841.2010.01993.x

Punshon T, Li Z, Marsit CJ, et al (2016) Placental Metal Concentrations in Relation to Maternal and Infant Toenails in a U.S. Cohort. Environ Sci Technol 50:1587–94. doi: 10.1021/acs.est.5b05316

Qian X, Lu Z, Tan M, et al (2007) A meta-analysis of association between C677T polymorphism in the methylenetetrahydrofolate reductase gene and hypertension. Eur J Hum Genet 15:1239–1245. doi: 10.1038/sj.ejhg.5201914

Qin X, Huo Y, Xie D, et al (2013) Homocysteine-lowering therapy with folic acid is effective in cardiovascular disease prevention in patients with kidney disease: A meta-analysis of randomized controlled trials. Clin Nutr 32:722–727. doi: 10.1016/j.clnu.2012.12.009

Qin X, Li J, Cui Y, et al (2012) Effect of folic acid intervention on the change of serum folate level in hypertensive Chinese adults: do methylenetetrahydrofolate reductase and methionine synthase gene polymorphisms affect therapeutic responses? Pharmacogenet Genomics 22:421–8. doi: 10.1097/FPC.0b013e32834ac5e8

Qiu A, Jansen M, Sakaris A, et al (2006) Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. Cell 127:917–28. doi: 10.1016/j.cell.2006.09.041

Quadros E V, Jacobsen DW (1995) The dynamics of cobalamin utilization in L-1210 mouse leukemia cells: a model of cellular cobalamin metabolism. Biochim Biophys Acta 1244:395–403.

Quinlivan EP, McPartlin J, McNulty H, et al (2002) Importance of both folic acid and vitamin B12 in reduction of risk of vascular disease. Lancet 359:227–228. doi: 10.1016/S0140-6736(02)07439-1

Qvist I, Abdulla M, Jägerstad M, Svensson S (1986) Iron, zinc and folate status during pregnancy and two months after delivery. Acta Obstet Gynecol Scand 65:15–22.

Rah H, Choi YS, Jeon YJ, et al (2012) Solute Carrier Family 19, member 1 (SLC19A1) polymorphisms (-43T>C, 80G>A, and 696C>T), and haplotypes in idiopathic recurrent spontaneous abortion in a Korean population. Reprod Sci 19:513–9. doi: 10.1177/1933719111426604

Rai R, Regan L (2006) Recurrent miscarriage. Lancet 368:601–611.

Raja KB, Simpson RJ, Peters TJ (1989) Membrane potential dependence of Fe(III) uptake by mouse duodenum. Biochim Biophys Acta 984:262–6.

Rakhit R, Chakrabartty A (2006) Structure, folding, and misfolding of Cu,Zn superoxide

dismutase in amyotrophic lateral sclerosis. Biochim Biophys Acta 1762:1025–37. doi: 10.1016/j.bbadis.2006.05.004

Ralston NVC, Ralston CR, Blackwell JL, Raymond LJ (2008) Dietary and tissue selenium in relation to methylmercury toxicity. Neurotoxicology 29:802–811. doi: 10.1016/j.neuro.2008.07.007

Rankama K (1941) On the composition of the residue from silica in rock analysis. Bull la Comm géologique Finlande 14:3–35.

Rao PV, Garrow TA, John F, et al (1998) Betaine-homocysteine methyltransferase is a developmentally regulated enzyme crystallin in rhesus monkey lens. J Biol Chem 273:30669–30674. doi: 10.1074/jbc.273.46.30669

Rasmussen K, Møller J (2000) Total homocysteine measurement in clinical practice. Ann Clin Biochem 37 (Pt 5):627–48.

Rasola A, Galietta LJ, Barone V, et al (1995) Molecular cloning and functional characterization of a GABA/betaine transporter from human kidney. FEBS Lett 373:229–33.

Reddy YS, Y A, Ramalaksmi BA, Kumar BD (2014) Lead and trace element levels in placenta, maternal and cord blood: A cross-sectional pilot study. J Obstet Gynaecol Res 40:2184–2190. doi: 10.1111/jog.12469

Refsum H, Helland S, Ueland PM (1985) Radioenzymic determination of homocysteine in plasma and urine. Clin Chem 31:624–8.

Refsum H, Smith AD, Ueland PM, et al (2004) Facts and Recommendations about Total Homocysteine Determinations : An Expert Opinion. 32:3–32. doi: 10.1373/clinchem.2003.021634

Reichard JF, Schnekenburger M, Puga A (2007) Long term low-dose arsenic exposure induces loss of DNA methylation. Biochem Biophys Res Commun 352:188–92. doi: 10.1016/j.bbrc.2006.11.001

Reid DJ, Barr SI, Leichter J (1992) Effects of folate and zinc supplementation on patients undergoing chronic hemodialysis. J Am Diet Assoc 92:574–579.

Relton CL, Pearce MS, Burn J, Parker L (2005) An investigation of folate-related genetic factors in the determination of birthweight. Paediatr Perinat Epidemiol 19:360–367. doi: 10.1111/j.1365-3016.2005.00662.x

Renuka I V, Sasank R, Devi SI, Vasundhara M (2009) Exencephaly in a live, full term fetus. J Pediatr Neurosci 4:134–6. doi: 10.4103/1817-1745.57332

Reynolds EH (2014) The neurology of folic acid deficiency. Handb Clin Neurol 120:927–43. doi: 10.1016/B978-0-7020-4087-0.00061-9

Reynolds RM, Jacobsen GH, Drake AJ (2013) What is the evidence in humans that DNA methylation changes link events in utero and later life disease? Clin Endocrinol (Oxf) 78:814–22. doi: 10.1111/cen.12164

Ridgway ND, Vance DE (1987) Purification of phosphatidylethanolamine N-methyltransferase from rat liver. J Biol Chem 262:17231–17239. doi: 10.1016/0076-6879(92)09045-5

Robinson JL, McBreairty LE, Randell EW, et al (2016) Restriction of dietary methyl donors limits methionine availability and affects the partitioning of dietary methionine for creatine and phosphatidylcholine synthesis in the neonatal piglet. J Nutr Biochem 35:81–6.

Rodríguez-Guillén MDR, Torres-Sánchez L, Chen J, et al (2009) Maternal MTHFR polymorphisms and risk of spontaneous abortion. Salud Publica Mex 51:19–25. doi: 10.1590/S0036-36342009000100006

Rodríguez IT, Ballart JF, Pastor GC, et al (2008) [Validation of a short questionnaire on frequency of dietary intake: reproducibility and validity]. Nutr Hosp 23:242–52.

Roels H, Hubermont G, Buchet JP, Lauwerys R (1978) Placental transfer of lead, mercury, cadmium, and carbon monoxide in women. Environ Res 16:236–247. doi: 10.1016/0013-9351(78)90159-7

Ronco AM, Arguello G, Muñoz L, et al (2005) Metals content in placentas from moderate cigarette consumers: correlation with newborn birth weight. Biometals 18:233–41.

Ronco AM, Garrido F, Llanos MN (2006) Smoking specifically induces metallothionein-2 isoform in human placenta at term. Toxicology 223:46–53. doi: 10.1016/j.tox.2006.03.002

Rosenberg IH, Zimmerman J, Selhub J (1985) Folate transport. Chemioterapia 4:354–8.

Roskoski R, Lim CT, Roskoski LM (1975) Human brain and placental choline acetyltransferase: purification and properties. Biochemistry 14:5105–10. doi: S0040-1951 (99) 00285-1

Rotter I, Kosik-Bogacka D, Dołęgowska B, et al (2015) Relationship between the Concentrations of Heavy Metals and Bioelements in Aging Men with Metabolic Syndrome. Int J Environ Res Public Health 12:3944–3961. doi: 10.3390/ijerph120403944

Rozen R (2001) Homocysteine in health and disease. In: Carmel R, Jacobsen D (eds) 1st edn. Cambridge, pp 259–269

Ruiz F, Corrales FJ, Miqueo C, Mato JM (1998) Nitric oxide inactivates rat hepatic methionine adenosyltransferase in vivo by S-nitrosylation. Hepatology 28:1051–1057. doi:

10.1002/hep.510280420

Rükgauer M, Neugebauer RJ, Plecko T (2001) The relation between selenium, zinc and copper concentration and the trace element dependent antioxidative status. J Trace Elem Med Biol 15:73–78. doi: 10.1016/S0946-672X(01)80046-8

Rupasree Y, Naushad SM, Rajasekhar L, Kutala VK (2014) Epigenetic modulation of RFC1, MHC2TA and HLA-DR in systemic lupus erythematosus: Association with serological markers and six functional polymorphisms of one-carbon metabolic pathway. Gene 536:45–52. doi: 10.1016/j.gene.2013.11.094

Sakamoto M, Yasutake A, Domingo JL, et al (2013) Relationships between trace element concentrations in chorionic tissue of placenta and umbilical cord tissue: Potential use as indicators for prenatal exposure. Environ Int 60:106–111. doi: 10.1016/j.envint.2013.08.007

Salojin K V., Cabrera RM, Sun W, et al (2011) A mouse model of hereditary folate malabsorption: Deletion of the PCFT gene leads to systemic folate deficiency. Blood 117:4895–4904. doi: 10.1182/blood-2010-04-279653

Santamaría R, Verdú L, Martín C, García G (1998) Neonatal weight according to gestational age Spanish tables [Tablas españolas de pesos neonatales según edad gestacional].

Schramel P, Lill G, Hasse S, Klose BJ (1988) Mineral- and trace element concentrations in human breast milk, placenta, maternal blood, and the blood of the newborn. Biol Trace Elem Res 16:67–75. doi: 10.1007/BF02795335

Schwab U, Törrönen A, Toppinen L, et al (2002) Betaine supplementation decreases plasma homocysteine concentrations but does not affect body weight, body composition, or resting energy expenditure in human subjects. Am J Clin Nutr 76:961–7.

Schwahn BC, Laryea MD, Chen Z, et al (2004) Betaine rescue of an animal model with methylenetetrahydrofolate reductase deficiency. Biochem J 382:831–840. doi: 10.1042/BJ20030822

Scott JM, Dinn JJ, Wilson P, Weir DG (1981) Pathogenesis of subacute combined degeneration: a result of methyl group deficiency. Lancet 2:334–7.

Scott JM, Weir DG (1981) The methyl folate trap. A physiological response in man to prevent methyl group deficiency in kwashiorkor (methionine deficiency) and an explanation for folic-acid induced exacerbation of subacute combined degeneration in pernicious anaemia. Lancet 2:337–40.

Seetharam B, Christensen EI, Moestrup SK, et al (1997) Identification of rat yolk sac target protein of teratogenic antibodies, gp280, as intrinsic factor-cobalamin receptor. J Clin Invest 99:2317–2322. doi: 10.1172/JCI119411

Selhub J, Jacques PF, Bostom AG, et al (2000) Relationship between plasma homocysteine and vitamin status in the Framingham study population. Impact of folic acid fortification. Public Health Rev. 28:117–145.

Selhub J, Jacques PF, Dallal G, et al (2008) The use of blood concentrations of vitamins and their respective functional indicators to define folate and vitamin B12 status. Food Nutr Bull 29:S67-73.

Selhub J, Rosenberg IH (1981) Folate transport in isolated brush border membrane vesicles from rat intestine. J Biol Chem 256:4489–93.

Seligman PA, Allen RH (1978) Characterization of the receptor for transcobalamin II isolated from human placenta. J Biol Chem 253:1766–72.

Selley M (2003) Increased concentrations of homocysteine and asymmetric dimethylarginine and decreased concentrations of nitric oxide in the plasma of patients with Alzheimer's disease. Neurobiol Aging 24:903–907.

Sengupta S, Wehbe C, Majors AK, et al (2001) Relative roles of albumin and ceruloplasmin in the formation of homocystine, homocysteine-cysteine-mixed disulfide, and cystine in circulation. J Biol Chem 276:46896–904. doi: 10.1074/jbc.M108451200

Serafim A, Company R, Lopes B, et al (2012) Assessment of essential and nonessential metals and different metal exposure biomarkers in the human placenta in a population from the south of Portugal. J Toxicol Environ Health A 75:867–77. doi: 10.1080/15287394.2012.690704

Shahzad K, Hai A, Ahmed A, et al (2013) A Structured-based Model for the Decreased Activity of Ala222Val and Glu429Ala Methylenetetrahydrofolate Reductase (MTHFR) Mutants. Bioinformation 9:929–36. doi: 10.6026/97320630009929

Shan X, Wang L, Hoffmaster R, Kruger WD (1999) Functional characterization of human methylenetetrahydrofolate reductase in Saccharomyces cerevisiae. J Biol Chem 274:32613–32618. doi: 10.1074/jbc.274.46.32613

Shane B (2010) Folate in health and disease. In: Bailey L (ed) 2nd edn. CRC Press, pp 1–24

Shapiro (1953) Purification and properties of a lysolecithinase from pancreas. Biochem J 53:663–6.

Shaw GM, Carmichael SL, Yang W, et al (2004) Periconceptional dietary intake of choline and betaine and neural tube defects in offspring. Am J Epidemiol 160:102–9. doi: 10.1093/aje/kwh187

Shaw GM, Finnell RH, Blom HJ, et al (2009a) Choline and risk of neural tube defects in a folate-fortified population. Epidemiology 20:714–719. doi: 10.1097/EDE.0b013e3181ac9fe7

Shaw GM, Lu W, Zhu H, et al (2009b) 118 SNPs of folate-related genes and risks of spina bifida and conotruncal heart defects. BMC Med Genet 10:49. doi: 10.1186/1471-2350-10-49

Shaw GM, Yang W, Carmichael SL, et al (2014) One-carbon metabolite levels in mid-pregnancy and risks of conotruncal heart defects. Birth Defects Res Part A - Clin Mol Teratol 100:107–115. doi: 10.1002/bdra.23224

Shaw GM, Zhu H, Lammer EJ, et al (2003) Genetic variation of infant reduced folate carrier (A80G) and risk of orofacial and conotruncal heart defects. Am J Epidemiol 158:747–52.

Shayeghi M, Latunde-Dada GO, Oakhill JS, et al (2005) Identification of an intestinal heme transporter. Cell 122:789–801. doi: 10.1016/j.cell.2005.06.025

Sheikh-Hamad D, García-Pérez A, Ferraris JD, et al (1994) Induction of gene expression by heat shock versus osmotic stress. Am J Physiol 267:F28-34.

Shen L, Ji H-F (2015) Associations between Homocysteine, Folic Acid, Vitamin B12 and Alzheimer's Disease: Insights from Meta-Analyses. J Alzheimers Dis 46:777–790. doi: 10.3233/JAD-150140

Shen M, Chaudhry SH, MacFarlane AJ, et al (2016) Serum and red-blood-cell folate demonstrate differential associations with BMI in pregnant women. Public Health Nutr 1–8. doi: 10.1017/S1368980016000756

Shennan DB (1988) Selenium (selenate) transport by human placental brush border membrane vesicles. Br J Nutr 59:13. doi: 10.1079/BJN19880005

Shull S, Heintz NH, Periasamy M, et al (1991) Differential regulation of antioxidant enzymes in response to oxidants. J Biol Chem 266:24398–24403.

Sierra EE, Brigle KE, Spinella MJ, Goldman ID (1997) pH dependence of methotrexate transport by the reduced folate carrier and the folate receptor in L1210 leukemia cells. Further evidence for a third route mediated at low pH. Biochem Pharmacol 53:223–231. doi: 10.1016/S0006-2952(96)00730-7

Simmer K, Iles CA, James C, Thompson RPH (1987) Are iron-folate supplements harmful? Am J Clin Nutr 45:122–125.

Singh PR, Lele SS (2012) Folate gene polymorphisms MTR A2756G, MTRR A66G, and BHMT G742A and risk for coronary artery disease: a meta-analysis. Genet Test Mol Biomarkers 16:471–5. doi: 10.1089/gtmb.2011.0237

Singla PN, Chand S, Agarwal KN (1979) Cord serum and placental tissue iron status in maternal hypoferremia. Am J Clin Nutr 32:1462–1465.

Sirotnak FM, Tolner B (1999) Carrier-mediated membrane transport of folates in mammalian cells. Annu Rev Nutr 19:91–122. doi: 10.1146/annurev.nutr.19.1.91

Skiba WE, Taylor MP, Wells MS, et al (1982) Human hepatic methionine biosynthesis. Purification and characterization of betaine:homocysteine S-methyltransferase. J Biol Chem 257:14944–14948.

Smith AD, Kim YI, Refsum H (2008) Is folic acid good for everyone? Am. J. Clin. Nutr. 87:517–533.

Solanky N, Requena Jimenez a., D'Souza SW, et al (2010) Expression of folate transporters in human placenta and implications for homocysteine metabolism. Placenta 31:134–143. doi: 10.1016/j.placenta.2009.11.017

Sole-Navais P (2016) Prenatal one carbon metabolism and in utero programming of growth and adiposity in the offspring. Universitat Rovira i Virgili

Sonet J, Bulteau A, García-Barrera C, et al (2016) Metallomics: Analytical Techniques and Speciation Methods. In: Michalke B (ed) 1st edn. Wiley-VCH Verlag, pp 359–462

Sorkun HC, Bir F, Akbulut M, et al (2007) The effects of air pollution and smoking on placental cadmium, zinc concentration and metallothionein expression. Toxicology 238:15–22. doi: 10.1016/j.tox.2007.05.020

Southon S, Wright AJ, Fairweather-Tait SJ (1989) The effect of combined dietary iron, calcium and folic acid supplementation on apparent 65Zn absorption and zinc status in pregnant rats. Br J Nutr 62:415–23.

Stabler SP (2010) Clinical folate deficiency. In: Bailey LB (ed) Folate in Health and disease, Second. CRC Press Taylor and Francis Group,

Stamp LK, Chapman PT, O'Donnell JL, et al (2010) Polymorphisms within the folate pathway predict folate concentrations but are not associated with disease activity in rheumatoid arthritis patients on methotrexate. Pharmacogenet Genomics 20:367–376. doi: 10.1097/FPC.0b013e3283398a71

Stanislawska-Sachadyn A, Mitchell LE, Woodside J V, et al (2009) The reduced folate carrier

(SLC19A1) c.80G>A polymorphism is associated with red cell folate concentrations among women. Ann Hum Genet 73:484–91. doi: 10.1111/j.1469-1809.2009.00529.x

Stasenko S, Bradford EM, Piasek M, et al (2010) Metals in human placenta: focus on the effects of cadmium on steroid hormones and leptin. J Appl Toxicol 30:242–253. doi: 10.1002/jat.1490

Steegers-Theunissen RPM, Twigt J, Pestinger V, Sinclair KD (2013) The periconceptional period, reproduction and long-term health of offspring: The importance of one-carbon metabolism. Hum Reprod Update 19:640–655. doi: 10.1093/humupd/dmt041

Steenge GR, Verhoef P, Katan MB (2003) Betaine supplementation lowers plasma homocysteine in healthy men and women. J Nutr 133:1291–5.

Stevens BR, Wright EM (1985) Substrate specificity of the intestinal brush-border proline/sodium (IMINO) transporter. J Membr Biol 87:27–34.

Stillman M (1995) Metallothioneins. Coord Chem Rev 144:461–511. doi: 10.1016/0010-8545(95)01173-M

Stover P (2010a) Folate in health and disease. In: Bailey LB (ed) 2nd edn. CRC Press, pp 49–74

Stover PJ (2010b) Folate biochemical pathways and their regulation. In: Bailey LB (ed) Folates in Health and Disease, Second edi. CRC Press, Taylor and Francis Group, pp 49–74

Stoytcheva ZR, Vladimirov V, Douet V, et al (2010) Metal transcription factor-1 regulation via MREs in the transcribed regions of selenoprotein H and other metal-responsive genes. Biochim Biophys Acta - Gen Subj 1800:416–424. doi: 10.1016/j.bbagen.2009.11.003

Stralin P, Marklund SL (1994) Effects of oxidative stress on expression of extracellular superoxide dismutase, CuZn-superoxide dismutase and Mn-superoxide dismutase in human dermal fibroblasts. Biochem J 298:347–352.

Subramanian K (1996) Determination of metals in biofluids and tissues: sample preparation methods for atomic spectroscopic techniques. Spectrochim Acta Part B At Spectrosc 51:291–319.

Suh JR, Herbig AK, Stover PJ (2001) New perspectives on folate catabolism. Annu Rev Nutr 21:255–282. doi: 10.1146/annurev.nutr.21.1.255

Sukla KK, Raman R (2012) Association of MTHFR and RFC1 gene polymorphism with hyperhomocysteinemia and its modulation by vitamin B12 and folic acid in an Indian population. Eur J Clin Nutr 66:111–118. doi: 10.1038/ejcn.2011.152

Summers CM, Mitchell LE, Stanislawska-Sachadyn A, et al (2010) Genetic and lifestyle variables

associated with homocysteine concentrations and the distribution of folate derivatives in healthy premenopausal women. Birth Defects Res A Clin Mol Teratol 88:679–88. doi: 10.1002/bdra.20683

Sun QA, Wu Y, Zappacosta F, et al (1999) Redox regulation of cell signaling by selenocysteine in mammalian thioredoxin reductases. J Biol Chem 274:24522–24530. doi: 10.1074/jbc.274.35.24522

Sunden SL, Renduchintala MS, Park EI, et al (1997) Betaine-homocysteine methyltransferase expression in porcine and human tissues and chromosomal localization of the human gene. Arch Biochem Biophys 345:171–174. doi: 10.1006/abbi.1997.0246

Svardal A, Refsum H, Ueland PM (1986) Determination of in vivo protein binding of homocysteine and its relation to free homocysteine in the liver and other tissues of the rat. J Biol Chem 261:3156–63.

Svingen GFT, Ueland PM, Pedersen EKR, et al (2013) Plasma dimethylglycine and risk of incident acute myocardial infarction in patients with stable angina pectoris. Arterioscler Thromb Vasc Biol 33:2041–8. doi: 10.1161/ATVBAHA.113.301714

Szegedi SS, Castro CC, Koutmos M, Garrow TA (2008) Betaine-homocysteine S-methyltransferase-2 is an S-methylmethionine-homocysteine methyltransferase. J Biol Chem 283:8939–45. doi: 10.1074/jbc.M710449200

Szegedi SS, Garrow TA (2004) Oligomerization is required for betaine-homocysteine S-methyltransferase function. Arch Biochem Biophys 426:32–42. doi: 10.1016/j.abb.2004.03.040

Takanaga H, Mackenzie B, Suzuki Y, Hediger MA (2005) Identification of mammalian proline transporter SIT1 (SLC6A20) with characteristics of classical system imino. J Biol Chem 280:8974–84. doi: 10.1074/jbc.M413027200

Takimoto H, Mito N, Umegaki K, et al (2007) Relationship between dietary folate intakes, maternal plasma total homocysteine and B-vitamins during pregnancy and fetal growth in Japan. Eur J Nutr 46:300–6. doi: 10.1007/s00394-007-0667-6

Tal R, Taylor H, Burney R, et al (2015) Endocrinology of Pregnancy. Endotext, South Dartmouth (MA)

Tamura T, Goldenberg RL, Freeberg LE, et al (1992) Maternal serum folate and zinc concentrations and their relationships to pregnancy outcome. Am J Clin Nutr 56:365–370.

Tamura T, Goldenberg RL, Johnston KE, DuBard M (2000) Maternal plasma zinc concentrations

and pregnancy outcome. Am J Clin Nutr 71:109–13.

Tamura T, Kaiser LL, Watson JE, et al (1987) Increased methionine synthetase activity in zinc-deficient rat liver. Arch Biochem Biophys 256:311–6.

Tamura T, Picciano MF (2006) Folate and human reproduction. Am J Clin Nutr 83:993–1016.

Tamura T, Shane B, Baer MT, et al (1978) Absorption of mono- and polyglutamyl folates in zinc-depleted man. Am J Clin Nutr 31:1984–7.

Tamura T, Weekes EW, Birch R, et al (1994) Relationship between amniotic fluid and maternal blood nutrient levels. J Perinat Med 22:227–34.

Tanaka T, Scheet P, Giusti B, et al (2009) Genome-wide Association Study of Vitamin B6, Vitamin B12, Folate, and Homocysteine Blood Concentrations. Am J Hum Genet 84:477–482. doi: 10.1016/j.ajhg.2009.02.011

Tandon S, Magos L, Webb M (1986) The stimulation and inhibition of the exhalation of volatile selenium. Biochem Pharmacol Pharmacol 35:2763–6.

Tang K-F, Li Y-L, Wang H-Y (2015) Quantitative assessment of maternal biomarkers related to one-carbon metabolism and neural tube defects. Sci Rep 5:8510. doi: 10.1038/srep08510

Taparia S, Gelineau-Van Waes J, Rosenquist TH, Finnell RH (2007) Importance of folate-homocysteine homeostasis during early embryonic development. In: Clinical Chemistry and Laboratory Medicine. pp 1717–1727

Tapia L, González-Agüero M, Cisternas MF, et al (2004) Metallothionein is crucial for safe intracellular copper storage and cell survival at normal and supra-physiological exposure levels. Biochem J 378:617–624. doi: 10.1042/BJ20031174

Tavares E, Carreras O, Gomez-Tubio A, et al (2000) Effects of folic acid and amino acids supplementation on zinc intestinal absorption in the progeny of ethanol-treated rats. J Physiol Biochem 56:247–256.

Terrones-Saldivar M, Serrano-Díaz L, Avelar-Gonzalez F, et al (2008) Estudio comparativo entre las concentraciones de cadmio y de plomo en placentas de embarazos normales y placentas de embarazos con oligoamnios idiopático [in Spanish]. Investig Cienc 42:11–17.

Terruzzi I, Senesi P, Fermo I, et al (2007) Are genetic variants of the methyl group metabolism enzymes risk factors predisposing to obesity? J Endocrinol Invest 30:747–753.

The human protein atlas (2016) Antibody primary data. http://www.proteinatlas.org/.

Thoma NH, Meier TW, Leadlay PF (1998) Vitamin B12 and B12-Proteins. In: Krautler B, Arigoni D, Golding B (eds). Wiley-VCH, pp 227–236

Thomson SW, Heimburger DC, Cornwell PE, et al (2000) Correlates of total plasma homocysteine: folic acid, copper, and cervical dysplasia. Nutrition 16:411–6.

Tolner B, Roy K, Sirotnak FM (1998) Structural analysis of the human RFC-1 gene encoding a folate transporter reveals multiple promoters and alternatively spliced transcripts with 5' end heterogeneity. Gene 211:331–341. doi: 10.1016/S0378-1119(98)00123-1

Tong KK, McArdle HJ (1995) Copper uptake by cultured trophoblast cells isolated from human term placenta. Biochim Biophys Acta 1269:233–236. doi: 10.1016/0167-4889(95)00123-6

Torkanlou K, Bibak B, Abbaspour A, et al (2016) Reduced Serum Levels of Zinc and Superoxide Dismutase in Obese Individuals. Ann Nutr Metab 69:232–236. doi: 10.1159/000454894

Tsang BL, Devine OJ, Cordero AM, et al (2015) Assessing the association between the methylenetetrahydrofolate reductase (MTHFR) 677C>T polymorphism and blood folate concentrations: a systematic review and meta-analysis of trials and observational studies. Am J Clin Nutr 101:1286–1294. doi: 10.3945/ajcn.114.099994

Tsitsiou E, Sibley CP, D'Souza SW, et al (2011) Homocysteine is transported by the microvillous plasma membrane of human placenta. J Inherit Metab Dis 34:57–65. doi: 10.1007/s10545-010-9141-3

Tsitsiou E, Sibley CP, D'Souza SW, et al (2009) Homocysteine transport by systems L, A and y+L across the microvillous plasma membrane of human placenta. J Physiol 587:4001–4013. doi: 10.1113/jphysiol.2009.173393

Tsuchiya H, Mitani K, Kodama K, Nakata T (1984) Placental transfer of heavy metals in normal pregnant Japanese women. Arch Environ Health 39:11–17. doi: 10.1080/00039896.1984.10545827

Tungtrongchitr R, Pongpaew P, Phonrat B, et al (2003) Serum copper, zinc, ceruloplasmin and superoxide dismutase in Thai overweight and obese. J Med Assoc Thail 86:543–551.

Tupe RS, Agte VV (2010) Effect of water soluble vitamins on Zn transport of Caco-2 cells and their implications under oxidative stress conditions. Eur J Nutr 49:53–61. doi: 10.1007/s00394-009-0048-4

Tupe RS, Chiplonkar SA, Agte V V. (2007) Changes in zinc uptake in response to ascorbic acid and folic acid in rat liver slices under normal and oxidative stress conditions. BioFactors 30:27–34. doi: 10.1002/biof.5520300104

Turnbull AJ, Blakeborough P, Thompson RP (1990) The effects of dietary ligands on zinc uptake

at the porcine intestinal brush-border membrane. Br J Nutr 64:733–41. doi: 10.1079/BJN19900075

Ubbink JB, Vermaak WJ, van der Merwe A, Becker PJ (1992) The effect of blood sample aging and food consumption on plasma total homocysteine levels. Clin Chim Acta 207:119–28.

Ubeda N, Reyes L, González-Medina A, et al (2011) Physiologic changes in homocysteine metabolism in pregnancy: a longitudinal study in Spain. Nutrition 27:925–30. doi: 10.1016/j.nut.2010.10.017

Ueland PM (2011) Choline and betaine in health and disease. J Inherit Metab Dis 34:3–15. doi: 10.1007/s10545-010-9088-4

Ueland PM, Holm PI, Hustad S (2005) Betaine: A key modulator of one-carbon metabolism and homocysteine status. Clin Chem Lab Med 43:1069–1075. doi: 10.1515/CCLM.2005.187

Uji Y, Motomiya Y, Hanyu N, et al (2002) Protein-bound homocystamide measured in human plasma by HPLC. Clin Chem 48:941–4.

Umapathy NS, Gnana-Prakasam JP, Martin PM, et al (2007) Cloning and functional characterization of the proton-coupled electrogenic folate transporter and analysis of its expression in retinal cell types. Investig Ophthalmol Vis Sci 48:5299–5305. doi: 10.1167/iovs.07-0288

Undas A, Perła J, Łacinski M, et al (2004) Autoantibodies against N-homocysteinylated proteins in humans: Implications for atherosclerosis. Stroke 35:1299–1304. doi: 10.1161/01.STR.0000128412.59768.6e

Upchurch GR, Welche GN, Fabian AJ, et al (1997) Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. J Biol Chem 272:17012–17017. doi: 10.1074/jbc.272.27.17012

US Food and Drug Administration (1996) Food standards: amendment of standards of identity for enriched grain products to require addition of folic acid. Fed Regist 61 8781–8797.

Uthus EO, Ross S a (2007) Dietary selenium affects homocysteine metabolism differently in Fisher-344 rats and CD-1 mice. J Nutr 137:1132–6.

Uthus EO, Yokoi K, Davis CD (2002) Selenium deficiency in Fisher-344 rats decreases plasma and tissue homocysteine concentrations and alters plasma homocysteine and cysteine redox status. J Nutr 132:1122–8.

Utley CS, Marcell PD, Allen RH, et al (1985) Isolation and characterization of methionine synthetase from human placenta. J Biol Chem 260:13656–13665.

van Beynum IM, Kouwenberg M, Kapusta L, et al (2006) MTRR 66A>G polymorphism in relation to congenital heart defects. Clin Chem Lab Med 44:1317–23. doi: 10.1515/CCLM.2006.254

van den Bosch H, Postema NM, de Haas GH, van Deenen LL (1965) On the positional specificity of phospholipase A from pancreas. Biochim Biophys Acta 98:657–9.

van der Linden IJM, den Heijer M, Afman LA, et al (2006) The methionine synthase reductase 66A>G polymorphism is a maternal risk factor for spina bifida. J Mol Med (Berl) 84:1047–54. doi: 10.1007/s00109-006-0093-x

van der Put NM, Steegers-Theunissen RP, Frosst P, et al (1995) Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. Lancet (London, England) 346:1070–1.

van der Put NMJ, Gabreëls F, Stevens EMB, et al (1998) A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet 62:1044–1051. doi: 10.1086/301825

van Driel LM, Eijkemans MJC, de Jonge R, et al (2009) Body Mass Index Is an Important Determinant of Methylation Biomarkers in Women of Reproductive Ages. J Nutr 139:2315–2321. doi: 10.3945/jn.109.109710.2315

van Hattum B, de Voogt P, Copius Peereboom JW (1981) An analytical procedure for the determination of cadmium in human placentae. Int J Environ Anal Chem 10:121–33. doi: 10.1080/03067318108071537

van Lee L, Tint MT, Aris IM, et al (2016) Prospective associations of maternal betaine status with offspring weight and body composition at birth: the GUSTO (Growing Up in Singapore Toward healthy Outcomes) cohort study. Am J Clin Nutr. doi: 10.3945/ajcn.116.138818

van Meurs JBJ, Pare G, Schwartz SM, et al (2013) Common genetic loci influencing plasma homocysteine concentrations and their effect on risk of coronary artery disease. Am J Clin Nutr 98:668–76. doi: 10.3945/ajcn.112.044545

Vance D, Ridgway N (1988) The methylation of phosphatidylethanolamine. Prog Lipid Res 27:61–79.

Vandevijvere S, Amsalkhir S, Oyen H Van, Moreno-Reyes R (2012) Determinants of folate status in pregnant women: results from a national cross-sectional survey in Belgium. Eur J Clin Nutr 66111:1172–1177. doi: 10.1038/ejcn.2012.111

Vargas Zapata CL, Trugo NM, Donangelo CM (2000) Zinc uptake by human placental microvillous membrane vesicles: effects of gestational age and maternal serum zinc levels.

Biol Trace Elem Res 73:127–37.

Vaughn JD, Bailey LB, Shelnutt KP, et al (2004) Methionine synthase reductase 66A->G polymorphism is associated with increased plasma homocysteine concentration when combined with the homozygous methylenetetrahydrofolate reductase 677C->T variant. J Nutr 134:2985–90.

Vaught JB (2006) Blood collection, shipment, processing, and storage. Cancer Epidemiol Biomarkers Prev 15:1582–4. doi: 10.1158/1055-9965.EPI-06-0630

Vazquez A, Tedeschi PM, Bertino JR (2013) Overexpression of the mitochondrial folate and glycine- Serine pathway: A new determinant of methotrexate selectivity in tumours. Cancer Res. 73:478–482.

Velzing-Aarts F V., Holm PI, Fokkema MR, et al (2005) Plasma choline and betaine and their relation to plasma homocysteine in normal pregnancy. Am J Clin Nutr 81:1383–1389. doi: 10.1016/S1090-798X(08)70315-1

Venn BJ, Grant AM, Thomson CD, Green TJ (2003) Selenium supplements do not increase plasma total homocysteine concentrations in men and women. J Nutr 133:418–420.

Verkleij-Hagoort A, Bliek J, Sayed-Tabatabaei F, et al (2007) Hyperhomocysteinemia and MTHFR polymorphisms in association with orofacial clefts and congenital heart defects: a meta-analysis. Am J Med Genet A 143A:952–60. doi: 10.1002/ajmg.a.31684

Verkleij-Hagoort AC, van Driel LMJW, Lindemans J, et al (2008) Genetic and lifestyle factors related to the periconception vitamin B12 status and congenital heart defects: A Dutch case-control study. Mol Genet Metab 94:112–119. doi: 10.1016/j.ymgme.2007.12.002

Verrey F (2003) System L: heteromeric exchangers of large, neutral amino acids involved in directional transport. Pflugers Arch 445:529–533. doi: 10.1007/s00424-002-0973-z

Vesela K, Pavlikova M, Janosikova B, et al (2005) Genetic determinants of folate status in central Bohemia. Physiol Res 54:295–303.

Vester B, Rasmussen K (1991) High performance liquid chromatography method for rapid and accurate determination of homocysteine in plasma and serum. Eur J Clin Chem Clin Biochem 29:549–54.

Villanueva JA, Halsted CH (2004) Hepatic Transmethylation Reactions in Micropigs with Alcoholic Liver Disease. Hepatology 39:1303–1310. doi: 10.1002/hep.20168

Vinukonda G, Shaik Mohammad N, Md Nurul Jain J, et al (2009) Genetic and environmental influences on total plasma homocysteine and coronary artery disease (CAD) risk among

South Indians. Clin Chim Acta 405:127–131. doi: 10.1016/j.cca.2009.04.015

Vir SC, Love AH, Thompson W (1981) Zinc concentration in hair and serum of pregnant women in Belfast. Am J Clin Nutr 34:2800–7.

Visentin CE, Masih S, Plumptre L, et al (2015) Maternal Choline Status, but Not Fetal Genotype, Influences Cord Plasma Choline Metabolite Concentrations. J Nutr 145:1491–7. doi: 10.3945/jn.115.211136

Visser S, Hermes W, Ket JCF, et al (2014) Systematic review and metaanalysis on nonclassic cardiovascular biomarkers after hypertensive pregnancy disorders. Am J Obstet Gynecol 211:373.e1-373.e9. doi: 10.1016/j.ajog.2014.03.032

Vollset SE, Refsum H, Nygard O, Ueland PM (2001) Homocysteine in health and disease. In: Carmel R, Jacobsen DW (eds) 1st edn. Cambridge, pp 341–355

Waalkes MP, Poisner a M, Wood GW, Klaassen CD (1984) Metallothionein-like proteins in human placenta and fetal membranes. Toxicol Appl Pharmacol 74:179–184.

Wadwa J, Chu Y-H, Nguyen N, et al (2014) Effects of ATP7A overexpression in mice on copper transport and metabolism in lactation and gestation. Physiol Rep 2:n/a-n/a. doi: 10.1002/phy2.195

Wald DS, Law M, Morris JK (2002) Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. Bmj 325:1202. doi: 10.1136/bmj.325.7374.1202

Wallace JMW, Bonham MP, Strain JJ, et al (2008) Homocysteine concentration, related B vitamins, and betaine in pregnant women recruited to the Seychelles Child Development Study. Am J Clin Nutr 87:391–397.

Wallis D, Ballard J, Shaw GM, et al (2010) Folate in health and disease. In: Bailey LB (ed) 2nd edn. CRC Press, pp 155–178

Wang B, Liu M, Yan W, et al (2013) Association of SNPs in genes involved in folate metabolism with the risk of congenital heart disease. J Matern Fetal Neonatal Med. doi: 10.3109/14767058.2013.799648

Wang H, Chen B, He M, et al (2017) Selenocystine against methyl mercury cytotoxicity in HepG2 cells. Sci Rep 7:147. doi: 10.1038/s41598-017-00231-7

Wang H, Hu Y-F, Hao J-H, et al (2015a) Maternal zinc deficiency during pregnancy elevates the risks of fetal growth restriction: a population-based birth cohort study. Sci Rep 5:11262. doi: 10.1038/srep11262

Wang H, Yoshizumi M, Lai K, et al (1997) Inhibition of growth and p21(ras) methylation in

vascular endothelial cells by homocysteine but not cysteine. J Biol Chem 272:25380–25385. doi: 10.1074/jbc.272.40.25380

Wang HG, Wang JL, Zhang J, et al (2012) Reduced folate carrier A80G polymorphism and susceptibility to neural tube defects: A meta-analysis. Gene 510:180–184. doi: 10.1016/j.gene.2012.02.020

Wang Y, Liu Y, Ji W, et al (2015b) Analysis of MTR and MTRR Polymorphisms for Neural Tube Defects Risk Association. Medicine (Baltimore) 94:e1367. doi: 10.1097/MD.0000000000001367

Wang Y, Walsh SW (1996) Antioxidant activities and mRNA expression of superoxide dismutase, catalase, and glutathione peroxidase in normal and preeclamptic placentas. J Soc Gynecol Investig 3:179–184. doi: 10.1016/1071-5576(96)00015-9

Wang Y, Zhao R, Russell RG, Goldman ID (2001) Localization of the murine reduced folate carrier as assessed by immunohistochemical analysis. Biochim Biophys Acta - Biomembr 1513:49–54. doi: 10.1016/S0005-2736(01)00340-6

Wang Y, Zhao S (2010) Vascular Biology of the Placenta, 1st edn. Morgan & Claypool Life Sciences, San Rafael (CA)

Ward A, Marciello L, Carrara L, Luciano V (1980) Simultaneous Determination of Major, Minor, and Trace Elements in Agricultural and Biological Samples by Inductively Coupled Argon Plasma Spectrometry. Spectrosc Lett 13:803–831.

Ward N, Watson R, Bryce-Smith D (1987) Evidence for effects of cadmium, lead and zinc on fetal growth, and for smoking as a source of cadmium. Int J Biosoc Med Res 9:63–81.

Wasowicz W, Wolkanin P, Bednarski M, et al (1993) Plasma trace element (Se, Zn, Cu) concentrations in maternal and umbilical cord blood in Poland - Relation with birth weight, gestational age, and parity. Biol Trace Elem Res 38:205–215. doi: 10.1007/BF02784053

Weekes EW, Tamura T, Davis RO, et al (1992) Nutrient levels in amniotic fluid from women with normal and neural tube defect pregnancies. Biol Neonate 61:226–231. doi: 10.1159/000243747

Wehner F, Olsen H, Tinel H, et al (2003) Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. Rev Physiol Biochem Pharmacol 148:1–80. doi: 10.1007/s10254-003-0009-x

Weisberg IS, Park E, Ballman K V., et al (2003) Investigations of a common genetic variant in

betaine-homocysteine methyltransferase (BHMT) in coronary artery disease. Atherosclerosis 167:205–214. doi: 10.1016/S0021-9150(03)00010-8

Weissbach H, Taylor RT (1970) Roles of vitamin B 12 and folic acid in methionine synthesis. Vitam Horm 28:415–40.

Welsch F (1976) Studies on accumulation and metabolic fate of [N-Me3H]choline in human term placenta fragments. Biochem Pharmacol 25:1021–1030. doi: 10.1016/0006-2952(76)90490-1

Wenstrom KD, Johanning GL, Johnston KE, DuBard M (2001) Association of the C677T methylenetetrahydrofolate reductase mutation and elevated homocysteine levels with congenital cardiac malformations. In: American Journal of Obstetrics and Gynecology. pp 806–817

West AR, Oates PS (2008) Mechanisms of heme iron absorption: Current questions and controversies. World J. Gastroenterol. 14:4101–4110.

Whetstine JR, Flatley RM, Matherly LH (2002) The human reduced folate carrier gene is ubiquitously and differentially expressed in normal human tissues: identification of seven non-coding exons and characterization of a novel promoter. Biochem J 367:629–40. doi: 10.1042/BJ20020512

Whetstine JR, Gifford AJ, Witt T, et al (2001) Single nucleotide polymorphisms in the human reduced folate carrier: characterization of a high-frequency G/A variant at position 80 and transport properties of the His(27) and Arg(27) carriers. Clin Cancer Res 7:3416–22.

Wilcken DE, Dudman NP, Tyrrell PA (1985) Homocystinuria due to cystathionine beta-synthase deficiency--the effects of betaine treatment in pyridoxine-responsive patients. Metabolism 34:1115–21.

Wilcken DE, Wang XL, Adachi T, et al (2000) Relationship between homocysteine and superoxide dismutase in homocystinuria: possible relevance to cardiovascular risk. Arter Thromb Vasc Biol 20:1199–1202. doi: 10.1161/01.ATV.20.5.1199

Wilcken DE, Wilcken B, Dudman NP, Tyrrell PA (1983) Homocystinuria--the effects of betaine in the treatment of patients not responsive to pyridoxine. N Engl J Med 309:448–53. doi: 10.1056/NEJM198308253090802

Willems HP, Bos GM, Gerrits WB, et al (1998) Acidic citrate stabilizes blood samples for assay of total homocysteine. Clin Chem 44:342–5.

Williams FM, Flintoff WF (1998) Structural organization of the human reduced folate carrier

gene: evidence for 5' heterogeneity in lymphoblast mRNA. Somat Cell Mol Genet 24:143–156.

Wilson A, Platt R, Wu Q, et al (1999) A common variant in methionine synthase reductase combined with low cobalamin (vitamin B12) increases risk for spina bifida. Mol Genet Metab 67:317–23. doi: 10.1006/mgme.1999.2879

Wilson DC, Tubman R, Bell N, et al (1991) Plasma manganese, selenium and glutathione peroxidase levels in the mother and newborn infant. Early Hum Dev 26:223–226. doi: 10.1016/0378-3782(91)90162-V

Wilson R, Grieger J, Bianco-Miotto T, Roberts C (2016) Association between Maternal Zinc Status, Dietary Zinc Intake and Pregnancy Complications: A Systematic Review. Nutrients 8:641. doi: 10.3390/nu8100641

Windelberg A, Arseth O, Kvalheim G, Ueland PM (2005) Automated assay for the determination of methylmalonic acid, total homocysteine, and related amino acids in human serum or plasma by means of methylchloroformate derivatization and gas chromatography-mass spectrometry. Clin Chem 51:2103–9. doi: 10.1373/clinchem.2005.053835

Winkelmayer WC, Eberle C, Sunder-Plassmann G, Fodinger M (2003) Effects of the glutamate carboxypeptidase II (GCP2 1561C>T) and reduced folate carrier (RFC1 80G<A) allelic variants on folate and total homocysteine levels in kidney transplant patients. Kidney Int 63:2280–2285. doi: 10.1046/j.1523-1755.2003.00025.x

Wistow G (2012) The human crystallin gene families. Hum Genomics 6:26. doi: 10.1186/1479-7364-6-26

Wittwer AJ, Wagner C (1981) Identification of the folate-binding proteins of rat liver mitochondria as dimethylglycine dehydrogenase and sarcosine dehydrogenase. Flavoprotein nature and enzymatic properties of the purified proteins. J Biol Chem 256:4109–15.

Wollesen F, Brattstrom L, Refsum H, et al (1999) Plasma total homocysteine and cysteine in relation to glomerular filtration rate in diabetes mellitus. Kidney Int 55:1028–1035. doi: 10.1046/j.1523-1755.1999.0550031028.x

Wolthers KR, Scrutton NS (2007) Protein interactions in the human methionine synthase-methionine synthase reductase complex and implications for the mechanism of enzyme reactivation. Biochemistry 46:6696–709. doi: 10.1021/bi700339v

Wolvekamp MCJ, De Bruin RWF (1994) Diamine oxidase: An overview of historical, biochemical

and functional aspects. Dig Dis 12:2–14. doi: 10.1159/000171432

World Health Organization (2011) Global atlas on cardiovascular disease prevention and control. Geneve

World Health Organization (2012) Serum and red blood cell folate concentrations for assessing folate status in populations. Geneve

World Health Organization (2015) Guideline: optimal serum and red blood cell folate concentrations in women of reproductive age for prevention of neural tube defects. Geneve

World Health Organization (2016) International statistical classification of diseases and related health problems 10th revision (ICD-10). http://apps.who.int/classifications/icd10/browse/2016/en.

World Medical Association (2013) Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects. http://www.wma.net/en/30publications/10policies/b3/index.html.

Wright TL, Fitz JG, Weisiger RA (1988) Non-transferrin-bound iron uptake by rat liver. Role of membrane potential difference. J Biol Chem 263:1842–7.

Wu BTF, Innis SM, Mulder K a., et al (2013) Low plasma vitamin B-12 is associated with a lower pregnancy-associated rise in plasma free choline in Canadian pregnant women and lower postnatal growth rates in their male infants. Am J Clin Nutr 98:1209–1217. doi: 10.3945/ajcn.113.060269

Wu LL, Wu J, Hunt SC, et al (1994) Plasma homocyst(e)ine as a risk factor for early familial coronary artery disease. Clin Chem 40:552–61.

Xu C, Wu Y, Liu G, et al (2014) Relationship between homocysteine level and diabetic retinopathy: a systematic review and meta-analysis. Diagn Pathol 9:167. doi: 10.1186/s13000-014-0167-y

Xuan C, Li H, Zhao J-X, et al (2014) Association Between MTHFR Polymorphisms and Congenital Heart Disease: A Meta-analysis based on 9,329 cases and 15,076 controls. Sci Rep 4:7311. doi: 10.1038/srep07311

Yadav U, Kumar P, Yadav SK, et al (2015) “Polymorphisms in folate metabolism genes as maternal risk factor for neural tube defects: an updated meta-analysis”. Metab Brain Dis 30:7–24. doi: 10.1007/s11011-014-9575-7

Yamada K, Chen Z, Rozen R, Matthews RG (2001) Effects of common polymorphisms on the

properties of recombinant human methylenetetrahydrofolate reductase. Proc Natl Acad Sci U S A 98:14853–8. doi: 10.1073/pnas.261469998

Yamada K, Gravel RA, Toraya T, Matthews RG (2006) Human methionine synthase reductase is a molecular chaperone for human methionine synthase. Proc Natl Acad Sci U S A 103:9476–81. doi: 10.1073/pnas.0603694103

Yamada K, Strahler JR, Andrews PC, Matthews RG (2005) Regulation of human methylenetetrahydrofolate reductase by phosphorylation. Proc Natl Acad Sci U S A 102:10454–10459. doi: 10.1073/pnas.0504786102

Yamashita M, Yamashita Y, Ando T, et al (2013a) Identification and Determination of Selenoneine, 2-Selenyl-N α , N α , N α -Trimethyl-l-Histidine, as the Major Organic Selenium in Blood Cells in a Fish-Eating Population on Remote Japanese Islands. Biol Trace Elem Res 156:36–44. doi: 10.1007/s12011-013-9846-x

Yamashita M, Yamashita Y, Suzuki T, et al (2013b) Selenoneine, a Novel Selenium-Containing Compound, Mediates Detoxification Mechanisms against Methylmercury Accumulation and Toxicity in Zebrafish Embryo. Mar Biotechnol 15:559–570. doi: 10.1007/s10126-013-9508-1

Yan J, Jiang X, West AA, et al (2013) Pregnancy alters choline dynamics: results of a randomized trial using stable isotope methodology in pregnant and nonpregnant women. Am J Clin Nutr 98:1459–67. doi: 10.3945/ajcn.113.066092

Yancey PH, Clark ME, Hand SC, et al (1982) Living with water stress: evolution of osmolyte systems. Science 217:1214–22.

Yang F, Tan H-M, Wang H (2005) Hyperhomocysteinemia and atherosclerosis. Sheng Li Xue Bao 57:103–14.

Yang H-T, Lee M, Hong K-S, et al (2012a) Efficacy of folic acid supplementation in cardiovascular disease prevention: an updated meta-analysis of randomized controlled trials. Eur J Intern Med 23:745–54. doi: 10.1016/j.ejim.2012.07.004

Yang J, Chen H, Vlahov IR, et al (2007) Characterization of the pH of folate receptor-containing endosomes and the rate of hydrolysis of internalized acid-labile folate-drug conjugates. J Pharmacol Exp Ther 321:462–8. doi: 10.1124/jpet.106.117648

Yang J, Hu X, Zhang Q, et al (2012b) Homocysteine level and risk of fracture: A meta-analysis and systematic review. Bone 51:376–382. doi: 10.1016/j.bone.2012.05.024

Yang M, Gong T, Lin X, et al (2013) Maternal gene polymorphisms involved in folate metabolism

and the risk of having a Down syndrome offspring: a meta-analysis. Mutagenesis 28:661–71. doi: 10.1093/mutage/get045

Yang Q-H, Botto LD, Gallagher M, et al (2008) Prevalence and effects of gene-gene and gene-nutrient interactions on serum folate and serum total homocysteine concentrations in the United States: findings from the third National Health and Nutrition Examination Survey DNA Bank. Am J Clin Nutr 88:232–46.

Yang Y, Chen J, Wang B, et al (2015) Association between MTHFR C677T polymorphism and neural tube defect risks: A comprehensive evaluation in three groups of NTD patients, mothers, and fathers. Birth Defects Res A Clin Mol Teratol 103:488–500. doi: 10.1002/bdra.23361

Yara M, Iwao B, Hara N, et al (2015) Molecular and functional characterization of choline transporter in the human trophoblastic cell line JEG-3 cells. Placenta 36:631–7. doi: 10.1016/j.placenta.2015.03.010

Yasuda S, Hasui S, Yamamoto C, et al (2008) Placental Folate Transport during Pregnancy. Biosci Biotechnol Biochem 72:2277–2284. doi: 10.1271/bbb.80112

Yates Z, Lucock M (2003) Interaction between common folate polymorphisms and B-vitamin nutritional status modulates homocysteine and risk for a thrombotic event. Mol Genet Metab 79:201–213. doi: 10.1016/S1096-7192(03)00093-3

Yates Z, Lucock M (2005) G80A reduced folate carrier SNP modulates cellular uptake of folate and affords protection against thrombosis via a non homocysteine related mechanism. Life Sci 77:2735–2742. doi: 10.1016/j.lfs.2005.02.029

Yi P, Melnyk S, Pogribna M, et al (2000) Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation. J Biol Chem 275:29318–23. doi: 10.1074/jbc.M002725200

Yin D, Galivan J, Ao W, Yao R (2003) Characterization of the human gamma-glutamyl hydrolase promoter and its gene expression in human tissues and cancer cell lines. Gene 312:281–8.

Yoshida T, Kikuchi G (1970) Major pathways of glycine and serine catabolism in rat liver. Arch Biochem Biophys 139:380–92.

Youngdahl-Turner P, Mellman IS, Allen RH, Rosenberg LE (1979) Protein mediated vitamin uptake. Adsorptive endocytosis of the transcobalamin II - Cobalamin complex by cultured human fibroblasts. Exp Cell Res 118:127–134. doi: 10.1016/0014-4827(79)90590-1

Younoszai MK, Haworth JC (1969) Placental dimensions and relations in preterm, term, and

growth-retarded infants. Am J Obstet Gynecol 103:265–71.

Yu D, Yang L, Shen S, et al (2014) Association between methionine synthase reductase A66G polymorphism and the risk of congenital heart defects: Evidence from eight case-control studies. Pediatr. Cardiol. 35:1091–1098.

Zadorozhnaja TD, Little RE, Miller RK, et al (2000) Concentrations of arsenic, cadmium, copper, lead, mercury, and zinc in human placentas from two cities in Ukraine. J Toxicol Env Heal A 61:255–263.

Zadrozna M, Gawlik M, Nowak B, et al (2009) Antioxidants activities and concentration of selenium, zinc and copper in preterm and IUGR human placentas. J Trace Elem Med Biol 23:144–8. doi: 10.1016/j.jtemb.2009.02.005

Zaitseva I, Zaitsev V, Card G, et al (1996) The X-ray structure of human serum ceruloplasmin at 3.1 Å: nature of the copper centres. J Biol Inorg Chem 1:15–23.

Zampieri BL, Biselli JM, Goloni-Bertollo EM, et al (2012) Maternal risk for Down syndrome is modulated by genes involved in folate metabolism. Dis Markers 32:73–81. doi: 10.3233/DMA-2011-0869

Zeisel SH (2006) Choline: critical role during fetal development and dietary requirements in adults. Annu Rev Nutr 26:229–250. doi: 10.1146/annurev.nutr.26.061505.111156

Zeisel SH, Da Costa KA, Franklin PD, et al (1991) Choline, an essential nutrient for humans. FASEB J 5:2093–8.

Zeisel SH, Ellis AL, Sun XF, et al (1987) Dose-response relations in urinary excretion of trimethylselenonium in the rat. J Nutr 117:1609–1614.

Zeisel SH, Zola T, daCosta KA, Pomfret EA (1989) Effect of choline deficiency on S-adenosylmethionine and methionine concentrations in rat liver. Biochem J 259:725–9.

Zeng R, Xu C-H, Xu Y-N, et al (2015) The effect of folate fortification on folic acid-based homocysteine-lowering intervention and stroke risk: a meta-analysis. Public Health Nutr 18:1514–21. doi: 10.1017/S1368980014002134

Zetterberg H, Regland B, Palmér M, et al (2002) Increased frequency of combined methylenetetrahydrofolate reductase C677T and A1298C mutated alleles in spontaneously aborted embryos. Eur J Hum Genet 10:113–118. doi: 10.1038/sj.ejhg.5200862

Zhang B, Georgiev O, Hagmann M, et al (2003) Activity of metal-responsive transcription factor 1 by toxic heavy metals and H2O2 in vitro is modulated by metallothionein. Mol Cell Biol 23:8471–85. doi: 10.1128/mcb.23.23.8471-8485.2003

Zhang H, Tao X, Wu J (2014) Association of homocysteine, vitamin B12, and folate with bone mineral density in postmenopausal women: A meta-analysis. Arch Gynecol Obstet 289:1003–1009. doi: 10.1007/s00404-013-3075-6

Zhang L, Wong SC, Matherly LH (1998) Structure and organization of the human reduced folate carrier gene. Biochim Biophys Acta - Gene Struct Expr 1442:389–393. doi: 10.1016/S0167-4781(98)00168-7

Zhang T, Lou J, Zhong R, et al (2013) Genetic Variants in the Folate Pathway and the Risk of Neural Tube Defects: A Meta-Analysis of the Published Literature. PLoS One. doi: 10.1371/journal.pone.0059570

Zhao CQ, Young MR, Diwan BA, et al (1997) Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. Proc Natl Acad Sci U S A 94:10907–12.

Zhao M, Ren Y, Shen L, et al (2014) Association between MTHFR C677T and A1298C polymorphisms and NSCL/P risk in Asians: a meta-analysis. PLoS One 9:e88242. doi: 10.1371/journal.pone.0088242

Zhao R, Goldman ID (2013) Folate and thiamine transporters mediated by facilitative carriers (SLC19A1-3 and SLC46A1) and folate receptors. Mol. Aspects Med. 34:373–385.

Zhao R, Min SH, Wang Y, et al (2009) A role for the proton-coupled folate transporter (PCFT-SLC46A1) in folate receptor-mediated endocytosis. J Biol Chem 284:4267–4274. doi: 10.1074/jbc.M807665200

Zhao R, Russell RG, Wang Y, et al (2001) Rescue of embryonic lethality in reduced folate carrier-deficient mice by maternal folic acid supplementation reveals early neonatal failure of hematopoietic organs. J Biol Chem 276:10224–8.

Zhon IE, Sarkar AA (2008) Mouse genetic model of developmental genetic disease. In: Krauss RS (ed) 1st edn. Academic Press, pp 2–35

Zhou K, Zhao R, Geng Z, et al (2012) Association between B-group vitamins and venous thrombosis: systematic review and meta-analysis of epidemiological studies. J Thromb Thrombolysis 34:459–67. doi: 10.1007/s11239-012-0759-x

Zhou Y-H, Tang J-Y, Wu M-J, et al (2011) Effect of folic acid supplementation on cardiovascular outcomes: a systematic review and meta-analysis. PLoS One 6:e25142. doi: 10.1371/journal.pone.0025142

Zhu L (2015) Polymorphisms in the methylene tetrahydrofolate reductase and methionine

synthase reductase genes and their correlation with unexplained recurrent spontaneous abortion susceptibility. Genet Mol Res 14:8500–8. doi: 10.4238/2015.July.28.19

Zhu Y, He ZY, Liu HN (2011) Meta-analysis of the relationship between homocysteine, vitamin B 12, folate, and multiple sclerosis. J Clin Neurosci 18:933–938. doi: 10.1016/j.jocn.2010.12.022

Zinellu A, Sotgia S, Scanu B, et al (2015) N- and S-homocysteinylation reduce the binding of human serum albumin to catechins. Eur J Nutr. doi: 10.1007/s00394-015-1125-5

Zuchner T, Schliebe N, Schliebs R (2006) Zinc uptake is mediated by M1 muscarinic acetylcholine receptors in differentiated SK-SH-SY5Y cells. Int J Dev Neurosci 24:23–27. doi: 10.1016/j.ijdevneu.2005.11.018

Zwolak I, Zaporowska H (2012) Selenium interactions and toxicity: a review. Cell Biol Toxicol 28:31–46. doi: 10.1007/s10565-011-9203-9

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

APPENDICES

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

nutrients

MDPI

Article

Maternal Folate Status and the BHMT c.716G>A Polymorphism Affect the Betaine Dimethylglycine Pathway during Pregnancy

Jose M. Colomina [1,2], Pere Cavallé-Busquets [2,3], Sílvia Fernàndez-Roig [1,2], Pol Solé-Navais [1,2], Joan D. Fernandez-Ballart [1,2], Mónica Ballesteros [4], Per M. Ueland [5], Klaus Meyer [6] and Michelle M. Murphy [1,2,*]

1 Area of Preventive Medicine and Public Health, Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili, IISPV, C/Sant Llorenç 21, Reus 43201, Spain; josemaria.colomina@urv.cat (J.M.C.); silviafroig@gmail.com (S.F.-R.); pol.sole@urv.cat (P.S.-N.); joan.fernandez-ballart@urv.cat (J.D.F.-B.)
2 Ciberobn Fisiopatología de la Obesidad y Nutrición (CB06/03), Instituto Carlos III, Madrid 28029, Spain; pecavalle@grupsagessa.com
3 Area of Obstetrics and Gynaecology, Hospital Universitari Sant Joan, Reus and Universitat Rovira i Virgili, Reus 43204, Spain
4 Area of Obstetrics and Gynaecology, Hospital Universitari Joan XXIII, Tarragona and Universitat Rovira i Virgili, Tarragona 43005, Spain; mobape71@yahoo.es
5 Section for Pharmacology, Department of Internal Medicine, University of Bergen, Bergen N-5020, Norway; per.ueland@ikb.uib.no
6 Bevital A/S, Laboratory building, 9th floor, Bergen N-5021, Norway; klaus.meyer@bevital.no
* Correspondence: michelle.murphy@urv.cat; Tel.: +34-977-758924

Received: 11 August 2016; Accepted: 27 September 2016; Published: 9 October 2016

Abstract: The effect of the betaine: homocysteine methyltransferase BHMT c.716G>A (G: guanosine; A: adenosine) single nucleotide polymorphism (SNP) on the BHMT pathway is unknown during pregnancy. We hypothesised that it impairs betaine to dimethylglycine conversion and that folate status modifies its effect. We studied 612 women from the Reus Tarragona Birth Cohort from ≤12 gestational weeks (GW) throughout pregnancy. The frequency of the variant BHMT c.716A allele was 30.8% (95% confidence interval (CI): 28.3, 33.5). In participants with normal-high plasma folate status (>13.4 nmol/L), least square geometric mean [95% CI] plasma dimethylglycine (pDMG, µmol/L) was lower in the GA (2.35 [2.23, 2.47]) versus GG (2.58 [2.46, 2.70]) genotype at ≤12 GW ($p < 0.05$) and in the GA (2.08 [1.97, 2.19]) and AA (1.94 [1.75, 2.16]) versus GG (2.29 [2.18, 2.40]) genotypes at 15 GW ($p < 0.05$). No differences in pDMG between genotypes were observed in participants with possible folate deficiency (≤13.4 nmol/L) (p for interactions at ≤12 GW: 0.023 and 15 GW: 0.038). PDMG was lower in participants with the AA versus GG genotype at 34 GW (2.01 [1.79, 2.25] versus 2.44 [2.16, 2.76] and at labour, 2.51 [2.39, 2.64] versus 3.00 [2.84, 3.18], ($p < 0.01$)). Possible deficiency compared to normal-high folate status was associated with higher pDMG in multiple linear regression analysis (β coefficients [SEM] ranging from 0.07 [0.04], $p < 0.05$ to 0.20 [0.04], $p < 0.001$ in models from early and mid-late pregnancy) and the AA compared to GG genotype was associated with lower pDMG (β coefficients [SEM] ranging from −0.11 [0.06], $p = 0.055$ to −0.23 [0.06], $p < 0.001$). Conclusion: During pregnancy, the BHMT pathway is affected by folate status and by the variant BHMT c.716A allele.

Keywords: pregnancy; folate; betaine: homocysteine methyltransferase; BHMT c.716G>A; betaine; dimethylglycine

1. Introduction

The importance of one carbon metabolism and homocysteine regulation in foetal development and optimal pregnancy outcome is well established. Homocysteine remethylation contributes not only to homocysteine homeostasis but also to the provision of methyl groups essential for foetal development. Homocysteine is remethylated to methionine by the ubiquitous folate and cobalamin-dependent methionine synthase (MTR; EC 2.1.1.13) and by betaine:homocysteine methyltransferase (BHMT; EC 2.1.1.5) that occurs mainly in the kidney and liver [1]. The estimated consumption of homocysteine by MTR and BHMT is similar in rat liver [2] but globally the MTR reaction is thought to prevail in mammals based on the fact that BHMT activity has only been detected in some organs such as the liver and kidney [3,4] with conflicting reports regarding its presence in the brain [1,5,6]. To the best of our knowledge this information is not available in human studies to date but a study of healthy Dutch adults reported that the inverse association between folate and fasting plasma total homocysteine (tHcy) was stronger than that between betaine and tHcy, suggesting that the MTR reaction prevails under normal circumstances [7]. Some studies also provide indirect evidence for interaction between both remethylation pathways and upregulation of the BHMT pathway when folate status is low [8–12]. Serum folate was positively associated with betaine and inversely associated with dimethylglycine in a study of healthy Dutch adults [8] and serum dimethylglycine was higher in folate-deficient compared to normal status adults in a USA study [9]. Stronger negative associations between betaine and post-methionine load tHcy were observed when serum folate status was low [10] or in the absence of B vitamin supplement use [11]. We reported an apparent shift in the roles of folate and betaine in homocysteine homeostasis as folate status declines with advancing pregnancy and that low plasma folate status was associated with low plasma betaine and high dimethylglycine and a high dimethylglycine/betaine ratio [12].

BHMT c.716G>A (also known as 742G>A, rs3733890) is a common single nucleotide polymorphism (SNP) in which arginine is substituted by glutamine at position 239 [13]. Numerous human studies reported no effect of the SNP on tHcy [14–18]. However, a Norwegian study did report that plasma dimethylglycine decreased with increasing number of A alleles [17].

The BHMT c.716G>A SNP in pregnant women has been associated with increased risk of placental abruption [16]. There are conflicting reports of the effect of the SNP in the foetus on foetal development outcomes. The variant allele was associated with increased risk of grave neural tube defects (NTDs) [19–21] but reduced risk of orofacial cleft [22] and Down syndrome [23,24]. Interestingly, in Liu et al.'s study [19], the risk of NTDs associated with the variant A allele was only observed in pregnancies of women that did not take folic acid supplements.

We hypothesised that the BHMT c.716G>A polymorphism impedes the conversion of betaine to dimethylglycine during pregnancy. We tested how plasma folate status affects plasma betaine and dimethylglycine according to BHMT c.716G>A genotype throughout pregnancy.

2. Experimental Section

2.1. Participants

A total of 612 women that were recruited before November 2014 from the pregnancy phase of the Reus-Tarragona Birth Cohort (RTBC) were studied. The RTBC is an observational longitudinal study of maternal nutritional status and pregnancy that is being carried out by the Area of Preventive Medicine and Public Health, Universitat Rovira i Virgili and the Areas of Obstetrics and Gynaecology of the University Hospitals: Sant Joan, Reus and Joan XXIII, Tarragona (Spain). The study design and participant recruitment have been previously described [12]. Briefly, women attending their first antenatal visit with a viable singleton pregnancy confirmed by ultrasonography that provided a fasting blood sample at or before the 12th gestational week (GW) were eligible to participate in the study. Exclusion criteria included chronic diseases, surgical interventions affecting nutritional status or medication affecting folate or cobalamin metabolism. The study was carried out with ethical approval

from the ethics committees of both participating hospitals and the research has been registered at Clinical Trials. gov: NCT01778205. All participants were informed of the nature and aims of the study and provided signed consent.

Participant age and body mass index (BMI) were recorded at the first antenatal check-up. Data regarding lifestyle, habits and supplement use from periconception throughout pregnancy was collected by the study team using questionnaires at 20 and 32 GW. Socioeconomic status of the participants was defined as low, middle or high according to the family unit income, education level and occupation of both parents [25]. Participants were classified as smokers throughout pregnancy, smokers during the first trimester and non-smokers based on first and second trimester as well as cord plasma cotinine concentrations (see biochemical determinations). Since plasma cotinine is indicative of recent smoking data from questionnaires and prenatal check-ups regarding declared smoking activity by the participants was also considered.

In line with the Spanish Obstetrics and Gynaecology Society recommendations [26], women were advised to take daily prenatal supplements containing 400 μg folic acid during the first trimester. The specific supplements recommended for this study were composed of 400 μg folic acid and 2 μg cyanocobalamin.

2.2. Blood Sample Collection and Biochemical and Genetic Determinations

Fasting blood samples were collected at ≤12, 15, 24–27 and 34 GW and nonfasting samples on admission to hospital with confirmed labour and from the umbilical cord. Maternal blood was drawn from the antecubital vein, and cord blood from the vein, into vacutainers containing EDTA-K_2. Samples were kept at 4 °C for a maximum of 1 h before separating plasma of which aliquots were immediately stored at −80 °C in the Institut d'Investigació Sanitària Pere Virgili biobank (Reus (Tarragona), Spain). Leukocytes were isolated from the blood cells remaining after plasma separation and DNA extracted from these using the Puregene DNA extraction kit (Gentra Systems, Minneapolis, MN, USA). Samples were transported on dry ice to Bevital (www.bevital.no, Bergen, Norway), carrying out biochemical analyses in batches comprising complete pregnancies within 18 months of collection. Plasma folate was determined by microbiological assay with *Lactobacillus casei* [27]. Plasma concentrations of choline, betaine, dimethylglycine, total homocysteine (tHcy) were measured by liquid chromatography–tandem mass spectrometry [28,29]. Plasma cotinine, which is an indicator of recent nicotine exposure, was also determined by liquid chromatography–tandem mass spectrometry [30] and plasma creatinine by modified Jaffé method (Química Clínica Aplicada SA, Amposta, Tarragona, Spain). Classification as current smoker was based on plasma cotinine >10 ng/mL at ≤12 GW (first trimester smoker) and 24–27 GW or in the cord (smoker throughout pregnancy). The maternal BHMT c.716G>A SNP was determined by Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF/MS) as previously described [31].

2.3. Statistical Analysis

Previously, we reported a shift in folate status between early and mid-late pregnancy in line with the overall cessation of folic acid supplement use or change in supplement type between the first and second or third trimesters of pregnancy [12]. To account for change in plasma folate status during pregnancy, and to classify plasma folate status according to WHO criteria [32], participants were classified into possibly deficient (plasma folate ≤ 13.4 nmol/L) or normal-high (plasma folate > 13.4 nmol/L) status categories during early pregnancy (based on plasma folate at ≤12 or 15 GW) and again during mid-late pregnancy (based on plasma folate at 24–27 or 34 GW). In the event of falling into different categories at different time points within the corresponding phase of pregnancy, normal-high status was assigned during early pregnancy and the predominant status out of the 3 time points was assigned in mid-late pregnancy. In any analysis using plasma folate status, the category occurring during the corresponding phase of pregnancy was used.

Natural log transformation was applied to normalise the distribution of plasma variables as required for the application of parametric tests. Thus, geometric means (95% CI) are reported for plasma folate, betaine, choline, dimethylglycine and tHcy. In all other cases medians (25th percentile, 75th percentile) are reported and frequencies are reported as % (95% CI). Proportions were compared using the chi-square test and the same test was also used to test the Hardy Weinberg equilibrium of the observed allele frequencies.

Plasma folate, choline, betaine, dimethylglycine and dimethylglycine/betaine ratio and tHcy were compared between the different BHMT c.716G>A genotypes at each gestational period and in the cord by ANCOVA adjusting for gestational age (weeks) at the time of the blood sample, and plasma folate status (in all models except for plasma folate models). The dimethylglycine models were also adjusted for plasma betaine. Interaction between plasma folate status and BHMT c.716G>A genotype in their effects on plasma dimethylglycine, dimethylglycine/betaine ratio and tHcy was tested and if observed, stratified analysis according to folate status was performed.

Changes in plasma concentrations of folate, choline, betaine, dimethylglycine, the dimethylglycine/betaine ratio and tHcy during pregnancy were assessed using 2-factor repeated measures ANOVA (General linear model; Intrasubject factor time of pregnancy and intersubject factor BHMT c.716G>A genotype) with posthoc Bonferroni correction of p values to account for multiple comparisons. The first trimester time point was used as the reference.

Multiple linear regression analysis was used to investigate the effects of plasma folate status and of maternal BHMT c.716G>A genotype on plasma dimethylglycine at each stage of pregnancy and in the cord. The models were adjusted for plasma betaine and gestational age at corresponding time of blood sample. The usual diagnostic techniques for multiple linear regression analysis were applied (Cook's distance to identify influential cases and analysis of residuals). Interaction between plasma folate status and BHMT c.716G>A genotype was also tested. All analyses were carried out with SPSS (SPSS Inc., Chicago, IL, USA) for Windows, version 22.0.

3. Results

Participation in the study by eligible candidates and completion (defined as live birth) is illustrated in Figure 1. Of the women screened at their first prenatal check-up, 42.4% were eligible to participate in the study. Of the 624 participants that entered the study (93.8% of those eligible) first trimester data was collected from 612 and 562 went on to have live births.

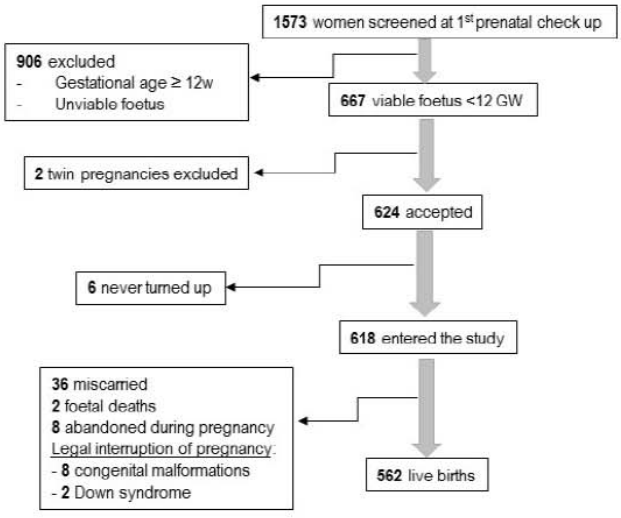


Figure 1. Flow chart of participation in the study.

Participant lifestyle, plasma folate, cobalamin, choline, tHcy and obstetrical characteristics are summarised in Table 1. This data shows that 80.9% of the women reported planning their pregnancy and 34.1% took folic acid supplements before becoming pregnant. Active smoking during the first trimester and throughout pregnancy was observed in 28.1% and in 17% of the participants respectively. The frequency of the BHMT c.716A allele was 30.8% (95% CI: 28.3, 33.5) and the genotypes were in Hardy Weinberg equilibrium (chi square for observed compared to expected genotype frequencies: 1.64).

Table 1. Participant characteristics (n = 612).

Age (Year) [1]		**32.0 (29.0, 35.0)**
Body mass index (kg/m^2) [1]		23.0 (20.9, 25.4)
Planned pregnancy [2]		80.9 (77.3, 84.0)
Previous pregnancy [2]		52.6 (48.6, 56.5)
Socioeconomic status [2,3]	High	43.6 (39.6, 47.6)
	Mid	49.6 (45.5, 53.6)
	Low	6.8 (5.1, 9.1)
Smoking during pregnancy [2]	First trimester	28.1 (24.7, 31.8)
	Throughout pregnancy	17.0 (14.2, 20.3)
Folic acid supplement use [2]	Preconception	34.1 (30.2, 38.2)
	First trimester	93.8 (91.5, 95.4)
	Mid-late pregnancy	53.9 (49.6, 58.1)
BHMT c.716G>A [2]	GG	48.9 (45.0, 52.9)
	GA	40.5 (36.6, 44.4)
	AA	10.6 (8.4, 13.3)

G: guanosine; A: adenosine. Values are [1] median (25th percentile, 75th percentile), [2] % (95% confidence interval); [3] based on total income, occupation and education level of both parents.

Plasma folate, choline, betaine and dimethylglycine concentrations, dimethylglycine/betaine ratios and tHcy at each time point of pregnancy and in the cord, according to maternal BHMT c.716G>A genotype are reported in Table 2. Plasma folate, choline, betaine or tHcy did not differ among genotypes at any time of pregnancy. Lower plasma dimethylglycine was observed in the heterozygote compared to homozygote common genotype at ≤12 GW and at labour. This was also true for the homozygote variant genotype during late pregnancy. A lower plasma dimethylglycine/betaine ratio was observed in the homozygote variant compared to heterozygote genotype at ≤12 GW and compared to the homozygote common genotype at 34 GW. This was also true for the heterozygote compared to the homozygote common genotype at 15 GW. Further adjustment of the ANCOVA models for plasma creatinine and smoking habit did not alter the results.

Plasma betaine gradually decreased in all of the genotypes throughout pregnancy. Plasma dimethylglycine fluctuated in a U shape pattern in the homozygote common and heterozygote genotypes where end of pregnancy concentrations were higher than in early pregnancy. However, in the homozygote variant genotype, plasma dimethylglycine concentrations remained lower than in early pregnancy, throughout pregnancy. The plasma dimethylglycine/betaine ratio gradually increased during pregnancy in all genotypes.

Interactions between plasma folate category and the effect of BHMT c.716G>A genotype on dimethylglycine were observed at ≤12 and 15 GW and on the dimethylglycine/betaine ratio at ≤12 GW.

A stratified analysis of plasma dimethylglycine for each genotype according to plasma folate status in early pregnancy is illustrated in Figure 2. In the normal-high category of plasma folate status, lower plasma dimethylglycine was observed in the heterozygote compared to the homozygote common genotype at both ≤12 and 15 GW and in the homozygote variant compared to homozygote common genotype at 15 GW.

Table 2. Plasma folate, choline, betaine, dimethylglycine and tHcy during pregnancy and in the cord according to maternal BHMT c.716G>A genotype.

	BHMT c.716G>A Genotype	≤12 GW [546] [1]	15 GW [440]	24–27 GW [500]	34 GW [485]	Labour [478]	Cord [465]
Folate (nmol/L)	GG	26.8 (24.5, 29.2)	25.6 (23.3, 28.1)	13.3 [a] (12.2, 14.6)	10.9 [a] (9.9, 12.1)	10.8 [a] (9.7, 11.9)	23.9 (22.2, 25.7)
	GA	25.5 (23.2, 28.0)	24.6 (22.3, 27.1)	12.9 [a] (11.7, 14.2)	10.7 [a] (9.7, 11.9)	10.8 [a] (9.7, 12.1)	24.2 (22.4, 26.1)
	AA	24.5 (20.2, 29.6)	25.4 (20.8, 31.1)	12.6 [a] (10.3, 15.3)	12.0 [a] (9.6, 15.1)	10.2 [a] (8.2, 12.9)	22.6 (19.1, 26.6)
	ANCOVA [3] *models*	NS	NS	NS	NS	NS	NS
Choline (µmo/L)	GG	7.6 (7.4, 7.9)	7.7 (7.5, 7.9)	9.1 [a] (8.9, 9.4)	10.3 [a] (10.1, 10.6)	11.7 [a] (11.3, 12.1)	28.1 (26.8, 29.5)
	GA	7.7 (7.4, 7.9)	7.7 (7.5, 8.0)	9.2 [a] (9.0, 9.5)	10.4 [a] (10.1, 10.7)	11.7 [a] (11.3, 12.2)	28.9 (27.5, 30.4)
	AA	7.6 (7.2, 8.1)	8.0 (7.5, 8.5)	9.5 [a] (9.0, 10.1)	10.5 [a] (9.9, 11.2)	11.6 [a] (10.7, 12.5)	29.8 (26.8, 33.1)
	ANCOVA [3] *models*	NS	NS	NS	NS	NS	NS
Betaine (µmol/L)	GG	20.9 (20.2, 21.6)	14.6 [a] (14.2, 15.1)	12.8 [a] (12.4, 13.1)	12.8 [a] (12.5, 13.1)	13.1 [a] (12.7, 13.5)	24.7 (24.0, 25.4)
	GA	21.8 (21.0, 22.6)	15.3 [a] (14.8, 15.8)	13.1 [a] (12.7, 13.4)	13.3 [a] (12.9, 13.7)	13.4 [a] (13.0, 13.8)	25.1 (24.3, 25.8)
	AA	20.2 (18.8, 21.8)	14.5 [a] (13.6, 15.5)	12.7 [a] (11.9, 13.4)	13.0 [a] (12.3, 13.8)	12.7 [a] (11.9, 13.5)	25.3 (23.8, 27.0)
	ANCOVA[3] *models*	NS	NS	NS	NS	NS	NS
Dimethylglycine (µmol/L)	GG	2.58 (2.47, 2.69)	2.29 [a] (2.19, 2.39)	2.22 [a] (2.12, 2.33)	2.51 (2.39, 2.64)	3.00 [a] (2.84, 3.18)	3.73 (3.55, 3.92)
	GA	2.37 (2.26, 2.48) *	2.12 [a] (2.02, 2.22)	2.11 [a] (2.00, 2.22)	2.39 (2.27, 2.52)	2.69 [a] (2.53, 2.86) *	3.54 (3.36, 3.73)
	AA	2.50 (2.28, 2.74)	2.08 [a] (1.88, 2.30)	2.02 [a] (1.82, 2.24)	2.01 [a] (1.79, 2.25) **,†	2.44 (2.16, 2.76) **	3.29 (2.95, 3.67)
	ANCOVA [4] *models*	$p = 0.026$	$p = 0.040$	NS	$p = 0.002$	$p = 0.002$	NS
	Folate genotype interaction	$p = 0.023$	$p = 0.058$	NS	NS	NS	NS
Dimethylglycine/betaine	GG	0.13 (0.12, 0.14)	0.17 [a] (0.16, 0.18)	0.19 [a] (0.18, 0.21)	0.22 [a] (0.20, 0.24)	0.26 [a] (0.23, 0.28)	0.17 (0.15, 0.18)
	GA	0.12 (0.11, 0.13)	0.15 [a] (0.14, 0.16) *	0.18 [a] (0.16, 0.19)	0.20 [a] (0.18, 0.22)	0.23 [a] (0.21, 0.26)	0.15(0.14, 0.16)
	AA	0.15 (0.13, 0.17) †	0.15 (0.13, 0.17)	0.18 [a] (0.15, 0.22)	0.16 (0.12, 0.20) *	0.22 [a] (0.17, 0.27)	0.14 (0.11, 0.16)
	ANCOVA [5] *models*	$p = 0.009$	$p = 0.018$	NS	$p = 0.020$	NS	$p = 0.044$
	Folate genotype interaction	$p = 0.038$	NS	NS	NS	NS	NS
Total homocysteine (µmol/L)	GG	5.2 (5.1, 5.4)	4.5 [a] (4.4, 4.7)	4.6 [a] (4.5, 4.8)	5.3 (5.1, 5.4)	6.2 [a] (6.0, 6.5)	4.8 (4.7, 5.0)
	GA	5.3 (5.1, 5.4)	4.4 [a] (4.3, 4.6)	4.7 [a] (4.5, 4.8)	5.4 (5.2, 5.6)	6.2 [a] (5.9, 6.4)	4.9 (4.7, 5.2)
	AA	5.3 (5.0, 5.6)	4.5 [a] (4.3, 4.8)	4.6 [a] (4.3, 4.9)	5.2 (4.9, 5.6)	6.0 [a] (5.6, 6.5)	4.9 (4.5, 5.3)
	ANCOVA [4] *models*	NS	NS	NS	NS	NS	NS

G: guanosine; A: adenosine; GW: gestational weeks; NS: non-significant. [1] *n* varies between time points due to participant loss due to complications, non-attendance of programmed blood draw or delivery elsewhere or failure to collect blood samples in the labour ward; [2] values are least square geometric means (95% confidence interval); [3] adjusting for plasma folate status and gestational age at time of blood draw; [4] adjusting for plasma folate status, plasma betaine and gestational age at time of blood draw. ANCOVA Bonferroni posthoc: * $p < 0.05$, ** $p < 0.01$ versus GG; † $p < 0.05$, versus GA; Two-factor repeated measures ANOVA versus ≤12 GW (intrasubject factor: gestational age; intersubject factor: BHMT c.716G>A genotype) followed by post hoc Bonferroni correction for multiple comparisons: [a] $p < 0.001$.

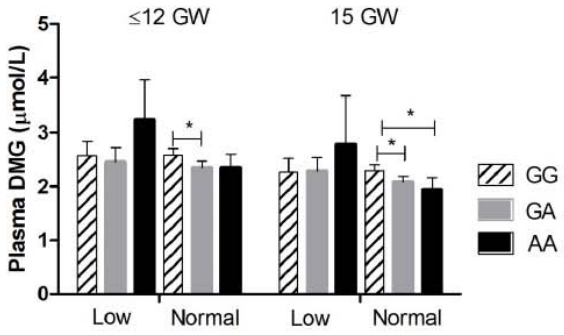


Figure 2. Plasma dimethylglycine according to BHMT c.716G>A genotype and folate status in early pregnancy. G: guanosine; A: adenosine; GW: Gestational weeks. Low: possibly deficient (plasma folate ≤ 13.4 nmol/L). Normal: normal-high (plasma folate > 13.4 nmol/L). At ≤12 GW, Low: GG (n = 57), GA (n = 48), AA (n = 14); Normal: GG (n = 231), GA (n = 193), AA (n = 47). At 15 GW, Low: GG (n = 37), GA (n = 40), AA (n = 6); Normal: GG (n = 169), GA (n = 141), AA (n = 34). The triple screening blood sample at 15 GW is optional and blood samples were available from less participants. Values are least square geometric means. Error bars represent 95% confidence interval. Comparisons between genotypes were made using ANCOVA adjusting for plasma betaine and gestational age at time of blood draw with posthoc Bonferroni correction for multiple comparisons of p values: * $p < 0.05$.

The associations between plasma folate status and BHMT c.716G>A genotype with plasma dimethylglycine throughout pregnancy and in the cord are reported in Table 3. Low compared to normal-high plasma folate status was positively associated with plasma dimethylglycine throughout pregnancy and in the cord. Compared to the homozygous common BHMT GG genotype, both variant GA and AA genotypes were associated with lower plasma dimethylglycine during early and late pregnancy. The observed associations were stronger in the case of the homozygote AA variant genotype during late pregnancy and in the cord. Further adjustment of the multiple linear regression models for plasma creatinine and smoking habit did not alter the results.

Table 3. Change in plasma dimethylglycine throughout pregnancy according to plasma folate category, and BHMT c.716G>A genotype.

		Model [1]		Plasma Folate Category [2]	BHMT c.716G>A Genotype	
		R^2	F (n)	Possibly deficient vs. normal-high	GA vs. GG	AA vs. GG
Early	≤12 GW	15.4	22.5 (592) ***	0.07 (0.04) [3,*]	−0.08 (0.03) **	−0.04 (0.05)
	15 GW	12.0	12.6 (427) ***	0.07 (0.04) [#]	−0.08 (0.03) *	−0.11 (0.06) [†]
Mid-late	24–27 GW	11.6	14.3 (507) ***	0.13 (0.03) ***	−0.06 (0.04)	−0.10 (0.06)
	34 GW	18.9	23.8 (492) ***	0.20 (0.04) ***	−0.06 (0.04)	−0.23 (0.06) ***
	Labour	13.2	15.5 (477) ***	0.15 (0.04) ***	−0.11 (0.04) *	−0.21 (0.07) **
	Cord	5.0	5.7 (447) ***	0.08 (0.04) *	−0.05 (0.04) *	−0.13 (0.06) *

F: analysis of variance F-test of overall significance; G: guanosine; A: adenosine; GW: Gestational weeks. [1] Multiple linear regression analysis: dependent variable plasma dimethylglycine. Adjusted for plasma betaine and gestational age at time of blood draw; [2] Pregnancy (possibly deficient: ≤13.4 nmol/L; normal-high: >13.4 nmol/L); [3] β coefficient (standard error of the mean). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, [†] $p = 0.055$, [#] $p = 0.087$.

4. Discussion

4.1. Principal Findings

This study reports for the first time that the A-allele of the BHMT c.716G>A SNP is associated with lower plasma dimethylglycine during pregnancy and in the cord. Low folate status, on the other

hand is associated with higher plasma dimethylglycine during mid and late pregnancy and in the cord. Folate status and genotype interacted during early pregnancy and stratification by plasma folate status category showed that the effect of the variant BHMT c.716A allele on dimethylglycine was limited to women with normal-high folate status. By late pregnancy, the variant allele's effect was independent of folate status.

4.2. Comparison with Previous Studies and Interpretation

We observed similar frequencies of the BHMT c.716G>A genotypes as previously reported in studies from Europe [14,17,33].

We interpret the lower plasma dimethylglycine concentrations observed in the presence of the variant BHMT c.716A allele to mean that the conversion of betaine to dimethylglycine is low in pregnant women carrying the A-allele. A similar observation was previously reported in a population study [17]. On the other hand, the higher plasma dimethylglycine concentrations when folate status is low, suggests BHMT upregulation when MTR activity is reduced. In early pregnancy when MTR activity was not reduced by low folate availability, the effects of lower BHMT activity in the presence of the BHMT c.716 A-allele are evident. We hypothesised that this effect would be more pronounced at mid-late pregnancy and with low folate status when the BHMT pathway is more active [12]. Our results showed decreased betaine to dimethylglycine conversion in the variant A-allele carriers, but in early pregnancy this effect was limited to women with normal-high folate status. In vitro experiments have shown that BHMT expression and activity may be modified by the molecular environment. SAM has been shown to inhibit BHMT transcription in human cells [34,35] and SAM [36] and to a lesser extent SAH [36,37] to inhibit BHMT activity in rat liver extracts. Methionine has also been shown to inhibit BHMT [38,39]. A study in MTHFR-deficient mice supplemented with very high doses of folic acid showed that despite greater betaine utilisation, likely for homocysteine remethylation, it was insufficient to maintain SAM concentrations [40]. Despite the differences in molecular environment between *in vivo* and *in vitro* studies, they shed light on potential mechanisms that may lead to our observed effects. A speculative suggestion from our data is that high folate status can lead to BHMT inhibition, with the inhibitory effect being stronger on the variant enzyme. Some studies have investigated the effect of folate on BHMT. Liver *BHMT* expression was reported to be lower in the offspring of rat dams supplemented with folic acid during pregnancy [41] but was unaffected by excessive folic acid intake in mice [40]. It has also been suggested that betaine is spared when folate status is replete [42]. It is possible that the combination of both mechanisms, replete folate status and the variant allele, lead to reduced conversion of betaine to dimethylglycine. Cessation of prenatal folic acid supplementation at the end of the first trimester was associated with a sharp reduction in folate status, and from mid pregnancy the genotype effect on dimethylglycine was independent of folate status.

No differences in plasma betaine concentrations between the different genotypes were observed. This is not surprising because a stable betaine pool is maintained even when the BHMT pathway is upregulated [9,43], possibly to spare betaine for its principal functions as an osmolyte [44] and in protein stabilisation [45].

4.3. Implications

BHMT expression and activity in foetal livers has been reported to increase with gestational age in human [5,46] and pig studies [47] which is in line with increased BHMT activity in late pregnancy that we suggest here. Interestingly, foetal liver MTR activity decreases in the third trimester [5]. This supports the idea of complementarity between the BHMT and MTR pathways in late pregnancy and our observations indirectly support this hypothesis.

Here we show that the BHMT c.716G>A polymorphism, previously associated with foetal developmental defects, affects the BHMT pathway during pregnancy. In situations of upregulation of the MTR and BHMT pathways, such as in pregnancy, the effect of the SNP on BHMT activity may

become more evident. Anomalies in homocysteine remethylation affect homocysteine homeostasis as well as methyl group supply to essential epigenetic reactions. Elevated maternal homocysteine has been associated with pregnancy complications affecting both maternal and foetal health as well as foetal development [48] and with lasting developmental effects into childhood [49]. Inhibition of BHMT has been shown to cause hyperhomocysteinaemia in mice [50]. We did not observe an effect of the variant allele on homocysteine. However, the extent of the effect of the SNP on BHMT activity in humans is unknown. Pregnancy itself has a profound effect on homocysteine [51] and may mask any potential effects of the SNP. Comparison of associations between betaine and homocysteine before and after the implementation of mandatory fortification of flour with folic acid in the USA showed that the inverse correlation reported in Framingham study participants with low folate status prior to fortification, was no longer observed post-fortification [52].

Potential modification of effects of folate status by the BHMT c.716G>A SNP should be considered when contrasting/interpreting results from fortified versus non-fortified populations or supplement versus non-supplement users. In addition, the importance of the BHMT pathway in homocysteine homeostasis may be less important in folate-replete populations.

4.4. Strengths and Limitations

A strength of this study is its longitudinal design and follow up from very early pregnancy. It was carried out in the absence of mandatory fortification with folic acid and therefore the effects of prenatal folic acid supplement use were observed. Samples were processed according to specific protocols to prevent artefacts in plasma analyte concentrations caused by temperature and time before separation of blood cells [53].

When folic acid supplementation ceased there was a large drop in plasma folate as we reported previously [12]. Sample power may have been limited in analyses of the effect of the BHMT c.716AA genotype during early pregnancy. However many significant effects of this genotype on dimethylglycine were observed in mid-late pregnancy and in the cord. Another potential limitation to the study was the difference in folic acid supplement use between women. They were recommended to take prenatal supplements containing 400 µg of folic acid during the first trimester. However some took other brands of folic acid containing higher doses or extended their folic acid use beyond the first trimester. We dealt with this in our analysis by classifying the women into plasma folate status categories in early and again in mid-late pregnancy to account for the difference in status that we expected to arise due to differences in patterns of supplement use.

Although there is increasing evidence that BHMT plays an important role in one carbon metabolism many aspects regarding the mechanisms involved are unclear. Information regarding the effect of the common BHMT c.716G>A polymorphism on BHMT activity and how it is affected by variations in molecular environment is lacking.

5. Conclusions

We conclude that dimethylglycine during pregnancy is affected by both folate status and the BHMT c.716G>A polymorphism.

Acknowledgments: This work was supported by funding from Comisión Interministerial de Ciencia y Tecnología, Spain [SAF 2005-05096]; Instituto de Salud Carlos III, Fondo de Investigaciones Sanitarias (cofinanced by FEDER) [FIS: 10/00335 and FIS: 13/02500] and Centros de Investigación Biomédica en Red CB06/03; Institut d'Investigació Sanitària Pere Virgili 2010/21; Agència de Gestió d'Ajuts Universitaris i de Recerca [SGR 2009-1237] and from Italfarmaco SA, Madrid, Spain. Jose M Colomina received a Universitat Rovira i Virgili (URV) Martí Franques predoctoral research grant.

Author Contributions: J.M.C., S.F.-R., J.D.F.-B. and M.M.M. developed the hypothesis, designed the study and analysed the data. P.C.-B. and M.B. recruited the participants and carried out the pregnancy phase of the study. S.F.-R., P.S.-N. and J.M.C. collected lifestyle and dietary data from the participants and processed samples for biobanking. P.M.U. and K.M. were responsible for the biochemical and genetic determinations. All authors participated in the writing of the paper and approved the final version.

Conflicts of Interest: The authors declare no conflicts of interests.

References

1. McKeever, M.P.; Weir, D.G.; Molloy, A.; Scott, J.M. Betaine-homocysteine methyltransferase: Organ distribution in man, pig and rat and subcellular distribution in the rat. *Clin. Sci.* **1991**, *81*, 551–556. [CrossRef] [PubMed]
2. Finkelstein, J.D.; Martin, J.J. Methionine metabolism in mammals. Distribution of homocysteine between competing pathways. *J. Biol. Chem.* **1984**, *259*, 9508–9513. [PubMed]
3. Finkelstein, J.D. Methionine metabolism in mammals. *J. Nutr. Biochem.* **1990**, *1*, 228–237. [CrossRef]
4. Lambert, B.D.; Titgemeyer, E.C.; Stokka, G.L.; DeBey, B.M.; Löest, C.A. Methionine supply to growing steers affects hepatic activities of methionine synthase and betaine-homocysteine methyltransferase, but not cystathionine synthase. *J. Nutr.* **2002**, *132*, 2004–2009. [PubMed]
5. Gaull, G.E.; Von Berg, W.; Räihä, N.C.; Sturman, J.A. Development of methyltransferase activities of human fetal tissues. *Pediatr. Res.* **1973**, *7*, 527–533. [PubMed]
6. Sunden, L.F.; Renduchintala, M.S.; Park, E.I.; Miklasz, S.D.; Garrow, T.A. Betaine-homocysteine methyltransferase expression in porcine and human tissues and chromosomal localization of the human gene. *Arch. Biochem. Biophys.* **1997**, *345*, 171–174. [CrossRef] [PubMed]
7. Melse-Boonstra, A.; Holm, P.I.; Ueland, P.M.; Olthof, M.; Clarke, R.; Verhoef, P. Betaine concentration as a determinant of fasting total homocysteine concentrations and the effect of folic acid supplementation on betaine concentrations. *Am. J. Clin. Nutr.* **2005**, *81*, 1378–1382. [PubMed]
8. Imbard, A.; Smulders, Y.M.; Barto, R.; Smith, D.E.C.; Kok, R.M.; Jakobs, C.; Blom, H.J. Plasma choline and betaine correlate with serum folate, plasma S-adenosyl-methionine and S-adenosyl-homocysteine in healthy volunteers. *Clin. Chem. Lab. Med.* **2013**, *51*, 683–692. [CrossRef] [PubMed]
9. Allen, R.H.; Stabler, S.P.; Lindenbaum, J. Serum betaine, *N*,*N*-dimethylglycine and *N*-methylglycine levels in patients with cobalamin and folate deficiency and related inborn errors of metabolism. *Metabolism* **1993**, *42*, 1448–1460. [CrossRef]
10. Holm, P.I.; Ueland, P.M.; Vollset, S.E.; Midttun, Ø.; Blom, H.J.; Keijzer, M.B.; den Heijer, M. Betaine and folate status as cooperative determinants of plasma homocysteine in humans. *Arterioscler. Thromb. Vasc. Biol.* **2005**, *25*, 379–385. [CrossRef] [PubMed]
11. Holm, P.I.; Bleie, Ø.; Ueland, P.M.; Lien, E.; Refsum, H.; Nordrehaug, J.E.; Nygård, O. Betaine as a determinant of postmethionine load total plasma homocysteine before and after B-vitamin supplementation. *Arterioscler. Thromb. Vasc. Biol.* **2004**, *24*, 301–307. [CrossRef] [PubMed]
12. Fernàndez-Roig, S.; Cavallé-Busquets, P.; Fernandez-Ballart, J.D.; Ballesteros, M.; Berrocal-Zaragoza, M.I.; Salat-Batlle, J.; Ueland, P.M.; Murphy, M.M. Low folate status enhances pregnancy changes in plasma betaine and dimethylglycine concentrations and the association between betaine and homocysteine. *Am. J. Clin. Nutr.* **2013**, *97*, 1252–1259.
13. Park, E.I.; Garrow, T. Interaction between dietary methionine and methyl donor intake on rat liver betaine-homocysteine methyltransferase gene expression and organization of the human gene. *J. Biol. Chem.* **1999**, *274*, 7816–7824. [CrossRef] [PubMed]
14. Heil, S.G.; Lievers, K.J.; Boers, G.H.; Verhoef, P.; den Heijer, M.; Trijbels, F.J.; Blom, H.J. Betaine-homocysteine methyltransferase (BHMT): Genomic sequencing and relevance to hyperhomocysteinemia and vascular disease in humans. *Mol. Genet. Metab.* **2000**, *71*, 511–519. [CrossRef] [PubMed]
15. Weisberg, I.S.; Park, E.; Ballman, K.V.; Berger, P.; Nunn, M.; Suh, D.S.; Breksa, A.P.; Garrow, T.A.; Rozen, R. Investigations of a common genetic variant in betaine-homocysteine methyltransferase (BHMT) in coronary artery disease. *Atherosclerosis* **2003**, *167*, 205–214. [CrossRef]
16. Ananth, C.V.; Elsasser, D.; Kinzler, W.L.; Peltier, M.R.; Getahun, D.; Leclerc, D.; Rozen, R.R. Polymorphisms in methionine synthase reductase and betaine-homocysteine *S*-methyltransferase genes: Risk of placental abruption. *Mol. Genet. Metab.* **2007**, *91*, 104–110. [CrossRef] [PubMed]
17. Fredriksen, A.; Meyer, K.; Ueland, P.M.; Vollset, S.E.; Grotmol, T.; Schneede, J. Large-scale population-based metabolic phenotyping of thirteen genetic polymorphisms related to one-carbon metabolism. *Hum. Mutat.* **2007**, *28*, 856–865. [CrossRef] [PubMed]

18. Liang, S.; Zhou, Y.; Wang, H.; Qian, Y.; Ma, D.; Tian, W.; Persaud-Sharma, V.; Yu, C.; Ren, Y.; Zhou, S.; et al. The effect of multiple single nucleotide polymorphisms in the folic acid pathway genes on homocysteine metabolism. *Biomed. Res. Int.* **2014**, *2014*, 560183. [CrossRef] [PubMed]
19. Liu, J.; Qi, J.; Yu, X.; Zhu, J.; Zhang, L.; Ning, Q.; Luo, X. Investigations of single nucleotide polymorphisms in folate pathway genes in Chinese families with neural tube defects. *J. Neurol. Sci.* **2014**, *337*, 61–66. [CrossRef] [PubMed]
20. Shaw, G.M.; Lu, W.; Zhu, H.; Yang, W.; Briggs, F.B.S.; Carmichael, S.L.; Barcellos, L.F.; Lammer, E.J.; Finnell, R.H. 118 SNPs of folate-related genes and risks of spina bifida and conotruncal heart defects. *BMC Med. Genet.* **2009**, *10*, 49. [CrossRef] [PubMed]
21. Boyles, A.L.; Billups, A.V.; Deak, K.L.; Siegel, D.G.; Mehltretter, L.; Slifer, S.H.; Bassuk, A.G.; Kessler, J.A.; Reed, M.C.; Nijhout, H.F.; et al. Neural tube defects and folate pathway genes: Family-based association tests of gene-gene and gene-environment interactions. *Environ. Health Perspect.* **2006**, *114*, 1547–1552. [CrossRef] [PubMed]
22. Mostowska, A.; Hozyasz, K.K.; Wojcicki, P.; Dziegelewska, M.; Jagodzinski, P.P. Associations of folate and choline metabolism gene polymorphisms with orofacial clefts. *J. Med. Genet.* **2010**, *47*, 809–815. [CrossRef] [PubMed]
23. Zampieri, B.L.; Biselli, J.M.; Goloni-Bertollo, E.M.; Vannucchi, H.; Carvalho, V.M.; Cordeiro, J.A.; Pavarino, E.C. Maternal risk for Down syndrome is modulated by genes involved in folate metabolism. *Dis. Markers* **2012**, *32*, 73–81. [CrossRef] [PubMed]
24. Amorim, M.R.; Moura, C.M.; Gomes, A.D.; Barboza, H.N.; Lopes, R.B.; Ribeiro, M.G.; Costa Lima, M.A. Betaine–homocysteine methyltransferase 742G>A polymorphism and risk of down syndrome offspring in a Brazilian population. *Mol. Biol. Rep.* **2013**, *40*, 4685–4689. [CrossRef] [PubMed]
25. Álvarez-Dardet, C.; Alonso, J.; Domingo, A.; Regidor, E. *La Medición de la Clase Social en Ciencias de la Salud*; SG Editores, SEE: Barcelona, Spain, 1995.
26. Dirección General de Salud Pública (Ministerio de Sanidad y Consumo—Gobierno de España). *Recomendaciones Sobre Suplementación con ácido Fólico para la Prevención de Defectos del Tubo Neural*; Dirección General de Salud Pública: Madrid, Spain, 2001; Volume 25.
27. Molloy, A.M.; Scott, J.M. Microbiological assay for serum, plasma, and red cell folate using cryopreserved, microtiter plate method. *Methods Enzymol.* **1997**, *281*, 43–53. [PubMed]
28. Holm, P.I.; Ueland, P.M.; Kvalheim, G.; Lien, E.A. Determination of choline, betaine, and dimethylglycine in plasma by a high-throughput method based on normal-phase chromatography-tandem mass spectrometry. *Clin. Chem.* **2003**, *49*, 286–294. [CrossRef] [PubMed]
29. Ueland, P.M.; Midttun, O.; Windelberg, A.; Svardal, A.; Skålevik, R.; Hustad, S. Quantitative profiling of folate and one-carbon metabolism in large-scale epidemiological studies by mass spectrometry. *Clin. Chem. Lab. Med.* **2007**, *45*, 1737–1745. [CrossRef] [PubMed]
30. Midttun, Ø.; Hustad, S.; Ueland, P.M. Quantitative profiling of biomarkers related to B-vitamin status, tryptophan metabolism and inflammation in human plasma by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 1371–1379. [CrossRef] [PubMed]
31. Meyer, K.; Fredriksen, A.; Ueland, P.M. High-level multiplex genotyping of polymorphisms involved in folate or homocysteine metabolism by matrix-assisted laser desorption/ionization mass spectrometry. *Clin. Chem.* **2004**, *50*, 391–402. [CrossRef] [PubMed]
32. World Health Organization. Serum and Red Blood Cell Folate Concentrations for Assessing Folate Status in Populations. Vitamin and Mineral Nutrition Information System. 2012. Available online: http://apps.who.int/iris/bitstream/10665/75584/1/WHO_NMH_NHD_EPG_12.1_eng.pdf (accessed on 6 September 2016).
33. Giusti, B.; Sestini, I.; Saracini, C.; Sticchi, E.; Bolli, P.; Magi, A.; Gori, A.M.; Marcucci, R.; Gensini, G.F.; Abbate, R. High-throughput multiplex single-nucleotide polymorphism (SNP) analysis in genes involved in methionine metabolism. *Biochem. Genet.* **2008**, *46*, 406–423. [CrossRef] [PubMed]
34. Castro, C.; Breksa, A.P.; Salisbury, E.; Garrow, T.A. Betaine-Homocysteine S-Methyltransferase (BHMT) Transcription Is Inhibited by S-Adenosylmethionine (ADOMET). In *Chemistry and Biology of Pteridines and Folates*; Milstien, S., Kapatos, G., Levine, R.A., Shane, B., Eds.; Springer: Bethesda, Spain, 2002; pp. 549–556.
35. Ou, X.; Yang, H.; Ramani, K.; Ara, A.I.; Chen, H.; Mato, J.M.; Lu, S.C. Inhibition of human betaine-homocysteine methyltransferase expression by S-adenosylmethionine and methylthioadenosine. *Biochem. J.* **2007**, *401*, 87–96. [CrossRef] [PubMed]

36. Finkelstein, J.D.; Martin, J.J. Inactivation of betaine-homocysteine methyltransferase by adenosylmethionine and adenosylethionine. *Biochem. Biophys. Res. Commun.* **1984**, *118*, 14–19. [CrossRef]
37. Finkelstein, J.D.; Kyle, W.E.; Harris, B.J. Methionine metabolism in mammals: Regulatory effects of S-adenosylhomocysteine. *Arch. Biochem. Biophys.* **1974**, *165*, 774–779. [CrossRef]
38. Finkelstein, J.D.; Harris, B.J.; Kyle, W.E. Methionine metabolism in mammals: Kinetic study of betaine-homocysteine methyltransferase. *Arch. Biochem. Biophys.* **1972**, *153*, 320–324. [CrossRef]
39. Szegedi, S.S.; Castro, C.C.; Koutmos, M.; Garrow, T.A. Betaine-homocysteine S-methyltransferase-2 is an S-methylmethionine-homocysteine methyltransferase. *J. Biol. Chem.* **2008**, *283*, 8939–8945. [CrossRef] [PubMed]
40. Christensen, K.E.; Mikael, L.G.; Leung, K.Y.; Lévesque, N.; Deng, L.; Wu, Q.; Malysheva, O.V.; Best, A.; Caudill, M.A.; Greene, N.D.; et al. High folic acid consumption leads to pseudo-MTHFR deficiency, altered lipid metabolism, and liver injury in mice. *Am. J. Clin. Nutr.* **2015**, *101*, 646–658. [CrossRef] [PubMed]
41. Chmurzynska, A.; Malinowska, A.M. Homocysteine homeostasis in the rat is maintained by compensatory changes in cystathionine β-synthase, betaine-homocysteine methyltransferase, and phosphatidylethanolamine N-methyltransferase gene transcription occurring in response to maternal protein and folic acid intake during pregnancy and fat intake after weaning. *Nutr. Res.* **2011**, *31*, 572–577. [PubMed]
42. Eussen, S.J.P.M.; Ueland, P.M.; Clarke, R.; Blom, H.J.; Hoefnagels, W.H.L.; van Staveren, W.; de Groot, L.C.P.G.M. The association of betaine, homocysteine and related metabolites with cognitive function in Dutch elderly people. *Br. J. Nutr.* **2007**, *98*, 960–968. [CrossRef] [PubMed]
43. Dominguez-Salas, P.; Moore, S.E.; Cole, D.; da Costa, K.A.; Cox, S.E.; Dyer, R.A.; Fulford, A.J.C.; Innis, S.M.; Waterland, R.A.; Zeisel, S.H.; et al. DNA methylation potential: dietary intake and blood concentrations of one-carbon metabolites and cofactors in rural African women. *Am. J. Clin. Nutr.* **2013**, *97*, 1217–1227. [CrossRef] [PubMed]
44. Yancey, P.H.; Clark, M.E.; Hand, S.C.; Bowlus, R.D.; Somero, G.N. Living with water stress: Evolution of osmolyte systems. *Science* **1982**, *217*, 1214–1222. [CrossRef] [PubMed]
45. Sheikh-Hamad, D.; García-Pérez, A.; Ferraris, J.D.; Peters, E.M.; Burg, M.B. Induction of gene expression by heat shock versus osmotic stress. *Am. J. Physiol.* **1994**, *267*, F28–F34. [PubMed]
46. Feng, Q.; Kalari, K.; Fridley, B.L.; Jenkins, G.; Ji, Y.; Abo, R.; Hebbring, S.; Zhang, J.; Nye, M.D.; Leeder, J.S.; et al. Betaine-homocysteine methyltransferase: Human liver genotype-phenotype correlation. *Mol. Genet. Metab.* **2011**, *102*, 126–133. [CrossRef] [PubMed]
47. Ganu, R.; Garrow, T.; Koutmos, M.; Rund, L.; Schook, L.B. Splicing variants of the porcine betaine-homocysteine S-methyltransferase gene: Implications for mammalian metabolism. *Gene* **2013**, *529*, 228–237. [CrossRef] [PubMed]
48. Murphy, M.M.; Fernandez-Ballart, J.D. Homocysteine in pregnancy. *Adv. Clin. Chem.* **2011**, *53*, 105–137. [PubMed]
49. Murphy, M.M.; Fernandez-Ballart, J.D.; Molloy, A.M.; Canals, J. Moderately elevated maternal homocysteine at preconception is inversely associated with cognitive performance in children 4 months and 6 years after birth. *Matern. Child. Nutr.* **2016**. [CrossRef] [PubMed]
50. Collinsova, M.; Strakova, J.; Jiracek, J.; Garrow, T.A. Inhibition of betaine-homocysteine S-methyltransferase causes hyperhomocysteinemia in mice. *J. Nutr.* **2006**, *136*, 1493–1497. [PubMed]
51. Murphy, M.M.; Scott, J.M.; McPartlin, J.M.; Fernandez-Ballart, J.D. The pregnancy-related decrease in fasting plasma homocysteine is not explained by folic acid supplementation, hemodilution, or a decrease in albumin in a longitudinal study. *Am. J. Clin. Nutr.* **2002**, *76*, 614–619. [PubMed]
52. Lee, J.E.; Jacques, P.F.; Dougherty, L.; Selhub, J.; Giovannucci, E.; Zeisel, S.H.; Cho, E. Are dietary choline and betaine intakes determinants of total homocysteine concentration? *Am. J. Clin. Nutr.* **2010**, *91*, 1303–1310. [CrossRef] [PubMed]
53. Fiskerstrand, T.; Refsum, H.; Kvalheim, G.; Ueland, P.M. Homocysteine and other thiols in plasma and urine: Automated determination and sample stability. *Clin. Chem.* **1993**, *39*, 263–271. [PubMed]

Nom: Data:

ENCUESTA 1 SOBRE HÁBITOS Y ESTILO DE VIDA

ANOTE LAS RESPUESTAS EN LOS ESPACIOS CORRESPONDIENTES A CADA PREGUNTA.
Estos datos servirán a la Universitat Rovira i Virgili para realizar un estudio comparativo entre diferentes poblaciones. En los resultados nunca aparecerá su nombre.

USO DE SUPLEMENTOS DE VITAMINAS / MINERALES

Por diferentes motivos, los suplementos de vitaminas y minerales recomendados no se toman siempre: por olvido, por sentimiento de que no son necesarios, por no encontrarse bien, porque dan molestias, etc. Por favor, conteste sinceramente estas preguntas para ayudarnos a valorar la realidad del uso de los suplementos.

- ¿Ha tomado por iniciativa propia o recetado por un médico algún tipo de suplemento vitamínico / mineral?
  Nunca he tomado ☐ Si he tomado ☐

En el caso que si, escriba el nombre del preparado e indique las veces a la semana que lo ha tomado marcando el cuadrado. Marque el cuadrado correspondiente a los meses que lo ha tomado.

Ejemplo, una mujer que ha tomado cada día FOLIDOCE durante los primeros 3 meses, escribiría:

Nombre del preparado	¿Cuántas veces a la semana?	Meses del embarazo 1	2	3	4	5
ÁCIDO FÓLICO	■ Cada día	■	■	■	☐	☐
	☐ La mayoría de los días (4-6 veces)	☐	☐	☐	☐	☐
¿Cuál? : FOLIDOCE	☐ Algunos días (1-3 veces)	☐	☐	☐	☐	☐

Nombre del preparado	¿Cuántas veces a la semana?	Meses del embarazo 1	2	3	4	5
ÁCIDO FÓLICO	☐ Cada día	☐	☐	☐	☐	☐
	☐ La mayoría de los días (4-6 veces)	☐	☐	☐	☐	☐
¿Cuál?:_____	☐ Algunos días (1-3 veces)	☐	☐	☐	☐	☐
	☐ Cada día	☐	☐	☐	☐	☐
HIERRO	☐ La mayoría de los días (4-6 veces)	☐	☐	☐	☐	☐
¿Cuál?:_____	☐ Algunos días (1-3 veces)	☐	☐	☐	☐	☐
MULTI-VITAMINAS	☐ Cada día	☐	☐	☐	☐	☐
	☐ La mayoría de los días (4-6 veces)	☐	☐	☐	☐	☐
¿Cuál?:_____	☐ Algunos días (1-3 veces)	☐	☐	☐	☐	☐

- ¿Tomó ácido fólico en los 3 meses antes de quedarse embarazada? **Sí ☐ No ☐**
- ¿Tomó hierro en los 3 meses antes de quedarse embarazada? **Sí ☐ No ☐**

DESAYUNO (durante el embarazo)

	Sí	No
¿Tiene la costumbre de desayunar?	☐	☐
¿Desayuna cereales inflados habitualmente (p.ej. tipo Kelloggs / Nestlé etc) ?	☐	☐
¿Toma café con cafeína?	☐	☐
¿Toma café descafeinado?	☐	☐

Nom: Data:

TABACO

- ¿Es fumadora pasiva (expuesta al humo habitualmente en casa o en el trabajo) ? **Sí** ☐ **No** ☐
- És fumadora activa? **Sí** ☐ **No** ☐

Sólo para fumadoras en los últimos 5 años

	0 cigs/día	1-5 cigs/día	6-10 cigs/día	> 10 cigs/día
Actualmente fumo	☐	☐	☐	☐
Fumaba durante los 12 meses antes del embarazo	☐	☐	☐	☐

	Antes de los 3 meses	Entre los 3 y los 6 meses	Después de los 6 meses
He dejado durante el embarazo durante los meses	☐	☐	☐

ALCOHOL

	Nunca / Ocasionalmente	< 3 copes / setmana	Cada día como aperitivo y/o con las comidas	> 7 copas / semana
Actualmente bebo alcohol	☐	☐	☐	☐
En los 12 meses antes del embarazo	☐	☐	☐	☐

	Antes de los 3 meses	Entre los 3 y los 6 meses	Después de los 6 meses
He dejado de beber alcohol durante el embarazo durante los meses	☐	☐	☐

- ¿Ha tomado algún otro tipo de sustancia tóxica (p.ej. marihuana, cocaína, heroína, etc...) en los últimos 5 años?

Sí ☐ **No** ☐

En el caso de que sí haya tomado alguna sustancia tóxica, especifique cuales:

	Ocasionalmente	Regularmente
Actualmente tomo sustancias tóxicas	☐	☐
En los 12 meses antes del embarazo tomaba sustancias tóxicas	☐	☐

	Antes de los 3 meses	Entre los 3 y los 6 meses	Después de los 6 meses
Lo he dejado durante el embarazo durante los meses	☐	☐	☐

Nom: Data:

ACTIVIDAD FÍSICA (durante el embarazo)

- ¿Qué actividad física hace en el trabajo, estudio o trabajo de casa?

  - Mi trabajo es básicamente de estar sentada y caminar poco (estudiante, docente, conductora de vehículos, dependienta, administrativa)..... ☐

  - En mi trabajo ando bastante pero no hago ningún esfuerzo vigoroso (ama de casa, fábrica, vendedora, cartera)..... ☐

  - Mi trabajo es básicamente de mucha actividad física (deportista)..... ☐

- ¿Qué actividad hace en el tiempo libre?

Si varia con la estación, escoger el grupo más representativo (sólo 1 grupo).

  - Lectura, televisión y actividades que no requieran actividad física importante..... ☐

  - Caminar, ir en bicicleta, jardinería (no se incluye el transporte de ir y volver del trabajo)..... ☐

  - Correr, esquiar, gimnástica, juegos de pelota o deportes vigorosos regularmente..... ☐

  - Entrenamiento deportivo regular para competición..... ☐

- ¿Cuántas veces por semana realiza las actividades referidas en la pregunta anterior? _____

- Durante los últimos 12 meses

	Nunca	Esporádicamente	Habitualmente
¿Ha tenido la costumbre de tomar el Sol?	☐	☐	☐

PLANIFICACIÓN DEL EMBARAZO

- ¿Ha buscado / planificado este embarazo? **Sí** ☐ **No** ☐

- Durante los 6 meses antes del embarazo

	Ninguno	DIU	Anticonceptivos orales	Pegados anticonceptivos	Anillo vaginal	Preservativo
¿Que método anticonceptivo ha utilizado?	☐	☐	☐	☐	☐	☐

- ¿Ciclos sin tomar anticonceptivos antes del embarazo? _____

(Número de reglas desde que dejó de tomar anticonceptivos hasta que se quedó embarazada)

Nom: Data:

DATOS SOCIODEMOGRÁFICOS

- Cual es su trabajo actual y que nivel de estudios ha completado

	Mare	Pare
Trabajo actual	☐	☐
Nivel de estudios	Primarios sin finalizar ☐ Primarios (ESO, EGB, ...) ☐ Secundarios (BUP, Bachillerato, FP, ...) ☐ Superiores (Universitarios) ☐	Primarios sin finalizar ☐ Primarios (ESO, EGB, ...) ☐ Secundarios (BUP, Bachillerato, FP, ...) ☐ Superiores (Universitarios) ☐ No aplicable (Familia monoparental) ☐

- Numero de personas que forman la unidad familiar _____

- Ingresos netos anuales totales en el hogar

Ejemplo, si la mujer tiene un sueldo de 20000 €, el hombre uno de 18000€ y hay un abuelo que vive con la familia y recibe una pensión de 6000 €

Menos de 9000 €	9000 € - 19000 €	19000 € - 25000 €	25000 € - 35000 €	Más de 35000 €
☐	☐	☐	☐	■

Menos de 9000 €	>9000 € - 19000 €	>19000 € - 25000 €	>25000 € - 35000 €	Más de 35000 €
☐	☐	☐	☐	☐

PESO Y ALTURA

- Peso (Kg) _____
- Altura (m) _____

Anote cualquier duda relacionada con esta encuesta:

NOM: DATA:

ENCUESTA 2 SOBRE HÁBITOS Y ESTILO DE VIDA

ANOTE LAS RESPUESTAS EN LOS ESPACIOS CORRESPONDIENTES A CADA PREGUNTA.

Estos datos servirán a la Universitat Rovira i Virgili para realizar un estudio comparativo entre diferentes poblaciones. En los resultados nunca aparecerá su nombre.

USO DE SUPLEMENTOS DE VITAMINAS / MINERALES

Por diferentes motivos, los suplementos de vitaminas y minerales recomendados no se toman siempre: por olvido, por sentimiento de que no son necesarios, por no encontrarse bien, porque dan molestias, etc. Por favor, conteste sinceramente estas preguntes para ayudarnos a valorar la realidad del seguimiento de los suplementos.

- ¿Ha tomado por iniciativa propia o recetado por un médico algún tipo de suplemento vitamínico / mineral?

  Nunca he tomado ☐ **Si he tomado** ☐

En el caso que sí, escriba el nombre del preparado e indique las veces a la semana que lo ha tomado. Rellene el cuadrado correspondiente a los meses del embarazo que lo ha tomado.

Ejemplo, una mujer que ha tomado la mayoría de los días FERPLEX durante los meses 6, 7, 8 y 9, escribiría:

Nombre del preparado	¿Cuántas veces a la semana?	Meses del embarazo 6	7	8	9
HIERRO	☐ Cada día	☐	☐	☐	☐
	■ La mayoría de los días (4-6 veces)	■	■	■	■
¿Cuál?: FERPLEX	☐ Algunos días (1-3 veces)	☐	☐	☐	☐

Nombre del preparado	¿Cuántas veces a la semana?	Meses del embarazo 6	7	8	9
HIERRO	☐ Cada día	☐	☐	☐	☐
	☐ La mayoría de los días (4-6 veces)	☐	☐	☐	☐
¿Cuál?:_____	☐ Algunos días (1-3 veces)	☐	☐	☐	☐
ÁCIDO FÓLICO	☐ Cada día	☐	☐	☐	☐
	☐ La mayoría de los días (4-6 veces)	☐	☐	☐	☐
¿Cuál?:_____	☐ Algunos días (1-3 veces)	☐	☐	☐	☐
MULTI-VITAMINAS	☐ Cada día	☐	☐	☐	☐
	☐ La mayoría de los días (4-6 veces)	☐	☐	☐	☐
¿Cuál?:_____	☐ Algunos días (1-3 veces)	☐	☐	☐	☐

- Si ha dejado de tomar el hierro, ¿cuál ha sido el motivo o motivos?

  ☐ Olvido
  ☐ No lo consideraba muy importante para la salud
  ☐ Le causaba molestias
  ☐ Otros (especificar) _____

DESAYUNO (durante el embarazo)

	Sí	No
¿Tiene la costumbre de desayunar?	☐	☐
¿Desayuna cereales inflados habitualmente (p.ej. tipo Kelloggs / Nestlé etc) ?	☐	☐
¿Toma café con cafeína?	☐	☐
¿Toma café descafeinado?	☐	☐

NOM: DATA:

TABACO

- ¿Es fumadora pasiva (expuesta al humo habitualmente en casa o en el trabajo)? **Sí** ☐ **No** ☐
- ¿Es fumadora activa? **Sí** ☐ **No** ☐

Sólo para fumadoras en los últimos 5 años

	0 cigs/día	1-5 cigs/día	6-10 cigs/día	> 10 cigs/día
Actualmente fumo	☐	☐	☐	☐
Fumaba durante los 12 meses antes del embarazo	☐	☐	☐	☐

	Antes de los 3 meses	Entre los 3 y los 6 meses	Después de los 6 meses
Ha dejado de fumar durante el embarazo durante los meses	☐	☐	☐

ALCOHOL

	Nunca / Ocasionalmente	< 3 copas / semana	Cada día como aperitivo y/o con las comidas	> 7 copas / semana
Actualmente bebe alcohol	☐	☐	☐	☐
En los 12 meses antes del embarazo	☐	☐	☐	☐

	Antes de los 3 meses	Entre los 3 y los 6 meses	Después de los 6 meses
Ha dejado de beber alcohol durante el embarazo	☐	☐	☐

- ¿Ha tomado algún otro tipo de sustancia tóxica (p.ej. marihuana, cocaína, etc...) en los últimos 5 años?
  Sí ☐ **No** ☐

En el caso de que sí haya tomado alguna substancia tóxica, especifique cuales:

	Ocasionalmente	Regularmente
Actualmente toma substancias tóxicas	☐	☐
En los 12 meses antes del embarazo tomaba substancias tóxicas	☐	☐

	Antes de los 3 meses	Entre los 3 y los 6 meses	Después de los 6 meses
Lo ha dejado durante el embarazo	☐	☐	☐

NOM: DATA:

ACTIVIDAD FÍSICA (durante el embarazo)

- ¿Qué actividad física hace en el trabajo, estudio o trabajo de casa?

  - Mi trabajo es básicamente de estar sentada y caminar poco (estudiante, docente, conductora de vehículos, dependienta, administrativa) ..... ☐

  - En mi trabajo ando bastante pero no hago ningún esfuerzo vigoroso (ama de casa, fábrica, agricultora, vendedora, cartera ...) ..... ☐

  - Mi trabajo es básicamente de mucha actividad física (deportista) ..... ☐

- ¿Qué actividad hace en el tiempo libre?

Si varia con la estación, escoger el grupo más representativo (sólo 1 grupo).

  - Lectura, televisión y actividades que no requieran actividad física importante..... ☐

  - Caminar, ir en bicicleta, jardinería (no se incluye el transporte de ir y volver del trabajo)..... ☐

  - Correr, esquiar, gimnástica, juegos de pelota o deportes vigorosos regularmente..... ☐

  - Entrenamiento deportivo regular para competición..... ☐

- ¿Cuántas veces por semana realiza las actividades referidas en la pregunta anterior? _____

- Durante los últimos 12 meses

	Nunca	Esporádicamente	Habitualmente
¿Ha tenido la costumbre de tomar el Sol?	☐	☐	☐

NOM: DATA:

DATOS SOCIODEMOGRÁFICOS (SOLO SI NO SE DISPONE DE LA ENCUESTA 1)

- Cual es su trabajo actual y que nivel de estudios ha completado

	Mare	Pare
Trabajo actual	☐	☐
Nivel de estudios	Primarios sin finalizar ☐ Primarios (ESO, EGB, ...) ☐ Secundarios (BUP, Bachillerato, FP, ...) ☐ Superiores (Universitarios) ☐	Primarios sin finalizar ☐ Primarios (ESO, EGB, ...) ☐ Secundarios (BUP, Bachillerato, FP, ...) ☐ Superiores (Universitarios) ☐ No aplicable (Familia monoparental) ☐

- Numero de personas que forman la unidad familiar _____

- Ingresos netos anuales totales en el hogar

Ejemplo, si la mujer tiene un sueldo de 20000 €, el hombre uno de 18000€ y hay un abuelo que vive con la familia y recibe una pensión de 6000 €

Menos de 9000 €	9000 € - 19000 €	19000 € - 25000 €	25000 € - 35000 €	Más de 35000 €
☐	☐	☐	☐	■

Menos de 9000 €	>9000 € - 19000 €	>19000 € - 25000 €	>25000 € - 35000 €	Más de 35000 €
☐	☐	☐	☐	☐

Anote cualquier duda relacionada con esta encuesta:

12 semanas

Estudio NUTCIR 1

Nombre..... *Fecha ....*

CUESTIONARIO DE FRECUENCIA DE CONSUMO ALIMENTARIO

INSTRUCCIONES PARA CONTESTAR

Procure contestar tranquilamente este cuestionario. Tómese el tiempo que considere necesario.

Este cuestionario le pregunta la frecuencia con que usted consume de forma **habitual** determinados alimentos.

La frecuencia de consumo se tiene que especificar en los recuadros de la derecha del listado de alimentos de este cuestionario. Para cada alimento del listado debe apuntar el **número de veces** que lo consume.

- Si lo consume **todos los días de la semana**, escriba un 7 en la columna **A LA SEMANA.**
- Si el consume **alguna vez a la semana,** escriba las veces: 1-2-3-4-5 o 6 en la columna **A LA SEMANA.**

  Piense siempre en sumar el consumo de todas las comidas del día (desayuno, almuerzo, merienda, cena, otros,…). Por ejemplo, si toma todos los días leche para desayunar y alguna vez a la semana para cenar: 7 + 4 = 11 veces a la semana.
- Si consume el alimento **alguna vez al mes**, escriba las veces: 1-2-3 etc… en la columna: **AL MES**
- Si no lo consume **nunca** o casi nunca, deje la casilla en blanco, sin escribir nada.

Ejemplo: Una mujer desayuna habitualmente un vaso de leche (7 veces) con magdalenas (7 veces), y para cenar a veces toma leche (4 veces) y a veces toma yogur (3 veces) de postres. Además, toma pescado algunas veces a la semana para almorzar (2 veces) y otras veces para cenar (4 veces). De legumbres consume alguna vez al mes (aproximadamente 4 veces). Si no consume nunca un alimento deje la casilla en blanco, sin contestar nada.
Este consumo lo apuntaría de la siguiente manera:

LISTADO DE ALIMENTOS	¿CUÁNTAS VECES COME…?	
	A LA SEMANA	AL MES
Leche	*11*	
Yogur	*3*	
Bizcocho, magdalenas, …	*7*	
…		
Pescado	*6*	
…		
Legumbres		*4*
…		
Queso de régimen		

CUESTIONARIO DE FRECUENCIA DE CONSUMO ALIMENTARIO

LISTADO DE ALIMENTOS	¿CUÁNTAS VECES COME...?	
	A LA SEMANA	AL MES
Leche		
Yogur		
Chocolate: tableta, bombones, "Kit-Kat", "Mars"…		
Cereales de desayuno ("Corn-Flakes" "Kellog's")		
Galletas tipo "maría"		
Galletas con chocolate, crema...		
Magdalenas, bizcocho ...		
Ensaimada, Donut, croissant...		

	A LA SEMANA	AL MES
Ensalada: lechuga, tomate, escarola…		
Judías verdes, acelgas, o espinacas		
Verduras de guarnición: berenjena, calabacín, champiñones…		
Patatas al horno, fritas o hervidas		
Legumbres: lentejas, garbanzos, judías blancas…		
Arroz blanco, paella		
Pasta: fideos, macarrones, espaguetis …		
Sopas y cremas		

	A LA SEMANA	AL MES
Huevos		
Pollo o pavo		
Ternera, cerdo, cordero (bistec, empanada…)		
Carne picada: longaniza, hamburguesa …		
Pescado blanco: merluza, mero…		
Pescado azul: sardinas, atún, salmón …		
Marisco: mejillones, gambas, langostinos, pulpo, calamares …		
Croquetas, empanadillas, pizza		
Pan (en bocadillos, en las comidas)		

	QUANTES VEGADES MENJA...?	
	A LA SEMANA	AL MES
Jamón, jamón dulce, embutidos		
Queso fresco (Burgos…) o bajo en calorías		
Quesos curados o semicurados, cremosos		

CUESTIONARIO DE PREFERENCIAS Y HÁBITOS ALIMENTARIOS

	A LA SEMANA	AL MES
Frutas cítricas: naranja, mandarina		
Otras frutas: manzana, pera, melocotón, albaricoque, plátano		
Frutas en conserva (en almíbar...)		
Zumos de fruta natural		
Zumos de fruta comercial		
Frutos secos: cacahuetes, avellanas, almendras		
Postres lácteos: natillas, flan, requesón		
Pasteles de crema o chocolate		
Bolsas de aperitivo ("chips", "cheetos", "fritos")		
Golosinas: gominolas, caramelos,…		
Helados		

	A LA SEMANA	AL MES
Bebidas azucaradas ("coca-cola", "Fanta")		
Bebidas bajas en calorías (coca-cola light…)		
Vino, sangría		
Cerveza		
Cerveza sin alcohol		
Bebidas destiladas (Whisky, ginebra, coñac…)		

Indique con una X la respuesta que usted desee señalar:

1.- ¿En la mesa, se añade sal a las comidas?

Nunca__ / Alguna vez__ / Frecuentemente__/ Casi siempre__

2.- ¿Cómo definiría su apetito? Mucho__ Bastante__ Normal __ Poco __ Ninguno__

3.- ¿Qué tipo de leche toma habitualmente?: Entera__ Semidesnatada__ Desnatada__

4.- ¿Qué tipo de yogur toma habitualmente?

a) Natural ___ b) Natural desnatado ___
c) De sabores ___ d) De sabores desnatado ___
e) Con trozos de fruta ___ f) Con trozos de frutas desnatado ___

5.- ¿Qué tipo de pan toma habitualmente?: Blanco __ Integral __

6.- ¿Unta el pan con tomate y aceite en los bocadillos?:

Siempre__ / Habitualmente__/ Alguna vez__/ Casi nunca_

Estudio NUTCIR 2

Nombre..... *Fecha ....*

CUESTIONARIO DE FRECUENCIA DE CONSUMO ALIMENTARIO

INSTRUCCIONES PARA CONTESTAR

Procure contestar tranquilamente este cuestionario. Tómese el tiempo que considere necesario.

Este cuestionario le pregunta la frecuencia con que usted consume de forma **habitual** determinados alimentos.

La frecuencia de consumo se tiene que especificar en los recuadros de la derecha del listado de alimentos de este cuestionario. Para cada alimento del listado debe apuntar el **número de veces** que lo consume.

- Si lo consume **todos los días de la semana**, escriba un 7 en la columna **A LA SEMANA.**
- Si el consume **alguna vez a la semana,** escriba las veces: 1-2-3-4-5 o 6 en la columna **A LA SEMANA.**

  Piense siempre en sumar el consumo de todas las comidas del día (desayuno, almuerzo, merienda, cena, otros,…). Por ejemplo, si toma todos los días leche para desayunar y alguna vez a la semana para cenar: 7 + 4 = 11 veces a la semana.
- Si consume el alimento **alguna vez al mes**, escriba las veces: 1-2-3 etc… en la columna: **AL MES**
- Si no lo consume **nunca** o casi nunca, deje la casilla en blanco, sin escribir nada.

Ejemplo: Una mujer desayuna habitualmente un vaso de leche (7 veces) con magdalenas (7 veces), y para cenar a veces toma leche (4 veces) y a veces toma yogur (3 veces) de postres. Además, toma pescado algunas veces a la semana para almorzar (2 veces) y otras veces para cenar (4 veces). De legumbres consume alguna vez al mes (aproximadamente 4 veces). Si no consume nunca un alimento deje la casilla en blanco, sin contestar nada.
Este consumo lo apuntaría de la siguiente manera:

LISTADO DE ALIMENTOS	¿CUÁNTAS VECES COME…?	
	A LA SEMANA	AL MES
Leche	11	
Yogur	3	
Bizcocho, madalenas, …	7	
…		
Pescado	6	
…		
Legumbre		4
…		
Queso de régimen		

CUESTIONARIO DE FRECUENCIA DE CONSUMO ALIMENTARIO

LISTADO DE ALIMENTOS	¿CUANTAS VECES COME...?	
	A LA SEMANA	AL MES
Leche		
Yogur		
Chocolate: tableta, bombones, "Kit-Kat", "Mars",…		
Cereales de desayuno ("Corn-Flakes", "Kellog's")		
Galletas tipo "maría"		
Galletas con chocolate, crema...		
Magdalenas, bizcocho ...		
Ensaimada, Donut, croissant...		

	A LA SEMANA	AL MES
Ensalada: lechuga, tomate, escarola…		
Judías verdes, acelgas, o espinacas		
Verduras de guarnición: berenjena, calabacín, champiñones…		
Patatas al horno, fritas o hervidas		
Legumbres: lentejas, garbanzos, judías blancas…		
Arroz blanco, paella		
Pasta: fideos, macarrones, espaguetis …		
Sopas y cremas		

	A LA SEMANA	AL MES
Huevos		
Pollo o pavo		
Ternera, cerdo, cordero (bistec, empanada…)		
Carne picada: longaniza, hamburguesa …		
Pescado blanco: merluza, mero…		
Pescado azul: sardinas, atún, salmón …		
Marisco: mejillones, gambas, langostinos, pulpo, calamares …		
Croquetas, empanadillas, pizza		
Pan (en bocadillos, en las comidas)		

CUESTIONARIO DE FRECUENCIA DE CONSUMO ALIMENTARIO

	¿CUÁNTAS VECES COME...?	
	A LA SEMANA	AL MES
Jamón, jamón dulce, embutidos		
Queso fresco (Burgos…) o bajo en calorías		
Quesos curados o semicurados, cremosos		

	A LA SEMANA	AL MES
Frutas cítricas: naranja, mandarina		
Otras frutas: manzana, pera, melocotón, albaricoque, plátano		
Frutas en conserva (en almíbar...)		
Zumos de fruta natural		
Zumos de fruta comercial		
Frutos secos: cacahuetes, avellanas, almendras		
Postres lácteos: natillas, flan, requesón		
Pasteles de crema o chocolate		
Bolsas de aperitivo (“chips”, “cheetos”, “fritos”)		
Golosinas: gominolas, caramelos,…		
Helados		

	A LA SEMANA	AL MES
Bebidas azucaradas (“coca-cola”, “Fanta”)		
Bebidas bajas en calorías (coca-cola light…)		
Vino, sangría		
Cerveza		
Cerveza sin alcohol		
Bebidas destiladas (Whisky, ginebra, coñac…)		

Indique con una X la respuesta que usted desee señalar:

1.- ¿En la mesa, se añade sal a las comidas?

Nunca__ / Alguna vez__ / Frecuentemente__/ Casi siempre__

2.- ¿Cómo definiría su apetito? Mucho__ Bastante__ Normal __ Poco __ Ninguno__

3.- ¿Qué tipo de leche toma habitualmente?: Entera__ Semidesnatada__ Desnatada__

4.-¿Qué tipo de yogur toma habitualmente?

a) Natural ___ b) Natural desnatado ___
c) De sabores ___ d) De sabores desnatado ___
e) Con trozos de fruta ___ f) Con trozos de frutas desnatado ___

5.- ¿Qué tipo de pan toma habitualmente?: Blanco __ Integral __

6.- ¿Unta el pan con tomate y aceite en los bocadillos?:

Siempre__ Habitualmente__ Alguna vez__ Casi nunca_

7.- ¿Cómo acostumbra a tomar el suplemento de hierro durante este embarazo? *(marque con una x)*

- ☐ No lo he tomado nunca
- ☐ Con agua
- ☐ Con zumo de naranja
- ☐ Con leche
- ☐ Otros (especificar)_____

8.- ¿Durante el embarazo ha tenido náuseas? ☐ Si ☐ No Y vómitos? ☐ Si ☐ No

En caso que sí haya sufrido vómitos durante el embarazo, especifique en qué meses:

☐ **1-3** ☐ **4** ☐ **5** ☐ **6** ☐ **7** ☐ **8**

¿Con qué frecuencia ha tenido estos vómitos?

- ☐ Regularmente
- ☐ De vez en cuando
- ☐ Muy pocas veces

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela

UNIVERSITAT ROVIRA I VIRGILI
PRENATAL ONE CARBON METABOLISM-GENE INTERACTIONS, PLACENTA TRACE ELEMENT CONTENT AND THEIR EFFECT ON PREGNANCY OUTCOMES
Jose Maria Colomina Muela