PRENATAL GENE-NUTRIENT INTERACTIONS AND THEIR RELATIONSHIP WITH DEVELOPMENT AND HEALTH IN THE OFFSPRING

Alejandra Rojas Gómez

UNIVERSITAT ROVIRA i VIRGILI

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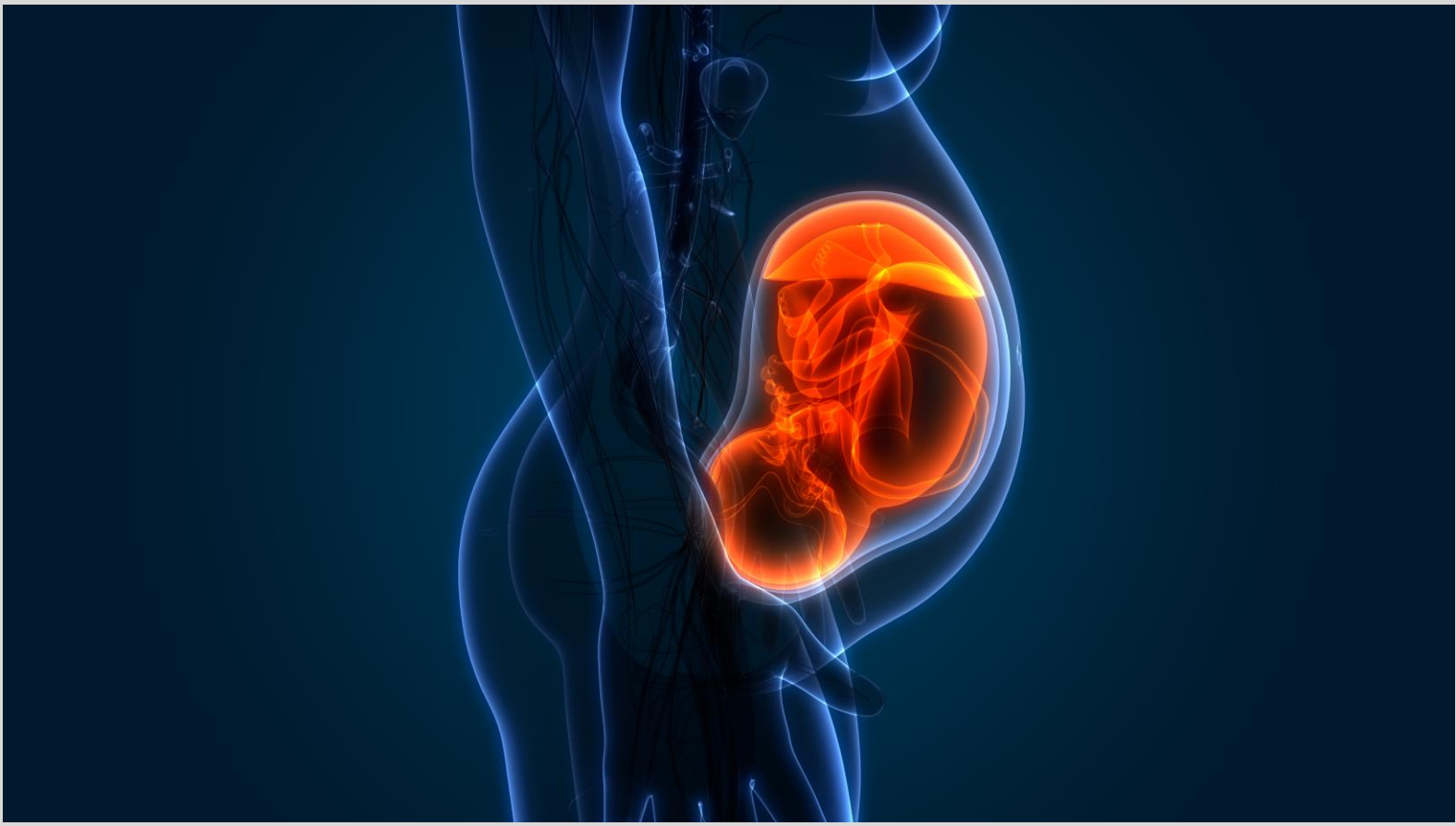

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Alejandra Rojas Gómez

Prenatal gene-nutrient interactions and their relationship with development and health in the offspring

Doctoral thesis

Thesis supervised by Dra. Michelle Murphy

Department of Basic Medical Sciences

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FAIG CONSTAR que aquest treball, titulat “Prenatal gene-nutrient interactions and their relationship with development and health in the offspring”, que presenta Alejandra Rojas Gómez per a l’obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament Ciències Mèdiques Bàsiques d’aquesta universitat.

HAGO CONSTAR que el presente trabajo, titulado “Prenatal gene-nutrient interactions and their relationship with development and health in the offspring”, que presenta Alejandra Rojas Gómez para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento Ciencias Médicas Básicas de esta universidad.

I STATE that the present study, entitled “Prenatal gene-nutrient interactions and their relationship with development and health in the offspring”, presented by Alejandra Rojas Gómez for the award of the degree of Doctor, has been carried out under my supervision at the Department Basic Medical Sciences of this university.

Reus, 31 de octubre de 2022
Reus, 31 de octubre de 2022
Reus, 31st October 2022

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Dra. Michelle Murphy

If I have seen further, it is by standing on the shoulders of giants.

Isaac Newton

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Abstract

Elevated pregnancy fasting plasma total homocysteine (tHcy) has been associated with foetal growth below the normal ranges and low birthweight has been linked to diseases in adulthood. In regions where cobalamin deficiency is frequent, low pregnancy cobalamin status has been associated with impaired glucose metabolism in the offspring during childhood. Cobalamin deficiency is less prevalent in European women and few studies outside Asia have investigated how pregnancy one-carbon (1C) metabolism status affects childhood metabolic and growth outcomes.

Less studied is the betaine - dimethylglycine pathway during pregnancy regarding foetal growth and infant health beyond birth.

The aims of the thesis are to investigate the effects of one carbon nutrient/metabolites during pregnancy on birthweight and mid-childhood metabolic outcomes.

METHODS: Associations between pregnancy tHcy, cobalamin status (plasma cobalamin, holotranscobalamin (holoTC), methylmalonic acid (MMA)), folate and mid-childhood metabolic score (MetSco) ((including fat mass index (zFMI), homeostatic model assessment of insulin resistance (zHOMA-IR) and dyslipidemia (zTG − zHDLc)/2) z-scores)) were investigated through multiple linear regressions in a prospective study of 293 mother–child dyads (from two cohorts: 213 from the Reus-Tarragona Birth Cohort (RTBC) and 80 from the Preconception Study Cohort (PreC).

Associations between pregnancy fasting plasma betaine, dimethylglycine (DMG)/betaine ratio and betaine - homocysteine S-methyltransferase (BHMT) c.716G>A polymorphism and foetal growth were investigated in a prospective study of 748 mother–neonate dyads (RTBC) through multiple linear and logistic regressions. Associations between pregnancy fasting plasma betaine, DMG/betaine ratio and mid-childhood MetSco were investigated in a prospective study of 213 mother–child dyads (RTBC) through multiple linear regressions.

Also, associations between pregnancy cobalamin and tHcy status with child plasma metabolite status were investigated.

Bivariate correlations between child nutrient/metabolites at 7.5 years and different anthropometric measurements in 238 RTBC children were studied to identify potentially relevant biomarkers for the metabolic syndrome.

RESULTS: Highest versus low–mid pregnancy tHcy tertile was associated with higher mid-childhood MetSco (1st trimester: B=0.418 standard error (SE)=0.189 p=<0.05. 3rd trimester B=0.435 SE=0.183, p=<0.05), specifically with higher child zFMI. Stratifying by sex, the maternal tHcy–child MetSco association was limited to boys and confirmed for zFMI and zHOMA-IR. The maternal tHcy-child zFMI association was not mediated by birthweight z-score. 1st trimester plasma cobalamin was not associated with child outcomes, but other indicators of cobalamin status were. Lowest versus mid–high plasma holoTC tertile was associated with MetSco (1st trimester B=0.897, SE=0.328, p=<0.01) (specifically zFMI and zHOMA-IR) and highest versus low–mid plasma MMA tertile was associated with higher MetSco (B=0.529, SE=0.241, p<0.05) and dyslipidemia in boys.

Pregnancy plasma betaine at 12 gestational weeks (GW) was not associated with birthweight or small for gestational age (SGA). At 34 GW, plasma betaine was inversely associated with birthweight and increased risk of SGA in the crude model, but lost significance in the adjusted models. Cord plasma betaine was inversely associated with birthweight (B=-7.76 SE=2.60, p=0.003) and increased risk of SGA (OR=1.08, CI=1.02; 1.15). Stratifying by sex, the increased risk was only observed in boys. When betaine-birthweight analyses were repeated stratifying by median plasma folate, we now observed that the inverse association between cord betaine and birthweight was significant only when plasma folate was above the median.

Plasma DMG/betaine at 12 GW and in the cord were positively associated with birthweight (B=656.5, SE=292.2, p=0.025; B=1153.5, SE=281.2, p=<0.001 respectively). Stratifying by sex, the cord association was only significant in boys.

The risk of SGA was higher in babies born to mothers with the homozygote variant *BHMT* c.716 AA genotype compared with the GG genotype (OR=4.02, CI=1.20; 13.52) or the GA + GG genotypes (OR=3.50; CI=1.17; 10.49). Also, each copy of A increased the risk of SGA by

an additive amount (OR=1.87 CI=1.00; 3.49). The polymorphism in the baby was not associated with risk of SGA.

Neither pregnancy plasma betaine nor DMG/betaine ratio were associated with child MetSco.

Child plasma betaine at 7.5 years was highest in the lowest vs mid-high maternal pregnancy plasma cobalamin tertile, while tryptophan at 7.5 years was lowest in the highest vs low-mid maternal pregnancy plasma tHcy tertile.

Regarding the association between child biomarkers and adiposity measures, plasma betaine, total cysteine, 2 metabolites produced during tryptophan catabolism (kynurenic acid, 3-hydroxyanthranilic acid (HAA)) as well as histidine and asymmetric dimethylarginine (ADMA) were positively correlated with adiposity. On the other hand, plasma picolinic acid (tryptophan catabolism), trigonelline and neopterin concentrations were negatively correlated with adiposity in the children.

CONCLUSIONS: Moderately elevated pregnancy tHcy and low cobalamin status were associated with mid-childhood metabolic score in boys. The pregnancy tHcy–child zFMI association was not mediated by birthweight.

Child plasma betaine was highest in the lowest vs mid-high pregnancy plasma cobalamin tertiles, while tryptophan at 7.5 years was lowest in the highest vs low-mid pregnancy plasma tHcy tertiles.

Cord plasma betaine was inversely associated with foetal growth. There was also a positive association between cord plasma DMG/betaine ratio and birthweight.

Babies born to mothers with the *BHMT* c.716 AA genotype have a higher risk of SGA than GG and than GA+GG genotypes. Also, each copy of A increased the risk of SGA in an additive fashion.

Neither pregnancy maternal betaine nor DMG/betaine ratio were associated with MetSco in the child.

Correlation of child plasma metabolites with adiposity measures were found for betaine, cysteine, three metabolites from the kynurenine pathway, histidine, trigonelline, ADMA and neopterin.

Keywords: pregnancy - cobalamin - betaine - dimethylglycine - homocysteine - birth weight - child - metabolic syndrome - adiposity - *BHMT* c.716 G>A polymorphism - developmental origins of health and disease - *in utero* programming - mother-child relations

PREGNANCY COBALAMIN STATUS, HOMOCYSTEINE, FOLATE AND MID CHILDHOOD OUTCOMES.		
What is known	**What is unknown**	**What this thesis adds**
Inadequate pregnancy cobalamin status has been associated with adverse offspring metabolic health in studies from India (Yajnik et al., 2008) and Nepal (Stewart et al., 2011).	Whether pregnancy cobalamin status is associated with adverse mid-childhood health outcomes in countries with low prevalence of cobalamin deficiency.	New evidence that impaired one carbon metabolism during pregnancy is associated with negative health outcomes in the offspring, in a population with low prevalence of cobalamin deficiency.
		tHcy during pregnancy and low cobalamin status during early pregnancy are associated with mid-childhood metabolic score and its components (fat mass, insulin resistance and dyslipidemia) in the offspring. Stratification by sex showed that these findings were limited to male offspring.
		The maternal-offspring associations were observed in the functional markers of cobalamin status (holotranscobalamin and methylmalonic acid) and tHcy, but not with the routine clinical measurement of cobalamin status, plasma cobalamin concentration.

BETAINE AND THE DIMETHYLGLYCINE PATHWAY. Effect on foetal growth and mid childhood outcomes.		
What is known	**What is unknown**	**What this thesis adds**
The association between pregnancy betaine status and birthweight is unclear. Previously, no association was reported in Dutch studies (Hogeveen et al., 2013; Moltó-Puigmartí et al., 2021), while Asian studies reported an inverse association (Du et al., 2019; L. van Lee et al., 2016) and one of them reported it in males only (Du et al., 2019).	Whether or not the association occurs in other populations and whether early pregnancy plasma betaine status is associated with birthweight.	We did not observe an association between 1st trimester plasma betaine and birthweight. 3rd trimester plasma betaine was inversely associated with birthweight in a crude model but the significance of the association was lost in the adjusted models.
Cord plasma betaine was reported to be inversely associated with birthweight in a Dutch study (Hogeveen et al., 2013).	Whether cord betaine is associated with birthweight in other populations. Whether the cord plasma DMG/betaine ratio is associated with birthweight.	Cord plasma betaine was inversely associated with birthweight and associated with increased risk of small for gestational age (SGA). The cord DMG/betaine ratio was positively associated with birthweight. Stratifying by sex showed that the increased risk of SGA and the DMG/betaine association with birthweight were only observed in boys.
The *BHMT* c.716G>A polymorphism has been associated with pregnancy complications including placental abruption, neural tube defects, orofacial clefts and Down syndrome, although not all studies are in agreement.	No previous study has investigated the association between the *BHMT* c.716 G>A polymorphism and risk of SGA.	Maternal AA genotype compared to GG (codominant model) and compared to GG+GA (recessive model) was associated with increased risk of SGA. Also, each copy of the A allele additively increased the risk of SGA. Cord genotype was not associated with risk of SGA.
Third trimester plasma betaine has been reported to be associated with being overweight in the 6-8-year-old male offspring (Moltó-Puigmartí et al., 2021). In contrast, breast milk betaine was negatively correlated with infant growth at 12 months in two different cohorts (Ribo et al., 2021).	Whether these associations are replicated in other populations and with other outcomes in the offspring.	Neither pregnancy or cord plasma betaine or the DMG/betaine ratio were associated with MetSco during infancy. Pregnancy betaine was positively associated with child zFMI but the association was not significant.

BETAINE AND DIMETHYLGLYCINE PATHWAY. Child plasma betaine and other metabolites: correlations with adiposity and other anthropometric measurements.		
What is known	**What is unknown**	**What this thesis adds**
Plasma total cysteine has been associated with obesity in children (da Silva et al., 2013), adolescents (Elshorbagy et al., 2012) and adults (Elshorbagy et al., 2009).	Whether plasma betaine and related 1C metabolites are associated with different adiposity measures in 7.5-year-old children.	Child total plasma cysteine was associated with 3 out of 5 measurements of adiposity (waist circumference, waist/height ratio and percentage fat mass). Child plasma betaine was positively correlated with all 5 adiposity measures, and the betaine-adiposity associations were stronger than those for cysteine, especially in boys. Child plasma choline was associated with only one adiposity measure. Child plasma methionine, folate (as well as red blood cell folate), cobalamin and methylmalonic acid were not associated with any adiposity measurement.
	Whether plasma betaine is associated with anthropometric measurements other than fat mass in 7.5-year-old children.	Child plasma betaine was positively correlated with measurements of muscle mass, circumferences (head, arm, trunk), and height. Stratifying by sex, the correlations were significant in girls. In boys the only significant correlation was with arm circumference. Plasma choline was correlated with the 4 measurements. Stratifying by sex, the correlations were significant in girls only. Cysteine was only weakly associated with arm circumference in girls and boys together.

IDENTIFICATION OF METABOLITES ASSOCIATED WITH ADIPOSITY IN 7.5 YEAR-OLD CHILDREN.		
What is known	**What is unknown**	**What this thesis adds**
Biomarkers (e.g. adipokines, inflammatory molecules etc) have been correlated with adipose tissue. Recently, the kynurenine pathway has been proposed to link obesity and inflammation and there are few studies on this pathway in children (Barat et al., 2016; Butte et al., 2015; Lischka et al., 2022; Mangge et al., 2014; Tan, Tint, Kothandaraman, Yap, et al., 2022).	New biomarkers associated with adiposity in childhood.	An exploration of the association between 20 child plasma metabolites and 5 different adiposity measurements showed that 7 were correlated ($p<0.1$) with at least 3 adiposity measures. Three of them (3-hydroxyanthranilic acid (HAA), kynurenic acid and picolinic acid) are from the kynurenine pathway. HAA and kynurenic acid were positively correlated, while picolinic acid was inversely correlated with adiposity measures. Another 2 plasma metabolites that positively correlated with adiposity measures were: histidine (anti-inflammatory properties) and asymmetric dimethylarginine (ADMA) (associated with endothelial dysfunction). Two other metabolites were inversely correlated with adiposity measures: trigonelline (a type of betaine often used as a marker of coffee consumption) and neopterin (reflects cellular immune activation).

Abbreviations

1C	One carbon
ADMA	Asymmetric dimethylarginine
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
BHMT	Betaine-homocysteine S-methyltransferase
BMI	Body mass index
CI	Confidence interval
DHF	Dihydrofolate
FMI	Fat mass index
GW	Gestational weeks
Hcy	Homocysteine
HDLc	High density lipoprotein cholesterol
HoloTC	Holotranscobalamin
HOMA-IR	Homeostatic model assessment of insulin resistance
LDLc	Low density lipoprotein cholesterol
MetSco	Metabolic score
MMA	Methylmalonic acid
RBCF	Red blood cell folate
RTBC	Reus-Tarragona Birth Cohort
SAM	S-adenosylmethionine
SGA	Small for gestational age
SNP	Single nucleotide polymorphism
TG	Triglycerides
tHcy	Fasting plasma total homocysteine

1. Introduction

There is increasing evidence that research into healthy ageing needs to start with early life because plasticity is more prominent during cell differentiation. This period is sensitive to factors such as nutrient availability, among other exposures (Barouki et al., 2012). Most studies of the chronic diseases that have increased in prevalence in recent decades do not begin until adulthood, when the disease is already manifested. To understand the development of biological lesions, present for years and long before the symptoms of the disease appear, research focusing on the early stages of development is required. Studying the Developmental Origins of Health and Disease (DOHaD) can lead to new strategies for research and disease prevention (Barouki et al., 2012).

Animal studies have shown the importance of the one carbon metabolic network in epigenetic regulation and in establishing and maintaining an optimal state of health (Sinclair et al., 2007). Interaction between prenatal and postnatal exposures to variations in nutrient supply have been shown to be associated with pathophysiological anomalies in the offspring in both animal and human studies (Sebert et al., 2011). Severe intrauterine nutritional deprivation leads to metabolic adaptations to save energy and facilitate the growth of the developing foetus (Franks et al., 2010; Gardner et al., 2005). However, this scenario programmed as an adaptive response to limited access to nutrients *in utero* seems likely to be altered in the presence of nutrient abundance after birth. Generalised alterations of metabolism caused by intrauterine growth restrictions subsequently lead to morbidity when there is an accelerated postnatal recovery of growth ("catch up") (Symonds et al., 2009). Human studies have shown that limited intrauterine growth resulting from maternal malnutrition during pregnancy, leads to increased risk of metabolic syndrome in offspring (Barker et al., 2005; Robinson et al., 2006; Yajnik et al., 1995).

1.1. One carbon metabolism: general view

One carbon (1C) metabolism, involves the intracellular transfer of 1C units and is summarised in **Figure 1**. This metabolic network supports multiple processes including the biosynthesis of purines and thymidine, amino acid homeostasis (glycine, serine, methionine and homocysteine), epigenetic support (through S-adenosyl-methionine (SAM), the second most common enzymatic cofactor after ATP), and redox defence (glutathione synthesis) (Ducker & Rabinowitz, 2017).

There are different 1C units: methyl (-CH3), methylene (-CH2-), methenyl (-CH=), formyl (-CHO) and formimino (HN=CH-) (Shane, 2010) and folate molecules function as carriers for these. The active form of folate is tetrahydrofolate (THF) and almost all natural folate in the diet and in the human body is present in the reduced form, 5-methyl-THF. Folic acid, a synthetic food additive, has to be reduced to dihydrofolate (DHF) and then to THF to enter the folate cycle (Ducker & Rabinowitz, 2017). Other 1C unit donors include formate, histidine, serine and glycine, and methyl-glycine species (sarcosine and dimethylglycine that are derived from the choline oxidation pathway) (Ducker & Rabinowitz, 2017).

Most plants, bacteria and yeast can synthesise folate and are a good source for animals that require dietary folate intake (Gorelova et al., 2019). In adults, insufficient dietary folate leads to anaemia (Shane, 2010) and during periconception it increases the risk of pregnancies affected by neural tube defects (NTD) (MRC Vitamin Study Research Group, 1991).

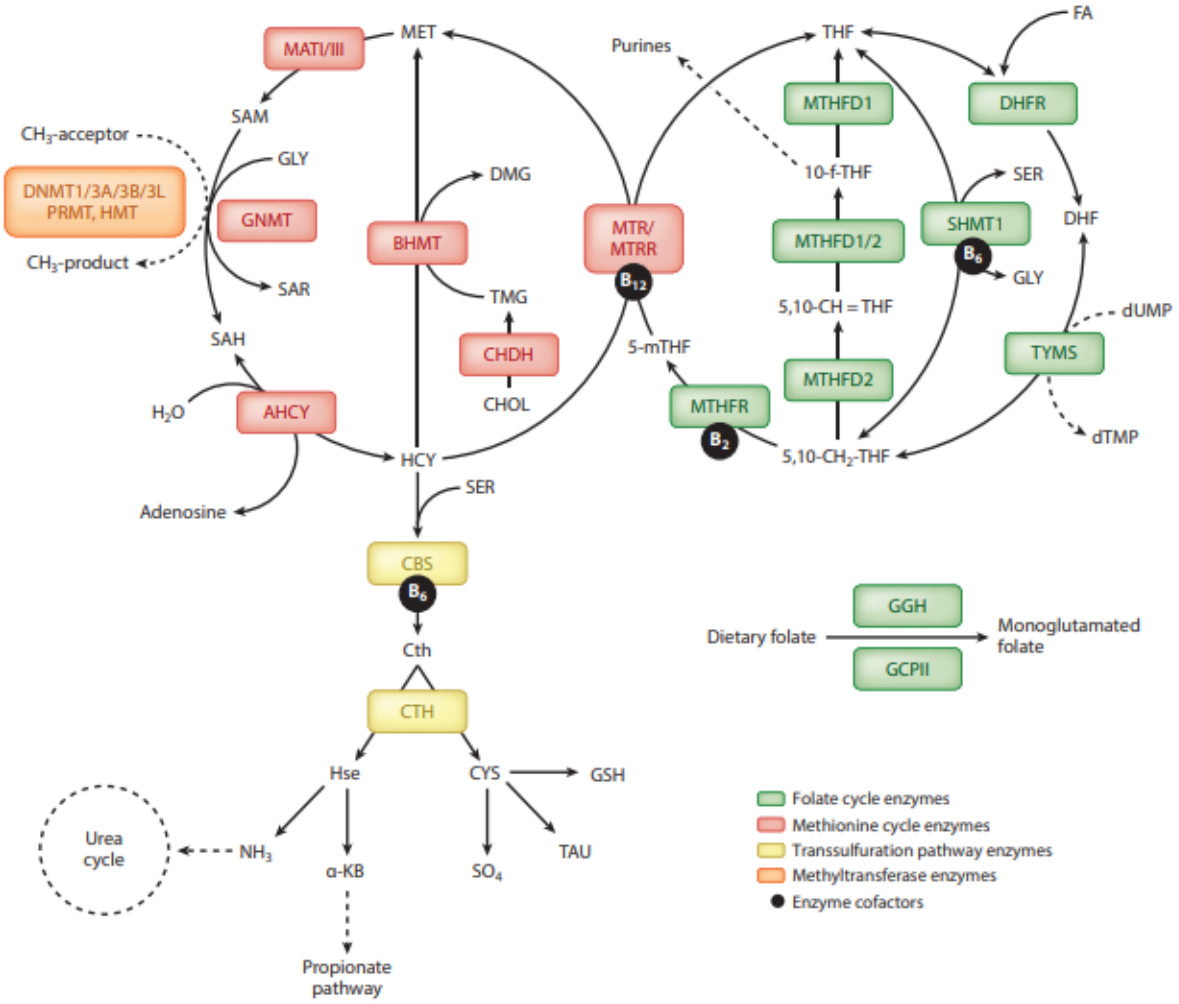


Figure 1. One-carbon (1C) metabolic pathways.

Folate cycle enzymes (green boxes): DHFR, dihydrofolate reductase; GCPII, glutamate carboxypeptidase; GGH, γ-glutamyl hydrolase; MTHFD1/2, methylenetetrahydrofolate dehydrogenase; MTHFR, 5,10-methylenetetrahydrofolate reductase; SHMT, serine hydroxymethyltransferase; TYMS, thymidylate synthase. Methionine cycle enzymes (red boxes): AHCY; S-adenosyl-L-homocysteine hydrolase; BHMT, betaine-homocysteine S-methyltransferase; CHDH, choline dehydrogenase; GNMT, glycine N-methyltransferase; MATI/III, methionine adenosyltransferase; MTR, methionine synthase; MTRR, methionine synthase reductase. Transsulfuration pathway enzymes (yellow boxes): CBS, cystathionine β-synthase; CTH, cystathionine γ-lyase. Key methyltransferase enzymes (orange box): DNMT1/3A/3B/3L, de novo and maintenance DNA methyltransferases; HMT, histone methyltransferase; PRMT, protein arginine methyltransferase. Enzyme cofactors (black circles): vitamin B2, B6, and B12. Substrates: 5,10-CH = THF, 5,10-methenyl-tetrahydrofolate; 5,10-CH2-THF, 5,10-methylenetetrahydrofolate; 5-mTHF, 5-methyltetrahydrofolate; 10-f-THF, 10-formyl-tetrahydrofolate; α-KB, α-ketobutyrate; CHOL, choline; Cth, cystathionine; CYS, cysteine; DHF, dihydrofolate; DMG, dimethylglycine; dTMP, thymidine monophosphate; dUMP, deoxyuridine monophosphate; FA, folic acid; GLY, glycine; GSH, glutathione; HCY, homocysteine; Hse, homoserine; MET, methionine; NH3, ammonia; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SAR, sarcosine; SER, serine; SO4, sulphate; TAU, taurine; THF, tetrahydrofolate; TMG, trimethylglycine/betaine. From (Clare et al., 2019)

1.1.1. Differences between males and females in one-carbon metabolism

Men and women of child-bearing age differ in some 1C metabolites. For example, men have higher tHcy due to their higher muscle mass and creatinine, and the difference is also explained in part by the influence of oestrogen in women (Dierkes et al., 2001).

A mathematical model suggests that the expression of 5 key enzymes in hepatic one-carbon metabolism are different between males and females: betaine-homocysteine S-methyltransferase (BHMT) and MTHFR are downregulated in females and methionine synthase (MS), serine hydroxymethyltransferase (SHMT), and phosphatidylethanolamine-N-methyltransferase (PEMT) are upregulated (Sadre-Marandi et al., 2018).

One of the nutrients in which hormonal influence has been detected is choline. It is obtained from the diet and also endogenously in small quantities from the biosynthesis of phosphatidylcholine, which is catalysed by PEMT. In humans and mice, primary hepatocyte PEMT is induced by oestrogen (Resseguie et al., 2007). This enzyme does not function equally in all the population. Deprivation of dietary choline led to signs of subclinical organ dysfunction (fatty liver or muscle damage) in 77% of the men, 80% of the postmenopausal women and less than half of premenopausal women. The damage was reversed when they consumed a high-choline diet (Fischer et al., 2010). Genetics also appear to contribute to the differences in dietary needs for choline. Women with the variant allele of the rs12325817 polymorphism have a higher dietary choline requirement (Fischer et al., 2010).

The differences in one-carbon metabolism between men and women are larger during pregnancy. Taking choline as an example, its plasma or serum concentrations are higher in pregnant women, compared to nonpregnant women and are around 7-fold higher in the foetus and newborn than they are in adults. In women, maternal-foetal choline transport can lead to decrease of maternal plasma choline (Zeisel & da Costa, 2009).

People vary greatly from one another in how they utilise and metabolise nutrients. More knowledge regarding the role of sex in these differences is required. In the case of the PEMT enzyme, the mechanism is better understood, but this is not the case for other enzymes.

1.1.2. Folate

1.1.2.1. Historical background

In **Table 1** we summarise some important events of the history of folate (Hoffbrand & Weir, 2001). The discovery of its function started when Dr. Lucy Wills arrived in India in 1928 to investigate tropical macrocytic anaemia in pregnancy. She found how pregnancy-related anaemia can be treated with Marmite (folate-rich yeast extract) and provided the first evidence of the role of folate for maternal health (Wills, 1931). Other important contributions to the folate knowledge were done by Victor Herbert, challenging previous recommendations not to use folic acid in any macrocytic anaemia. He experimented on himself by consuming a triple-boiled diet for 20 weeks and induced folate deficiency (Stabler, 2010). Later, Herbert defined the relationship between folate and cobalamin, which he termed the methylfolate trap hypothesis. This hypothesis explains how folic acid/folate temporarily overcomes a deficit of cobalamin (Lichtman & Spivak, 2000). Shortly after Herbert's experiments, Bryan M. Hibbard was the first to report an association between folate deficiency not only for maternal health during pregnancy but with foetal malformation (Hibbard, 1964).

Table 1. The discovery of folic acid. Adapted from (Hoffbrand & Weir, 2001).

1931	Wills	Yeast (Marmite) prevents macrocytic anaemia of pregnancy
1938	Wills & Evans	Highly purified liver extracts that are an effective treatment for pernicious anaemia, do not correct tropical macrocytic anaemia. Crude liver or autolysed yeast extracts should be used for the treatment of tropical macrocytic anaemia.
1941	Mitchell *et al.*	"Folic acid" gets its name.
1945	Angier *et al.*	Synthesis of folic acid and called pteroylglutamic acid.
1947	Editorial	*The New England Journal of Medicine* recommended folic acid not to be employed in any macrocytic anaemia.
1962	Herbert	Victor Herbert shows that pure nutritional deficiency of folate was present in humans, challenging 1947 recommendations.
1964	Hibbard	First report of an association between folate deficiency and foetal health (abruptio placentae, foetal malformation).

1.1.2.2. Structure, characteristics and functions

All natural folates are reduced forms of pteroylmonoglutamate while "folic acid" is used to refer to the most oxidised form found in supplements and fortified foods or when dietary folates are oxidised (see **Figure 2**). Folic acid is generally more stable than reduced folates whose stability depends on the one-carbon substitution (Shane, 2010). Folic acid first needs to be reduced to dihydrofolate (DHF) and then to tetrahydrofolate (THF) before it can enter the folate cycle. The latter are the biologically active reduced species. In humans, almost all natural folate in the diet and in the body are in the form of 5-methyl-THF (Ducker & Rabinowitz, 2017; Wright et al., 2007).

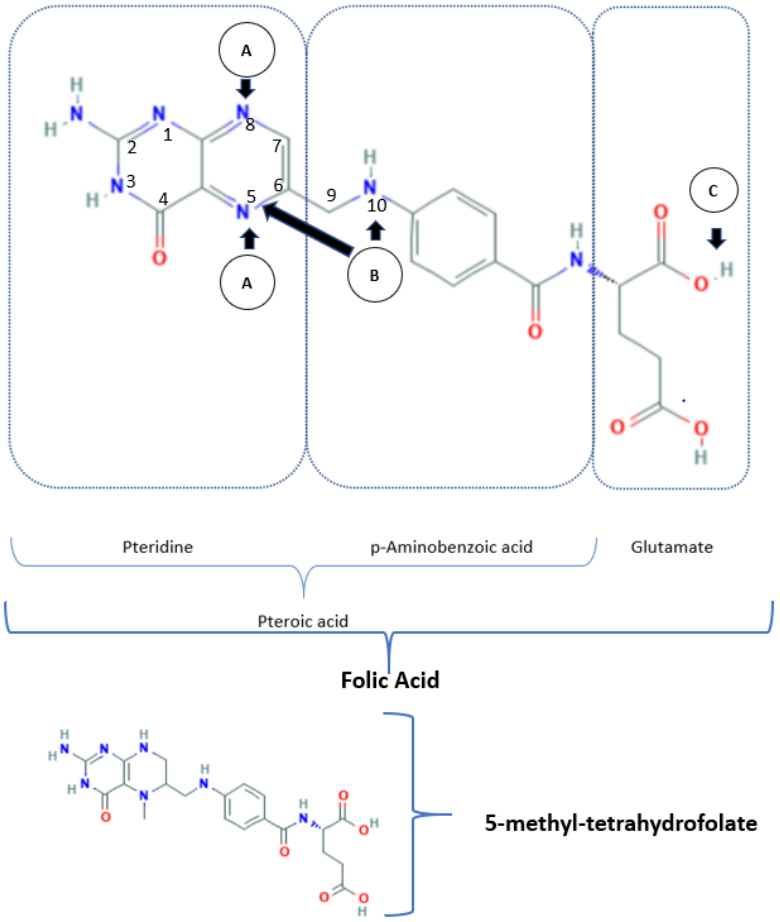


Figure 2. Folic acid and 5-methyl-THF structure.

Atom positions are shown as numbers. Folic acid modifications: A. reduction of the N-8 and N-5 of the pterin moiety B. acquisition and oxidation or reduction of 1C units at N-5 and/or N-10 position C. Elongation of the glutamate chain via gamma-peptide linkage. Adapted from (National Center for Biotechnology Information., 2020)

Evidence for folate functions throughout the lifecycle was reviewed by (McNulty, Pentieva, et al., 2012) and is replicated in **Table 2**.

Table 2. Role of folate throughout the lifecycle (McNulty, Pentieva, et al., 2012).

Role	**Evidence**
Prevention of maternal folate deficiency (anaemia) during pregnancy	Conclusive
Foetal development	Conclusive
Cognitive health in childhood	Early evidence
Prevention of heart disease/stroke	Convincing
Cancer prevention	Promising
Bone health	Possible
Cognitive function in ageing	Possible

1.1.2.3. Sources, bioavailability and requirements

Folates are essential for all organisms, except methanogenic and sulphate-reducing archaea (Gorelova et al., 2019). Most bacteria, yeast, and plants can synthesise folates de novo but animals require it from the diet (Ducker & Rabinowitz, 2017).

Body folate stores last around 2-3 months, and deficiency can arise at any age (Bailey et al., 2015). Bioavailability depends on intestinal, sex and genetic factors, among others (McNulty & Pentieva, 2010).

In Spain, a study of 996 females and 1013 males aged 9–75 years, found that the highest contribution to total folate intakes was vegetables (21.7–24.9%) and cereals (10.7–11.2%). The total median folate intake (156.3 µg/d in women and 163.6 µg/d in men) was below the recommended daily intakes for the Spanish population (Moreiras et al., 2015). In general, few participants had adequate folate intakes (women 3.0% and men 6.6%) opposite to the B12 adequacy (women 93.4% and men 96.6%). Adequacy was defined as the percentage of population above 80% of the recommended dietary intakes (Partearroyo et al., 2017).

1.1.2.4. Folate status and its determinants

World Health Organization (WHO) guidelines regarding red blood cell folate cut-off for preventing neural tube defects (World Health Organization, 2015) are shown in **Table 3**.

Table 3. Folate concentrations in red blood cells for preventing neural tube defect-affected pregnancies in women of reproductive age at the population level.[a]

Red blood cell folate, ng/mL (nmol/L).[b]	**Interpretation**
> 400 (>906)	Folate sufficiency
< 400 (<906)	Folate insufficiency

[a] These thresholds should not be used at an individual level for determination of risk of a neural tube defect-affected pregnancy.
[b] Folic acid conversion factor: 1 ng/mL = 2.265 nmol/L.

The World Health Organization in 2015 did not recommend a serum folate threshold for the prevention of neural tube defects in women of reproductive age at the population level. However, some researchers later in 2019 proposed that a threshold of 25.5 nmol/L for optimal NTD prevention may be appropriate in populations without vitamin B-12 insufficient status. They found that the relationship between RBCF and plasma folate concentrations was modified by BMI and genotype and substantially by low plasma vitamin B12 (M.-Y. Chen et al., 2019).

1.1.2.4.1. MTHFR

The 5,10-methylenetetrahydrofolate reductase (*MTHFR)* gene encodes a protein that converts 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate that is a methyl donor needed for the conversion of homocysteine to methionine (Goyette et al., 1996). MTHFR enzyme is dependent on the coenzyme flavin adenine dinucleotide (FAD) in which riboflavin (vitamin B2) is an integral component. The electron donor of the MTHFR is nicotinamide adenine dinucleotide phosphate (NADPH). The MTHFR enzyme is strongly inhibited by S-adenosylmethionine (SAM) and this is partially reversed by S-adenosylhomocysteine (Kutzbach & Stokstad, 1971).

A common polymorphism in this gene called C677T also known as Ala222Val (rs1801133) and A222V, in the cytogenetic location 1p36.22 (National Center for Biotechnology Information, n.d.), changes a highly-conserved alanine for a valine. It correlates with a reduction in enzyme activity and increase in thermolability in lymphocyte extracts. Also, the polymorphism is associated with higher homocysteine concentrations (Frosst et al., 1995).

1.1.2.5. Folate and pregnancy

The original evidence that folate deficiency, apart from being important for maternal health (megaloblastic anaemia), could also cause NTDs was proposed by Brian Hibbard, who demonstrated an association between folate deficiency and congenital malformation and drew attention to the fact that the role of folic acid on the mother attracted the interest of many researchers but less attention was paid to the possible effects on the foetus and its placenta (Hibbard, 1964). Later other studies (Smithells et al., 1976) found that women with megaloblastic anaemia during pregnancy had a high incidence of NTDs. Afterwards, some of the main clinical trials that led to the mandatory fortification policies were done in the United Kingdom using 360 ug/d folic acid in a multivitamin (Smithells et al., 1980), other study with population from United Kingdom, Hungary, Israel, Australia, Canadá, USSR and France using 4000 ug/day folic acid alone or in combination with other vitamins (MRC Vitamin Study Research Group, 1991) and one in Hungary using 800 ug/day in a multivitamin (Czeizel & Dudás, 1992). All these studies led to mandatory folic acid fortification of staple food that was fully implemented by January 1998 in the USA (U.S. Food and Drug Administration (FDA)., 1996) and November of the same year in Canada (J. G. Ray, 2004). The objective of the fortification was the prevention of neural tube defects. There are many other countries that have implemented mandatory folic acid fortification as shown in **Figure 3**.

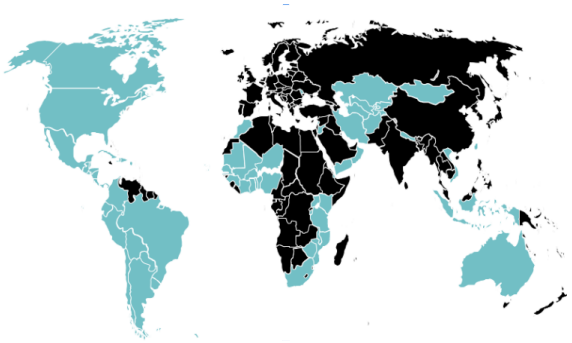


Figure 3. Countries with mandatory folic acid policy are shown in blue, without in black. Source: FFI (Food Fortification Initiative, September 2020).

A lower dose than most of the previous clinical trials is used as current recommendations for women capable of becoming pregnant to prevent NTD's: 400 ug/d of folic acid from supplements or fortified food in addition to consuming food folate from a varied diet (Institute of Medicine et al., 1998). This is the same concentration used in a Chinese trial of 247,831 women that evaluated the outcomes of pregnancy in women who were asked to take a supplement containing 400 ug/d from the time of their premarital examination until the end of their 1st trimester of pregnancy vs. women who did not take folic acid. There was an 85 % reduction in risk of NTD's in the Northern region of China and 40 % reduction in the Southern region (Berry et al., 1999).

The World Health Organization recommends taking 5 mg/d in pregnancies with a high risk of neural tube defects. The United Kingdom has the same recommendation and in the United States the recommendation is 4 mg/d (Gomes et al., 2016).

Similarly in Spain, the Ministry of Health, Social Policy and Equality recommends woman planning a pregnancy to take 400 ug/d of folic acid (López et al., 2010) from at least one month before conception, and throughout the first three months of pregnancy (Departament de Salut, Generalitat de Catalunya, 2018). Women with a history of a previous child with a neural tube defect, taking anticonvulsants or being treated with folic acid antagonists should take at least 4 mg/day. However, in Spain there is no preparation with this dose, the closest one contains 5 mg/tablet and is the current recommendation (López et al., 2010).

1.1.3. Cobalamin

1.1.3.1. Historical background

The discovery of this vitamin, also known as vitamin B12, and the treatment of its deficiency started in the XIX century. The vitamin was isolated by two groups simultaneously and was crystallised and characterised in the laboratory of Dorothy Hodgkin, contributing to her Nobel Prize in 1964 (Scott & Molloy, 2012). In a review by Scott and Molloy they mention some key events such as the discovery of liver consumption as a treatment of pernicious anaemia. However, the disease was not due to a poor diet but due to the lack of an 'intrinsic factor' of the stomach that was a key component of the system, a breakthrough made by Castle in 1926 (Wadsworth, 1988).

1.1.3.2. Structure, characteristics and functions

Cobalamin is a general term that refers to a group of compounds that contain cobalt (corrinoids) with a specific structure that has ribose, phosphate and a base (see structure in **Figure 4**). Vitamin B12, a term that is usually restricted to cyanocobalamin (a commercial form used in supplements), can be converted to two active forms in humans: methylcobalamin and 5-deoxyadenosylcobalamin. Other authors use cobalamin and vitamin B12 as synonyms and throughout this thesis will be used in the same way (Green et al., 2017). This vitamin is water soluble and needs an intrinsic factor (a glycoprotein that is secreted by the parietal cells of the stomach after food consumption) for its absorption in the intestine. It is a coenzyme for two reactions, in the first as methylcobalamin in a methyl transfer reaction that transforms homocysteine into methionine and in a second unrelated reaction as 5-deoxyadenosylcobalamin that converts l-methylmalonyl-coenzyme A (CoA) to succinyl-CoA. Its cellular functions are linked to those of folate. It is involved in hematopoiesis, neural metabolism, nucleotides synthesis and carbohydrate, fat and protein metabolism. Vitamin B12 deficiency leads to pernicious anaemia, megaloblastic anaemia and neurologic problems (Institute of Medicine et al., 1998; National Center for Biotechnology Information., 2020)

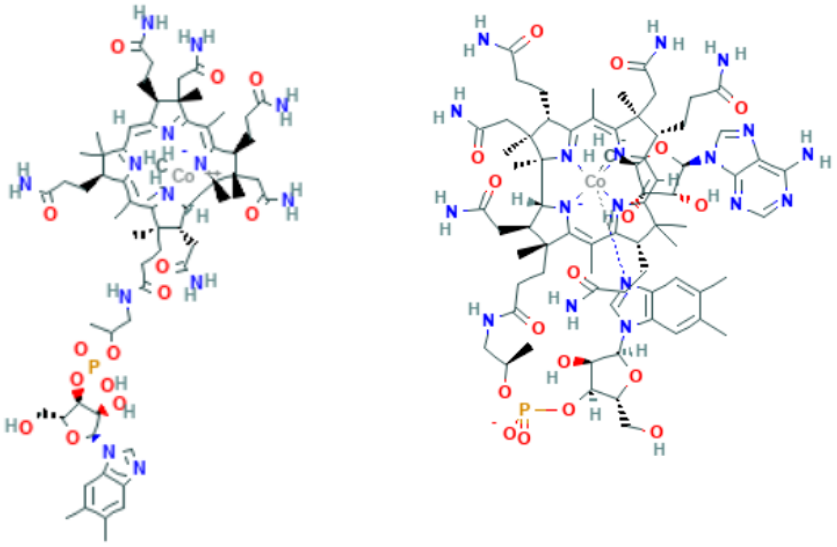


Figure 4. Structure of methylcobalamin (left) and adenosylcobalamin (right) (National Center for Biotechnology Information., 2020).

1.1.3.3. Sources, bioavailability and requirements

Only some microorganisms and some algae synthesise Vitamin B12 (Z. Schneider & Stroinski, 1987) that later is concentrated in the body of higher organisms of the food chain system. For this reason animal foods are the main sources of this vitamin (Watanabe, 2007).

The main contributors to cobalamin intake in a Spanish population study are milk and dairy products (27.3%) and meat and meat products (26.4%). The total median cobalamin intake was 4.0 ug/day in women and 4.5 ug/day in men (Partearroyo et al., 2017), exceeding the recommended daily intakes (Moreiras et al., 2015). In general, most of the participants had adequate cobalamin intake (Partearroyo et al., 2017).

1.1.3.4. Cobalamin status and its determinants

Cobalamin deficiency can present with megaloblastic anaemia and neurologic symptoms, but for some patients it can manifest with only one of the two for unknown reasons. Some misconceptions regarding the diagnosis of deficiency include that it always causes anaemia, does not occur when serum or plasma status is above 140 pmol/L, does not occur if serum or plasma methylmalonic acid is normal and never occurs in children. It has been suggested that for some patients the diagnosis can be made when considering the improvement of the clinical symptoms after parenteral cobalamin therapy even when the biomarkers appear normal. It is important to consider that serum cobalamin might not be informative due to intrinsic factor antibody interference with the assay (Wolffenbuttel et al., 2019). Also, people with cobalamin deficiency can have serum concentrations above 140 pmol/L, due to the use of oral supplements that increase serum concentrations but it is not enough to reach the tissues (Hill et al., 2013; Wolffenbuttel et al., 2019).

The cut off for serum or plasma cobalamin concentrations of <148 pmol/L (200 ng/L), is recommended to identify cobalamin deficiency in the presence of a strong clinical suspicion (Devalia et al., 2014). However, serum or plasma deficiency in cobalamin or folate does not lead to an equivalent prevalence of anaemia (Metz, 2008), the correlation between cobalamin status and symptoms is low and clinical signs may not be obvious (Hunt et al., 2014). For those reasons, the exact cut-offs to classify deficiency remain debated (Green et al., 2017).

Using only blood cobalamin status for the diagnosis of deficiency is usually not enough, and many investigators recommend the use of serum or plasma MMA and tHcy measurements, especially for the identification of subtler states of deficiency (Green et al., 2017).

1.1.3.5. Cobalamin and pregnancy

Our group, as well as others, have reported that cobalamin blood concentrations drop during pregnancy drop (Milman et al., 2006; Murphy et al., 2002).

Our group proposed that the decrease in plasma cobalamin during pregnancy may be partly due to the mobilisation of maternal cobalamin reserves as suggested by the rise in MMA, a functional marker of low cobalamin status, as shown in **Figure 5**. This occurs in the face of the physiological changes that occur during pregnancy such as haemodilution and increased glomerular filtration that are associated with lower circulating concentration. The rise in plasma MMA was greater in women that started pregnancy with lower cobalamin status (Murphy et al., 2007). Also during pregnancy, there is an inverse correlation between cobalamin and MMA and tHcy (Solé-Navais et al., 2018).

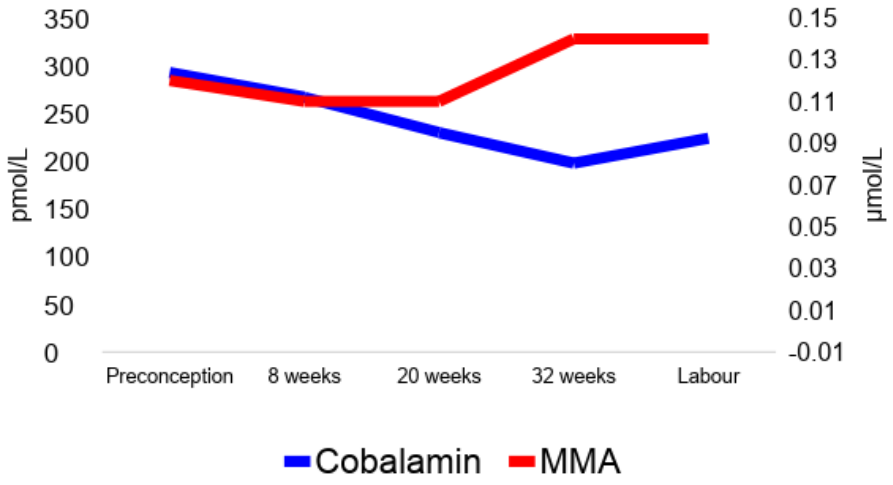


Figure 5. Plasma cobalamin and MMA status during preconception, pregnancy and at labour (Murphy et al., 2007).

1.1.4. Betaine and the dimethylglycine pathway

1.1.4.1. Historical background

The German chemist Carl Scheibler isolated an organic base from sugar beet (*Beta vulgaris*) that he coined as betaine in the 1860's (Takis et al., 2015a) reflecting its original source. Betaine is the term used hereafter but it is also known as N,N,N-trimethylglycine, glycine betaine, lycine, and oxyneurine (Craig, 2004). The plural name "betaines" refers in general to methylamine compounds and metabolites (Takis et al., 2015b).

Betaine was considered an impurity of the beet sugar industry; however, now betaine is also commercialised (Takabe et al., 2015).
In 1947 Borsook and Dubnoff described for the first time the use of betaine for the methylation of homocysteine in the liver (Garrow, 2015).

1.1.4.2. Structure, characteristics and functions

Betaine's structure is shown in **Figure 6** as well as the structure of the related metabolites dimethylglycine, sarcosine and glycine whose interconnection is explained later in the betaine-homocysteine S-methyltransferase (BHMT) section.
Betaine is a methyl derivative of glycine and its molecule is a zwitterion (a positively charged tri-methylammonium group and a negatively charged carboxyl group, therefore electrically neutral) (Patel & Mehta, 2015). It is highly soluble in water and increases the water retention of cells (Craig, 2004).

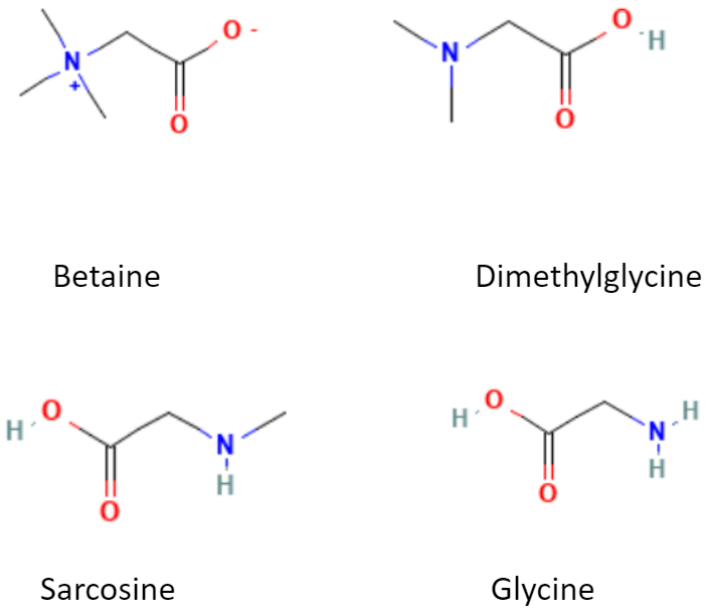


Figure 6. Structure of betaine and related metabolites (National Center for Biotechnology Information., 2020).

Betaine has three functions in mammals. Firstly, it is an osmolyte in the renal medullary cells and other tissues. An example of this function is found also in vegetables like spinach that are grown in saline soil causing betaine accumulation representing up to 3% of its fresh weight (Craig, 2004). Secondly, under denaturing conditions it acts as a chaperone to stabilise protein structure and thirdly, it functions as a methyl donor for the conversion of homocysteine to methionine. Functions of other betaines found in food like proline betaine and trigonelline are less understood (Ueland et al., 2005).

Regarding the function of betaine as a methyl donor, it seems to be more important when folate status (Holm et al., 2005) or intake is low and the same was found for low cobalamin intake (J. E. Lee et al., 2010). Betaine is interconnected with methylenetetrahydrofolate (methyleneTHF*)* because it can be regenerated via betaine metabolites (sequential demethylation of dimethylglycine and sarcosine produces methyleneTHF) (Finkelstein, 1998). Changes in one of those metabolites can result in compensatory changes of others (Craig, 2004).

1.1.4.3. Sources, bioavailability and requirements

In the human diet, betaine is obtained from food as choline or directly as betaine. Intake depends on food composition, but might be related to its production including osmotic conditions (Ueland et al., 2005). Foods with the highest content of betaine (mg/100 g) include wheat bran (1339), wheat germ (1241), spinach (645), beet canned (296), pretzels (237), prawn (218) and wheat bread (201) (Zeisel et al., 2003). Average dietary betaine intake ranges from 1 - 2.5 g/day in a diet rich in whole wheat and seafood (Craig, 2004). Seeds of many medicinal plants also contained betaine (Patil & Tatke, 2015). Betaine is not currently included in national food composition databases in Europe but in Spanish pregnant women it is known that the main food sources are cereals and derivatives (85.3%) (Requejo et al., 2021).

1.1.4.4. Betaine status and its determinants

In adults, plasma or serum 10-90 percentile varies between 20 to 40 mmol/L and the 50 percentile is around 30 mmol/L. Women have about 15% lower blood betaine concentrations than men. Plasma choline at any concentration is a good predictor of plasma betaine. Betaine produces DMG. Above the 80th DMG percentile, betaine does not increase further. Long-term folate supplementation is likely to decrease betaine's role in the DMG pathway (Ueland et al., 2005).

The DMG/betaine (product/precursor) ratio has been used as an inference for the betaine-dependent remethylation of homocysteine (Hcy) (Gillies et al., 2022).

1.1.4.4.1. Betaine-homocysteine S-methyltransferase (BHMT)

Betaine-homocysteine S-methyltransferase (BHMT) is a zinc metalloprotein that accounts for most of the bound zinc in the liver (Lever & Slow, 2010). This enzyme is present mainly in the human liver, kidney and lenses (Park & Garrow, 1999) but it is expressed in other specialised cells during embryonic development in mice (Anas et al., 2008). There is also a significant

expression in the pancreas of sheep and guinea pigs. In the cell, the enzyme has been found only in the cytoplasm and part of it, related to the microtubules (Garrow, 2015).

Betaine donates its methyl group to Hcy through catalysis with BHMT (Velzing-Aarts et al., 2005) forming DMG (from betaine demethylation) and methionine (from Hcy methylation) (Park & Garrow, 1999). In a negative feedback reaction, DMG strongly inhibits the BHMT reaction because it can replace betaine in a tight complex with BHMT and Hcy (Garrow, 2015). DMG can be transformed mainly to sarcosine and sequentially to glycine or be excreted in urine (less frequently) (Swierczynski et al., 2015). The only pathway to generate DMG is this one, so it is a marker of the methylation of Hcy to methionine by BHMT. On the other hand, sarcosine is generated also by another reaction (methylation of glycine by S-adenosylmethionine) and therefore is not a specific marker of the BHMT reaction (Lever et al., 2011).

The *BHMT* gene localised in humans in the position 5q13.1– q15 (Sunden et al., 1997), has a polymorphism called c.716G>A (G: guanosine; A: adenosine), also known as 742G>A; p.Arg239Gln, or rs3733890 (Cunningham et al., 2022), first reported in 1999 (Park & Garrow, 1999). The allele frequency of this polymorphism is shown in **Figure 7**. AA genotype frequency in the European population is around 10.5% while in the African population it is the lowest around 4.8% (Cunningham et al., 2022).

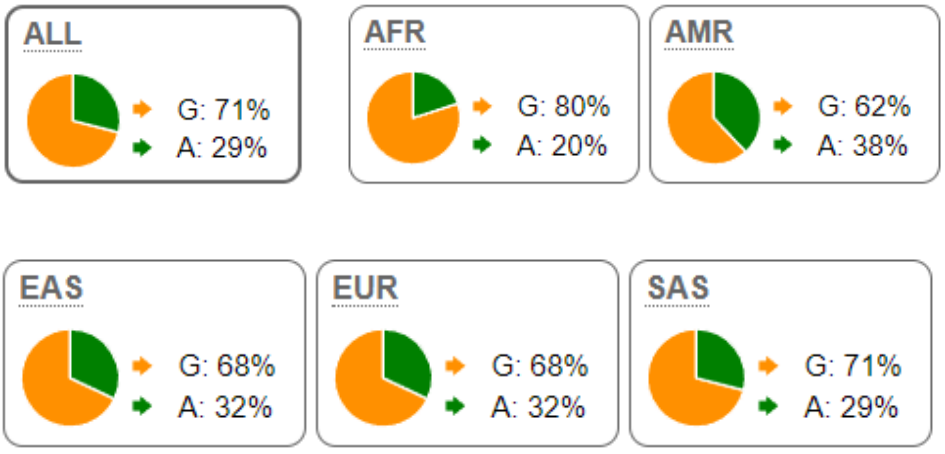


Figure 7. *BHMT* c.716G>A allele frequency (Cunningham et al., 2022).
AFR: African, AMR: American, EAS: East Asian, EUR: European, SAS: South Asian.

Some studies did not see any effect of the SNP on tHcy (Fredriksen et al., 2007; Heil et al., 2000) but one study reported a decrease in plasma DMG concentrations with increasing A allele presence (Fredriksen et al., 2007). Upregulation of the *BHMT* gene catalyses one carbon transfer in the methionine cycle, while downregulation conserves betaine to be used as an osmolyte (Craig, 2015).

1.1.4.5. Betaine and pregnancy

In our Reus-Tarragona Birth Cohort study (Fernàndez-Roig et al., 2013), as well in another (Velzing-Aarts et al., 2005), plasma betaine concentrations decreased until around 20-24 gestational weeks and remained stable until labour (**Figure 8**). We have observed that the association between betaine and tHcy became stronger as blood folate status was lowered with advancing pregnancy (Fernàndez-Roig et al., 2013).

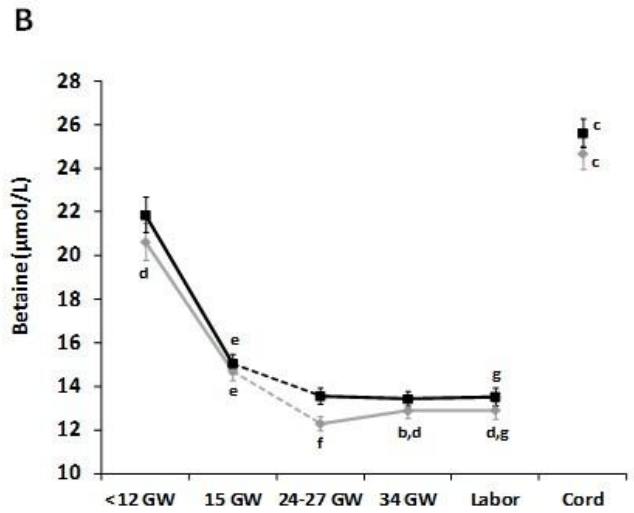


Figure 8. Betaine during pregnancy and in cord.
Grey: low folate status. Black high folate status. From (Fernàndez-Roig et al., 2013).

In a Seychelles study of pregnant women with high choline intake (from eggs), a stratified analysis according to low and high serum methionine (above and below the median), showed that non-fasting total Hcy and betaine were inversely associated in the low methionine group (univariate correlations r = −0.358, P <0.001) but not in the high group (r = −0.137, P = 0.149). The authors proposed that this association might be present only when

folate status is low given that 35% of their population had deficient serum folate concentrations. In populations with higher folate status, they do not expect to see the interaction between methionine and betaine. They also proposed that the interaction might occur in pregnancy due to the high methionine turnover (J. M. Wallace et al., 2008).

Regarding the *BHMT* c.716G>A polymorphism, in our cohort we found that the A allele was associated with lower plasma DMG both during pregnancy and in the cord, suggesting that this allele impairs the conversion of betaine to DMG during pregnancy. On the other hand, low folate status was associated with higher plasma DMG during pregnancy and in the cord. During early pregnancy, we saw an interaction between plasma folate and *BHMT* genotype, and stratification by plasma folate showed that the effect of the A allele on plasma DMG was limited to women with normal-high plasma folate status. While at the end of pregnancy, the allele effect was independent of plasma folate status (Colomina et al., 2016).

Regarding pregnancy outcomes, in a USA study in 2007 the AA genotype in the *BHMT* gene was associated with risk of placental abruption (Ananth et al., 2007). In a Chinese population without folic acid supplementation, the polymorphism was associated with neural tube defects (J. Liu et al., 2014). On the other hand, in a Polish population the AA genotype was associated with a lower risk of orofacial clefts (OR 0.15, 95% CI 0.04 to 0.50) (Mostowska, Hozyasz, Wojcicki, et al., 2010). Similarly, the A allele decreases the risk of Down syndrome in a Brazilian population and the protection is bigger in the case of the homozygous genotype (A allele: OR=0.61; 95% CI: 0.40-0.93; AA: R=0.17; 95% CI: 0.04-0.80) (Amorim et al., 2013).

1.1.5. Homocysteine

1.1.5.1. Historical background

As shown in **Table 4** the discovery of Hcy and related metabolism started in 1810 when it was first isolated from urinary bladder stones but only in 1932 it was first synthesised by Butz and Vincent du Vigneaud. The original interest of du Vigneaud was the study of insulin, discovered in 1921. He was specifically interested in the nature of the sulphur in the insulin molecule (Finkelstein, 2000).

Table 4. The discovery of Hcy and related substances 1810-1932 (Finkelstein, 2000).

1810	Wollaston	Isolated “cystic oxide” from two human bladder stones
1833, 1838	Berzelius and Thaulow	Established the correct elemental formula and the name “cystine.”
1894	Hofineister	Defined the term “transmethylation”
1899	Morner	First isolated cystine from animal protein
1902-1903	Neuberg and Friedmann	Working independently, defined the chemical structure of cystine.
1928	Barger and Coyne	Used a total synthesis to prove the structure of a new compound, which they named methionine.
1932	Butz and du Vigneaud	Treatment of methionine with concentrated acid yielded homocystine (oxidised disulfide).

In 1933, in parallel with Hcy synthesis, the New England Journal of Medicine published an unusual case of an 8-year-old boy of Irish-American ancestry evaluated in the Mass General Hospital after 4 days with a headache, vomiting and drowsiness as well as poor mental development and dislocation of lenses in both eyes. He also had a high temperature and high blood pressure. Subsequently, he showed signs of having suffered a stroke. The cause of death was reported as arteriosclerosis of the carotid artery with cerebral infarct (Kumar et al., 2017).

Later in 1963, the disease homocysteinuria was discovered in Belfast, Northern Ireland while studying the chemical composition of urine (Carson et al., 1963). In 1965 a case of a 9-year-old girl of Irish-American descent was evaluated for poor mental development again in the Mass General Hospital. As was the case with the boy, she had a dislocated eye lens and exhibited several similarities with cases of homocysteinuria. Her blood sample

confirmed hyperhomocysteinemia. After further investigations the paediatricians realised that the boy from the 1933 article was the girl's uncle (Carson et al., 1963; Kumar et al., 2017).

Four years later in 1969 McCully from Harvard treated 2 children with homocysteinuria. The first was a boy of 2 months of age with advanced arteriosclerosis. He had extremely high concentration of Hcy in the blood and urine with no lipid deposits in his vascular plaques. The second child was an 8-year-old who had died of a stroke. The autopsy revealed cardiovascular characteristics exactly like those of elderly men with arteriosclerosis. McCully hypothesised that the vascular pathology in these patients could be the result of exposure to elevated homocysteine, homocystine, or a derivative of homocysteine in the circulating blood. Based on these findings, he suggested for the first time that elevated Hcy is the common factor leading to arterial damage (McCully, 1969). The scientific community however did not accept his hypothesis for a long time until similar observations were confirmed many years later (Kumar et al., 2017).

1.1.5.2. Structure, characteristics and functions

In clinical biochemistry the term total homocysteine refers to the homocysteine that can be determined after a reducing treatment aimed to break disulphide bonds; therefore total homocysteine includes homocysteine molecules that are, prior to the treatment, free homocysteine (reduced), homocysteine disulfides and homocysteine mixed disulfides (bound to proteins and other molecules by disulphide bonds, see **Figure 9**). The words “homocysteine” and “homocystine” that designate, respectively, the reduced (sulfhydryl) and the oxidised (disulfide) forms; were coined by du Vigneaud and coworkers, who discovered these compounds in 1932 (Mudd & Levy, 1995; Rasmussen & Møller, 2000; Refsum et al., 2004).

Throughout the results of this thesis, with the exception of samples at labour that are not from a fasting state, “tHcy” will refer to fasting plasma total homocysteine concentrations.

Plasma homocysteine is about 1-2% in the sulfhydryl form; while the remaining 98% is in disulfide form (Mudd & Levy, 1995). As shown in **Figure 9**, homocysteine can be reduced or oxidised (as homocystine or as mixed disulfides). Clearly, the oxidised forms make up the bulk of circulating homocysteine (Carmel & Jacobsen, 2001).

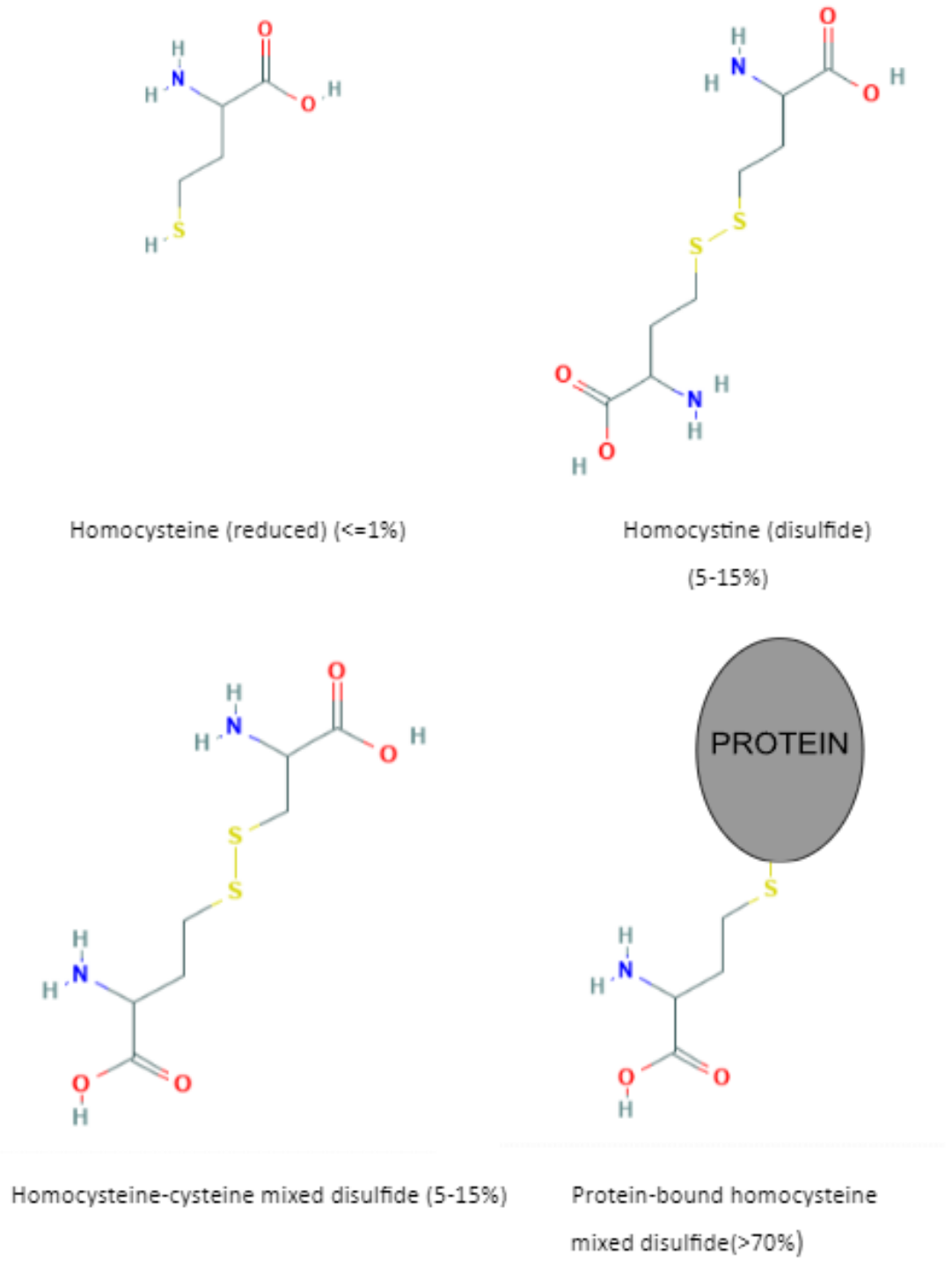


Figure 9. Structure of homocysteine.

Structure of homocysteine showing percent distribution of reduced and oxidised forms in human plasma. Adapted from (National Center for Biotechnology Information., 2020) and (Carmel & Jacobsen, 2001).

1.1.5.3. Physiological and clinical relevance

Homocysteine is a sulphur amino acid not found in food and is related to two metabolic pathways: remethylation to methionine and transsulfuration to cystathionine. S-adenosylmethionine (SAM) is involved in both pathways, and it can inhibit the MTHFR reaction and activate cystathionine β-synthase (Finkelstein, 2000; Selhub, 1999). Homocysteine remethylation to methionine needs 5-methyltetrahydrofolate as a methyl donor and methylcobalamin as a coenzyme and occurs in all mammalian cells. The liver of all mammals and the kidney of primates can also use betaine instead of 5-methyl-THF as the methyl donor (Finkelstein & Martin, 2000).

Regarding the transsulfuration pathway that converts methionine to cysteine, two enzymes are involved (cystathionine-β-synthase and γ-cystathionase) that require the pyridoxal phosphate as a cofactor. In contrast to the ubiquitous remethylation pathway, the transsulfuration pathway is only present in some tissues, and those without it require cysteine from the diet. Both enzymes are present in the liver, kidney, small intestine and pancreas, while the brain has only one enzyme (cystathionine) causing accumulation of cystathionine in the brain (Finkelstein & Martin, 2000).

Increase homocysteine has been associated with cardiovascular disease, however some clinical trials did not find a homocysteine lowering effect through the use of B vitamins for the prevention of cardiovascular disease (Albert et al., 2008; Bønaa et al., 2006; Ebbing et al., 2008; Lonn et al., 2006; Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH) Collaborative Group et al., 2010; Toole et al., 2004; VITATOPS Trial Study Group, 2010). Later, one of those trials (HOPE 2) reported that lowering homocysteine through B vitamins caused a reduction in risk of stroke (Saposnik et al., 2009); however, this study is not as recognised as the original report of the trial. As reviewed previously by McNulty and colleagues it is important also to mention that those trials were done in patients with advanced disease (McNulty, Strain, et al., 2012).

There are other conditions associated with hyperhomocysteinaemia (Kuo et al., 2005) some of them are cognitive decline, dementia, and Alzheimer's disease, but it has been claimed that a large intervention trial is still necessary to establish whether cognitive decline is slowed or prevented with B vitamin supplementation (Smith et al., 2018). Also, there was a causal association between homocysteine and type II diabetes in a mendelian randomization analysis (T. Huang et al., 2013). Similarly, there was a positive association between homocysteine and other comorbidities of the metabolic syndrome such as polycystic ovary syndrome (Murri et al., 2013) and obstructive sleep apnea (Niu et al., 2014).

1.1.5.4. Homocysteine and pregnancy

Our group studied the changes in tHcy from preconception throughout pregnancy in the PreC cohort as shown in **Figure 10**. The causes of reduction in plasma tHcy during pregnancy are not only due to folic acid supplements, hemodilution or decrease in serum albumin (Murphy et al., 2002). Maternal tHcy was correlated from preconception to different points during pregnancy (8, 20, 32 weeks and delivery) and also tHcy from preconception throughout pregnancy was correlated with tHcy in cord (Murphy et al., 2004).

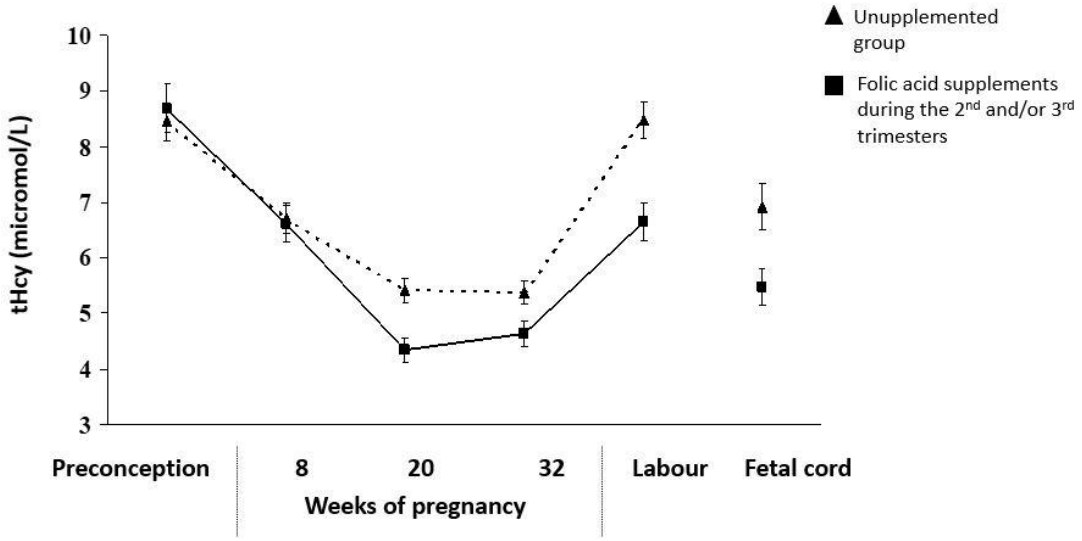


Figure 10. tHcy concentrations from preconception to labour and foetal cord tHcy (Murphy et al., 2004).
Geometric mean (SE; error bars)

Elevated homocysteine has been associated with numerous complications like recurrent pregnancy loss (Puri et al., 2013), early miscarriage (Cavallé-Busquets et al., 2020; Gris et al., 2003), preeclampsia (Cotter et al., 2001; Laskowska et al., 2013), placental abruption (Goddijn-Wessel et al., 1996), gestational diabetes mellitus (Seghieri et al., 2003), preterm delivery (Qiu et al., 2018), neural tube defects (Mills et al., 1995) and low birthweight (Murphy et al., 2004; Onalan et al., 2006).

Regarding low birthweight, we found in the PreC cohort through multiple linear regressions that maternal tHcy in the highest tertile compared with mid-low tertiles was negatively associated with birthweight throughout pregnancy but was significant only at birth. More precisely, maternal tHcy in the highest tertile at labour was associated with babies that weighed 227.98 g less than those born to mothers in the medium - low tertiles. Looking at the association with birthweight, maternal tHcy in the highest tertile at 8 weeks vs. mid-low tertiles was associated with a 3 times greater probability of giving birth to a neonate in the lowest birthweight tertile. Similar results were found when looking at tHcy at labour (Murphy et al., 2004), when tHcy was not in the nadir part of the curve, typical of mid-pregnancy, and possibly masked by the physiological factors that influence it at that point.

1.2. Foetal origins of adult cardiovascular health

1.2.1. General overview

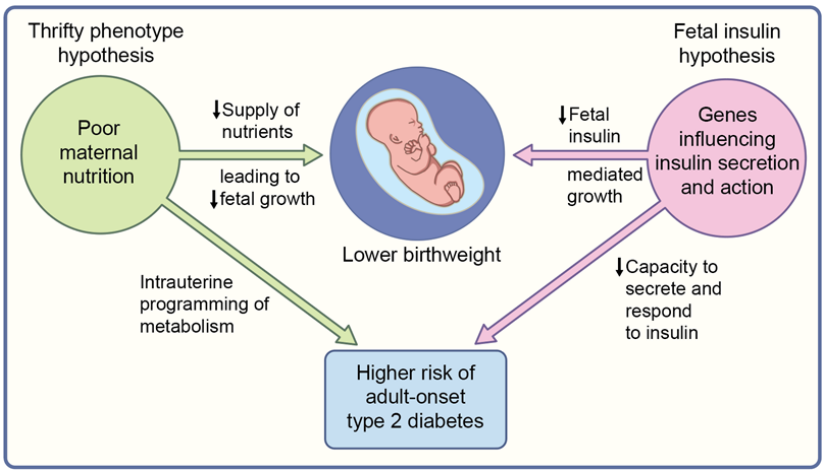
The work of David Barker and colleagues in 1989 linked low birthweight to later risk of cardiovascular disease, highlighting the relevance of the gestational environment to adult health (Barker, Osmond, et al., 1989; Barker, Winter, et al., 1989). There is now more evidence from both human and animal studies to indicate that the parental environment before and during pregnancy can determine the health of offspring. For example, other studies have included other conditions such as type 2 diabetes (Barker, Hales, et al., 1993; Curhan, Willett, et al., 1996), elevated triglycerides (Barker, Martyn, et al., 1993), and hypertension (Barker, Hales, et al., 1993; Curhan, Chertow, et al., 1996; Curhan, Willett, et al., 1996). Later on, the knowledge of relevant environmental characteristics during periconception was broadened to consider the consequences of paternal diet (Sinclair & Watkins, 2013), parental exposure to environmental chemicals (Heindel et al., 2017) and assisted reproduction (M. Chen & Heilbronn, 2017).

Following Barker's findings, in 1992 he and Nicholas Hales proposed the "thrifty phenotype" hypothesis postulating that poor maternal diet could lead to reduced foetal growth, producing stable modifications in glucose-insulin metabolism such as reduced capacity for insulin secretion and insulin resistance. If the foetal and postnatal environment are similar, those characteristics would be advantageous but in an energy-rich postnatal situation it might be deleterious (Fernandez-Twinn et al., 2019). The foetal modifications in the glucose-insulin metabolism in combination with obesity, ageing and poor physical activity later in life will determine type 2 diabetes. Almost 10 years later in 2001, the same authors affirmed that the evidence showed a clear relationship with insulin resistance but the relationship with insulin secretion was not so evident (Hales & Barker, 2001). The thrifty hypothesis evolved including other types of exposures during pregnancy and is now known as the Developmental Origins of Health and Disease (DOHaD) hypothesis (Fernandez-Twinn et al., 2019). Some potential epigenetic mechanisms in the development of metabolic disease were proposed, based on evidence from human and animal studies

(Fernandez-Twinn et al., 2019). In the case of maternal smoking it was proposed that it causes an increased risk of obesity in the offspring and that methylation at *GFI1* might be involved in the association. Maternal nutritional status was also proposed to be another factor involved, since famines have been associated with changes in methylation at *INSR, CPT1A* and at imprinted *IGF2.* Maternal obesity has been associated with macrosomia/large for gestational age and increased inflammatory markers in the offspring and this association might also be explained by changes in DNA methylation. Also, maternal obesity has been linked to hypermethylation of the *Pomc* gene that synthesises a polypeptide in the pituitary. Epigenetic changes in sperm cells might explain that paternal smoking and obesity increase offspring body fat and metabolic dysfunction. Pancreas failure or peripheral insulin resistance in the offspring might be explained by less acetylation of histone 3 and 4 in the uterine artery ligation. Other epigenetic changes include hypermethylation of the imprinted *Igf/H19* loci (Fernandez-Twinn et al., 2019).

In 1998 another explanation for Barker's findings was proposed by Andrew Hattersley and colleagues saying that lower birthweight and later insulin resistance, glucose intolerance, type 2 diabetes and hypertension are different phenotypes of the same foetal genotype. They explain the association of low birthweight and adult insulin resistance via genetic mediation (Hattersley & Tooke, 1999). In 2021, Hattersley and colleagues published a review of the evidence supporting this hypothesis that is summarised in **Figure 11** (Hughes et al., 2021).

A.



B.

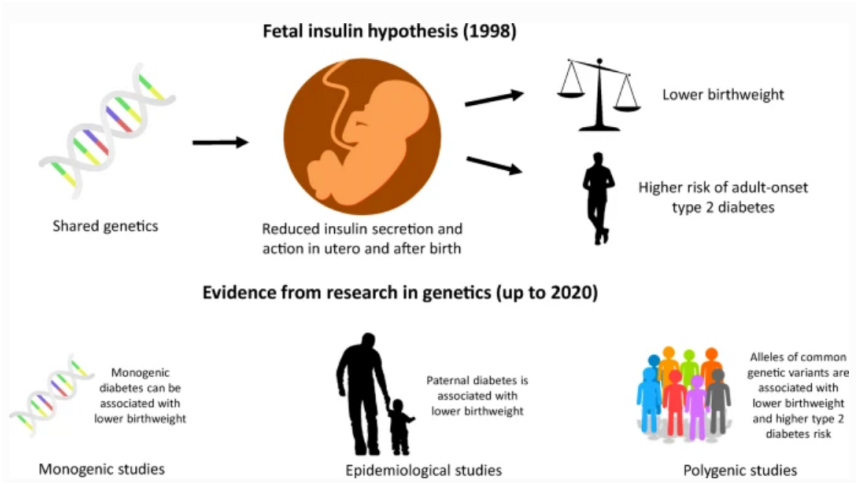


Figure 11. Thrifty hypothesis and foetal insulin hypothesis (Hughes et al., 2021).
A. Comparison between thrifty hypothesis and foetal insulin hypothesis B. Evidence supporting foetal insulin hypothesis.

The plausibility of DOHaD, foetal insulin hypothesis and other explanations studied through a mathematical model are reviewed by Baig and colleagues and proposed that one hypothesis alone is not enough to adequately explain obesity (Baig et al., 2011).

Another possible mechanism explaining foetal programming of diseases is a reduction in the number of cells causing changes in the structure and function of organs as seen in low

protein diet during pregnancy in animals and its effects on the offspring (Chmurzynska, 2010).

1.2.2. The role of one carbon metabolism in developmental origins of health and disease (DOHaD)

1.2.2.1. Animal models

In 2003 pregnant yellow agouti (A^{vy}) mice on a nutritionally adequate diet that additionally received methyl-donor supplementation, had increased DNA methylation and the body composition and metabolism of their offspring changed. This mouse has a metastable epiallele, resulting from the insertion of an intracisternal A particle (IAP). Metastable epialleles are genomic regions in which DNA methylation is established probabilistically in the embryo and then maintained in differentiated tissues through mitosis, leading to interindividual epigenetic variation that affects multiple cell types (Rakyan et al., 2002). The methylation of the epiallele was altered by the provision of methyl donors (e.g: folic acid, vitamin B12, choline chloride, and anhydrous betaine). The diets were provided for 2 weeks before the females were mated and throughout pregnancy and lactation (Waterland & Jirtle, 2003). This study provided the first evidence that modifications in 1C nutrients can alter the epigenome and metabolic phenotype of offspring (Clare et al., 2019).

Later, another study in animals showed the 1C metabolism again involved in the DOHaD. It was published in 2007 involving mature female sheep with a restricted supply of B12, folate and methionine in the periconceptional diet. Six days after conception, the embryos were transferred to surrogate ewes. The maternal diet led to adult offspring with increased adiposity, insulin resistance, altered immune function, and high blood pressure. These effects were most evident in males and over half of the affected loci were specific to males (Sinclair et al., 2007). A similar study in rats in 2011, also modified the periconceptional diet of dams. The intervention started 3 weeks prior to mating until the first 5 days of gestation and was methyl-deficient with no folic acid, 0.05% choline, and approximately one-half the recommended content of methionine. The deficient diet led to 32% higher HOMA-IR index

at 6 months of age in the male offspring and 39% higher peak insulin during an oral glucose tolerance test (oGTT) compared to offspring on a replete diet. There was no difference in the response to the oGTT in the female offspring at 6 months of age (Maloney et al., 2011). Both studies in sheep and rats show not only the importance of 1C in DOHaD but also suggest that the periconceptional period is particularly susceptible to maternal diet–mediated epigenetic alterations in gene regulation and both showed male-specific outcomes. On the other hand, the animal studies mentioned above, mainly consist of short interventions so the effects of extended periods still need to be tested to simulate human or animal populations (Clare et al., 2019).

A study of female rats fed a low protein diet during the preimplantation period of development only, before returning to the control diet, reported altered birthweight, postnatal growth rate, hypertension and organ/body-weight ratios in the offspring at up to 12 weeks of age (Kwong et al., 2000). Five years later, Lillycrop et al. reported that folic acid supplementation prevented the epigenetic changes of hepatic gene expression in the offspring of rats on a low protein diet throughout pregnancy (Lillycrop et al., 2005) and similar results were reported in 2013 (Altobelli et al., 2013). Low protein diets are based on casein, which is deficient in cysteine, leading to a 25% decline in cystathionine γ-lyase activity by approximately day 4 of gestation and causing high tHcy concentrations in the dams (Clare et al., 2019; Rees, 2002).

In a mouse model of maternal obesity, choline supplementation normalises foetal adiposity and the mechanism is not known (Jack-Roberts et al., 2017). A possible explanation could involve the role of choline in homocysteine methylation. In mice, methyl donor deficiency during pregnancy and lactation, followed by a high fat diet of the adult offspring, causes liver steatosis, a condition that is a consequence of central obesity (Bison et al., 2016). One possible mechanism that can link the one carbon metabolism with dyslipidemias, has been seen in sheep, in which the cobalamin-dependent liver disease causes an accumulation of MMA that inhibits the carnitine O-palmitoyltransferase oxidation of free fatty acids within the liver (Clare et al., 2019).

1.2.2.2. Human studies

The first report in humans to suggest that defects in 1C metabolism could be at the heart of intrauterine programming of adult disease came from a cohort in Pune, India (Yajnik et al., 2008).

1C metabolism and adiposity.

In two Indian cohorts there was an association between higher maternal tHcy and neonatal smaller mid–upper arm circumference, skinfold measurements (Krishnaveni et al., 2014; Yajnik et al., 2008), abdominal circumference (Yajnik et al., 2008) and weight (Krishnaveni et al., 2014). However, there was no association between pregnancy tHcy and measurements of adiposity in children at 5 (Krishnaveni et al., 2014) and 6 years (Yajnik et al., 2008).

Also in India higher maternal erythrocyte folate status at 28 weeks was reported to predict greater adiposity at 6 years (Yajnik et al., 2008). Outside of India, in a cohort from the United States sufficient plasma folate status, mainly in obese mothers, was associated with lower ofspring BMI z-scores and reduced probability of overweight or obesity (G. Wang et al., 2016).

1C metabolism and glucose metabolism

Maternal tHcy during pregnancy (30 GW) was positively associated with offspring postload glucose concentrations at 5 and 9.5 years in the Mysore cohort. Similarly, maternal pregnancy tHcy was positively associated with insulin concentrations at 5 years of age and there was a borderline positive association between maternal tHcy (30 GW) and HOMA-IR at 9.5 years of age. Furthermore, maternal plasma folate concentration was positively associated with HOMA-IR in the offspring (9.5 years of age) (Krishnaveni et al., 2014).

The Pune cohort reported that higher maternal erythrocyte folate at 28 GW status or lower cobalamin status at 18 GW, predicted higher insulin resistance in the children at 6 years (Yajnik et al., 2008). Moreover, it was found that children born to mothers with a

combination of high folate and low vitamin B12 concentrations were the most insulin resistant (Yajnik et al., 2008). Maternal plasma cobalamin status did not predict insulin resistance in Mysore cohort (Krishnaveni et al., 2014). Additionally, in Pune high maternal plasma MMA was associated with insulin resistance in the children in a conditional independence analysis (Yajnik et al., 2008).

The difference between the above cohorts, regarding a high folate/low B12 interaction or not, may be due to differences in the dose and duration of exposure of supplemental folic acid as well as the prevalence of vitamin B12 deficiency (Paul & Selhub, 2017).

1C metabolism and dyslipidemia

Studies on the Dutch winter famine, Jewish holocaust survivors and Chinese famine reported that malnutrition during pregnancy was associated with dyslipidemias in the adult offspring (Burdge & Lillycrop, 2010). Potential mechanisms to explain these findings involving 1C metabolites have been proposed, as described in the previous section on animal models (Clare et al., 2019).

1C metabolism and blood pressure

A U-shaped association between maternal plasma Hcy (48-72 hours after delivery) and systolic blood pressure in the 6-7 year old offspring was reported in a USA cohort. Maternal obesity combined with maternal plasma Hcy in the highest or the lowest quartile increased the risk of elevated systolic blood pressure in the offspring with ORs (CI) of 1.75 (1.03, 2.96) and 2.22 (1.35, 3.64) respectively, compared to non-obese mothers in the 2nd or 3rd Hcy quartile. The association between low maternal plasma Hcy and child blood pressure had not been previously reported and the authors proposed that it might be due to reduced ability to generate glutathione and taurine (in whose production homocysteine is an intermediate) and consequently it caused a reduced ability to respond to oxidative stress (H. Wang et al., 2017).

On the contrary, a study in the Netherlands of children of approximately the same age did not find an association between 1st trimester plasma Hcy and blood pressure in the children (van den Hil et al., 2013). Similarly, an Indian cohort did not find an association between maternal tHcy, plasma folate or vitamin B12 and offspring blood pressure (Krishnaveni et al., 2014).

1C metabolism and programming of other conditions related to the metabolic syndrome.

The Generation R study in the Netherlands reported that higher tHcy status during pregnancy was associated with smaller kidney volume (a measurement of kidney development) and lower childhood estimated glomerular filtration rate based on cystatin C at 6 years of age. In adults, elevated tHcy was associated with microalbuminuria, however in the birth cohort they did not find the same association, suggesting that microalbuminuria might not be detectable during childhood. In the same study they also found that higher maternal folate status was associated with larger kidney volume of the children, and similarly, higher maternal cobalamin status was associated with higher estimated glomerular filtration rate based on cystatin C in the children (Miliku et al., 2017). In the first formal definition of the metabolic syndrome, microalbuminuria was one of components proposed by WHO, as explained in the metabolic syndrome section of this thesis.

Possible mechanisms

Regarding molecular studies in humans explaining the mechanisms behind the association of 1C metabolism with the DOHaD, the first putative metastable epialleles were identified in Gambia seven years after the publication of the agouti mice study. DNA methylation at metastable epialleles was higher in individuals conceived during the nutritionally challenged rainy season. In the autopsy samples, they showed that DNA methylation at these loci is highly correlated across tissues and can be deduced from peripheral blood DNA (Waterland et al., 2010). Later in 2014, another study with the same population showed that DNA methylation was influenced by periconceptional maternal plasma status of micronutrients involved in one-carbon metabolism. This study in humans shows that maternal nutrition

around conception can impact the offspring epigenome, with probably long-lasting effects (Dominguez-Salas et al., 2014).

Final considerations

The importance of periconceptional parental lifestyle on offspring health is important enough for it to be taken into consideration in the preparation for pregnancy (Fleming et al., 2018). Following this idea and considering the effect of tHcy on pregnancy outcomes and offspring health, during 2018 a randomised control trial was conducted to test nutritional supplements to lower tHcy in non pregnant women in Gambia with the objective to use them in future trials for optimising offspring DNA methylation (James et al., 2019).

In summary, 1C metabolism is a key link between parental environment and early development. Future studies should consider the effects of maternal and paternal 1C metabolism alterations at the same time. Also, we need better understanding of sex-specific outcomes in the offspring (Clare et al., 2019).

1.3. Metabolic syndrome

The term 'metabolic syndrome' describes the clustering in an individual of several disorders that are risk factors for cardiovascular disease and type 2 diabetes mellitus (Alberti et al., 2009). During the XXI century many scientists have described the coexistence of various cardiovascular risk factors that are summarised below.

During the First World War in Vienna, two physicians, Karl Hitzenberger and Martin Richter-Quittner, discussed the link between blood pressure and diabetes mellitus. They were able to publish their work only after the end of the war in 1921. The same year a Swedish physician Eskil Kylin, and in 1922 a Spanish physician Gregorio Marañón published independently, with almost the same title, an article proposing a link between hypertension and diabetes mellitus. Again, Kylin in 1923, published an article whose title was similar to the current description of the metabolic syndrome: *"Studies of the hypertension - hyperglycemia - hyperuricemia syndrome"*. Later, in 1947, Vague from France was the first to

link upper body adiposity (mainly in men) with diabetes and cardiovascular issues, then the Italians Avogaro and Crepaldi in 1967 described a link between hypertension, hyperglycemia, and obesity (Sarafidis & Nilsson, 2006).

In 1988, Gerald Reaven in the Banting Lecture (series of presentations given by an expert in diabetes) proposed a link between insulin resistance (a characteristic of type 2 diabetes) and high blood pressure, high triglycerides, and other metabolic anomalies (central obesity was not included in the original description). Reaven named the cluster, syndrome X and the "X" was used to highlight that the importance of insulin resistance as a coronary heart disease risk was not well recognized. He hypothesised that insulin resistance was the common causal factor for the group of disorders mentioned (Reaven, 2001; Sarafidis & Nilsson, 2006).

The idea was controversial but today syndrome X, now called metabolic syndrome, is considered an important predictor of the risk for diabetes and cardiovascular disease. In the 1950s most of his colleagues believed that there was only one type of diabetes that resulted from the failure of insulin-secreting cells in the pancreas to make the hormone. But Reaven proposed another type characterised by a gradual loss of cell sensitivity to insulin. This controversial opinion was right. The confirmation came from the radioimmunoassay experiments of Solomon Berson and Rosalyn Yalow in 1959. They identified "insulin-dependent" and "non-insulin dependent" diabetes, the latter developed in people whose cells become insensitive to the hormone, even when they make sufficient insulin. From then on, Reaven became known as "the father of insulin resistance" (Alberti & Zimmet, 1998; Oransky & Marcus, 2018; Reaven, 2001).

1.3.1. Definition in adults

After Reaven's proposal of the syndrome X, it was named also as insulin resistance syndrome and the dysmetabolic syndrome (Magge et al., 2017). The first formal definition of metabolic syndrome was proposed by a consultation group for the World Health Organization in 1998. This group highlighted that insulin resistance was the major risk factor and was required for

diagnosis of the syndrome. A diagnosis according to WHO criteria could be made based on some markers of insulin resistance and two additional risk factors from obesity, hypertension, high triglycerides, low high-density lipoprotein cholesterol, or microalbuminuria. Later in 2001, the National Cholesterol Education Program Adult Treatment Panel III (ATP III) proposed a definition that did not require direct proof of insulin resistance. Besides, the diagnosis was made by the presence of any 3 of 5 risks among abdominal obesity (highly correlated with insulin resistance), high triglyceride, reduced high-density lipoprotein cholesterol, high blood pressure, and high fasting glucose (impaired fasting glucose or type 2 diabetes mellitus). In the absence of CVD or diabetes, the metabolic syndrome is a predictor of these conditions and the syndrome is frequently present in patients with CVD or diabetes (Alberti et al., 2009).

Later, other definitions were proposed. One from the International Diabetes Federation (IDF) and another from the Adult Treatment Panel III/American Heart Association/National Heart, Lung, and Blood Institute (ATP III/AHA/NHLBI) both in 2005. The measure of abdominal adiposity was the main difference between the two definitions, an obligatory component for the IDF and was lower than in the ATP III/AHA/NHLBI definition. In 2009 there was an attempt to unify the definition. It was decided that a person with 3 abnormal findings out of 5 would have metabolic syndrome. The 5 risk factors were included high blood pressure, high triglycerides, low high-density lipoprotein cholesterol, high fasting glucose, and central obesity (Alberti et al., 2009). In the **Table 5** a comparison between the WHO, IDF and the harmonised definition is shown.

Table 5. Metabolic syndrome defined by the WHO, IDF, and the harmonised IDF/NHLBI/AHA (Roberts et al., 2013).

Clinical measure	WHO (1998)	IDF (2005)	Harmonised IDF/NHLBI/AHA (2009)
Insulin resistance	IGT, IFT, T2DM, or lowered insulin sensitivity*	None	None
	Plus any two of the following		But any three of the following five features
Body metric	Men: waist-to-hip ratio >0.90 Women: waist-to-hip ratio >0.85 and/or BMI >30kg/m2	Increased WC (population specific) plus any two of the following	Population- and country-specific definitions
Lipids	TG 150 mg/dL and/or HDLc <35 mg/dL in men or <39 mg/dL in women	TG > 150 mg/dL or on TG Rx HDLc<40 mg/dL in men or <50 mg/dL in women or on HDLc prescription drugs	TG ≥150 mg/dL (1.7 mmol/L) HDLc<40 mg/dL (1.0 mmol/L) in males; <50 mg/dL (1.3 mmol/L) in females
Blood pressure	≥140/90 mmHg	≥130 mmHg systolic or 85 mmHg diastolic or on hypertension Rx	Systolic ≥130 and/or diastolic ≥85 mmHg
Glucose	IGT, IFG, or T2DM	≥100 mg/dL (includes diabetes)	≥100 mg/dL
Other	Microalbuminuria		

Abbreviations: BMI, body mass index; IFG, impaired fasting glucose; HDLc, high density lipoprotein cholesterol; IGF, impaired glucose tolerance; TG, triglycerides, T2DM type 2 diabetes mellitus; Rx: medical prescription; WC, waist circumferences.
*Insulin sensitivity measured under hyperinsulinemic euglycemic conditions, glucose uptake below lowest quartile for background population under investigation.

Regarding the mechanism involved in the metabolic syndrome, a question has been posed with considerable argument. Which comes first in metabolic syndrome, hyperinsulinemia (elevated insulin relative to glucose), or insulin resistance (a reduced responsiveness of a cell or an organism to the insulin concentration to which it is exposed)? (Alberti, 1993)

In vivo, insulin resistance is linked to hyperinsulinemia. However, there is a predominant perspective that considers a dominant role for insulin resistance. On the other hand, some researchers propose that hyperinsulinemia has a role, especially in the basal state, in sustaining, expanding, or initiating the insulin resistance. It has been hypothesised that hyperinsulinemia is frequently both a cause and a result of insulin resistance (Shanik et al., 2008). A study supporting the causal role measured basal hyperinsulinemia in normoglycemic adults and it ended up being an independent risk factor for developing dysglycemia over 24 years (Dankner et al., 2009). A study that would ideally answer questions such as the above, would be a human study on the role of maternal nutrition and habits during pregnancy although it would take 30 or 40 years to get answers (Clarivate, 2011).

In 2015, the Cardiometabolic Health Alliance think tank discussed some emergent concepts that require validation but have the potential to improve current treatment recommendations. Some of emerging priorities are the necessity to classify the Metabolic Syndrome according to subtype and stage and the definition of structured lifestyle interventions. The subtypes proposed are vascular dominant, adiposity dominant, lipid dominant, insulin resistance dominant and “other risk factors” that contain patients with hormonal dysfunction, chronic kidney disease and hyperuricemia. Additionally, they highlighted some key findings, one of them says that part of the variability of the metabolic syndrome in different ethnicities might be explained by the presence of ectopic fat and/or visceral adipose tissue and they also remarked that this characteristic is critical to the pathogenesis of the metabolic syndrome (Sperling et al., 2015).

1.3.2. Definition in children

Metabolic syndrome in children is a controversial topic because it is challenging to define. In adults, metabolic syndrome predicts cardiovascular disease and type 2 diabetes mellitus but in the paediatric population a large percentage of children defined as having the syndrome

do not have it on follow-up (Magge et al., 2017). For instance, a study of children and adolescents aged 6-12 yr with repeated measurements more than 1.5 yr apart, showed that metabolic syndrome diagnosis was unstable in 45.5% of cases (Gustafson et al., 2009). Even with such instability, the Lipid Research Clinics (LRC) Princeton Prevalence study that started between 1973-1976 found that after 25 to 30 years of follow-up, paediatric metabolic syndrome predicted adult type 2 diabetes mellitus with an OR (CI) of 11.5 (2.1, 63.7) and metabolic syndrome in adulthood with an OR of 9.4 (4.0, 22.2) (Morrison et al., 2008).

In 2007, the IDF developed a definition of the metabolic syndrome for the paediatric population during a consensus workshop of experts. The IDF suggested that below the age of 10 years, the metabolic syndrome cannot be diagnosed. They recommended that the paediatric definition should be based on the adult IDF definition and it should only apply to children 10 to <16 years old. The 90th percentile for waist circumference or adult cut off point (whichever was lower) should diagnose central obesity. Adult criteria should apply for those aged 16 years and older (Zimmet et al., 2007). Later, in 2009, the AHA refused to include a definition of or specific criteria for metabolic syndrome in children and mentioned the limitations of adapting definitions from adults and the need of a new approach for children. However, they made it clear from studies in children and young adults that the atherosclerotic process is stimulated in an exponential manner when there are more cardiovascular risk factors. They highlighted a report from the Bogalusa Heart Study that showed that BMI, insulin resistance, triglycerides/high density lipoprotein cholesterol (HDLc) ratio, and mean arterial pressure were clustered both in childhood and adulthood and, importantly, longitudinally. They point out the importance of identifying the paediatric cardiometabolic risk factors, only a few of which are associated with the proposed definitions of metabolic syndrome in children. The AHA also mentioned the need for studying the risk factors in longitudinal studies from childhood to adulthood to determine the factors that should be included in a future definition of the metabolic syndrome in youth. Nevertheless, as mentioned before, they recognized an instability in the categorical diagnosis of metabolic syndrome in the paediatric population (Steinberger et al., 2009).

Some definitions of metabolic syndrome in the paediatric population are shown in **Table 6**.

Table 6. Metabolic syndrome definitions in children and adolescents.

	Defining criterion	**Excess adiposity**	**Blood pressure**	**Blood lipids**			**Blood glucose/insulin**
(Cook et al., 2003) **(12 to 19 years)**	≥3 criteria out of the next 5 factors	WC ≥90th percentile (age and sex specific, NHANES III)	BP ≥90th percentile (age, sex, and height specific)	Triglycerides ≥110 mg/dL	HDLc ≤40 mg/dL (1.03 mmol/L; all ages and sexes, NCEP)		Fasting glucose ≥110 mg/dL (≥6.1 mmol/L)
(de Ferranti et al., 2004) **(12 to 19 years)**	≥3 criteria of the next 5 factors	WC >75th percentile for age and sex	BP >90th percentile percentile for age, sex, and height	Triglycerides ≥100 mg/dL (≥1.1 mmol/L)	HDLc <50 mg/dL (1.3 mmol/L)		≥110 mg/dL (≥6.1 mmol/L)
(Viner et al., 2005) **(2.3–18 years)**	≥3 criteria out of the next 4 components	BMI ⩾95th percentile for age and sex	SBP ⩾95th percentile for age and sex	Triglycerides ⩾1.75 mmol l − 1 (154 mg dl − 1) or	HDLc<0.91 mmol l − 1 (35 mg dl − 1) or	high total cholesterol ⩾95th percentile	Hyperinsulinaemia ⩾104.2 pmol l − 1 (15 mU l − 1) (prepubertal) or impaired fasting glucose ⩾6.11 mmol l − 1 (110 mg dl − 1)
(Zimmet et al., 2007) **(IDF Ages 10–16)**	Presence of central obesity plus any two of the other four factors.	WC ≥90th percentile or adult cutoff if lower	Systolic BP ≥130 mm Hg or diastolic BP ≥85 mm Hg	Triglycerides ≥150 mg/dL	HDLc <40 mg/dL (1.03 mmol/L)		Fasting glucose ≥100 mg/dL (>5.6 mmol/L) or known type 2 diabetes mellitus
(Ahrens et al., 2014) **IDEFICS - monitoring level (2.0 to 10.9 years)**	≥3 criteria out of the next 4 components	WC ⩾90th percentile	SBP ⩾90th percentile or DBP ⩾90th percentile	Triglycerides ⩾90th percentile or	HDLc ⩽10thpercentile		HOMA-insulin resistance ⩾90th percentile or fasting glucose ⩾90th percentile

A possible solution for the metabolic syndrome definition in children is not a dichotomous definition that results in loss of information but a scoring system that takes into account all of the risk factors, their interaction, and other important characteristics, that might include family history and ethnicity. Metabolic scores can be a more reliable alternative in predicting adult risk from early life (Magge et al., 2017; Steinberger et al., 2009). In the methods section are described some metabolic scores.

1.3.3. Pathophysiology

The pathogenesis of metabolic syndrome remains debated but insulin signalling is central and can promote cardiovascular disease indirectly through development of abnormal glucose and lipid metabolism, hypertension and a proinflammatory state (Rask-Madsen & Kahn, 2012). Multiple tissues including liver, fat, muscle, and blood vessels are reached by insulin, producing diverse effects (Magge et al., 2017). According to Rask-Madsen and Kahn (2012), the most important changes in insulin signalling that contribute to metabolic syndrome include insulin signalling in adipocytes and macrophages that promotes fat tissue expansion and inflammation, impaired insulin signalling in muscle that causes insulin resistance, hepatocyte insulin resistance leading to increased glucose output and dyslipidemia. Also, insulin resistance in the hypothalamus contributes to fasting hyperglycemia and impaired appetite regulation and at the same time hyperinsulinemia may cause sympathetic overactivity and hypertension.

1.3.4. Comorbidities

Comorbidities of metabolic syndrome include nonalcoholic fatty liver disease (NAFLD) that is a spectrum of damage to the liver from steatosis to fibrosis and cirrhosis. In NAFLD, patients have liver fat >5% of liver weight (not due to alcohol consumption) and is strongly associated with insulin resistance. Another comorbidity is polycystic ovary syndrome characterised by the absence of ovulation for an extended period, elevated concentration of androgens and numerous small collections of fluid (follicles) on the ovaries. Obstructive sleep apnea and mental health disorders are recognized as other comorbidities (Magge et al., 2017).

1.3.5. Treatment

Early treatment of obesity in children and adolescents is recommended as the first option to reduce cardiometabolic risk (Magge et al., 2017). Some useful interventions for obesity in the paediatric population are to increase daily levels of physical activity, consumption of

fruits and vegetables and water consumption, while decreasing time in front of television. It might also be helpful to reinforce the parent-child relationship and establish patterns of sleep duration (Kovács et al., 2014).

1.4. Nutrients/metabolites and adiposity

In order to more precisely understand the correlation of different nutrients/metabolites with body composition and to identify new biomarkers in childhood, a list of 20 nutrients/metabolites is presented in **Table 7**. Some of them do not have previous direct results associating them with adiposity in children. We will study them in association with childhood adiposity which is a key component of the metabolic syndrome.

Table 7. List of potential plasma nutrient/metabolite biomarkers to explore for an association with adiposity in children.

	Metabolite	What is? Indication(s)?	Pathway/ function
1	Tryptophan	Involved in protein, kynurenine and serotonin synthesis. Other functions include tryptamine, melatonin, nicotinamide adenine dinucleotide (NAD), NAD phosphate (NADP) and niacin synthesis (Richard et al., 2009). In children a positive association was reported between plasma tryptophan and BMI but not between tryptophan and body fat percentage (Tan, Tint, Kothandaraman, Yap, et al., 2022).	Tryptophan Metabolites-kynurenines
2	3-hydroxyanthranilic acid (HAA)	In children, plasma HAA has been positively associated with body fat percentage and metabolic syndrome scores (Tan, Tint, Kothandaraman, Yap, et al., 2022).	Tryptophan Metabolites-kynurenines
3	3-hydroxykynurenine (HK)	In children, plasma HK has been positively associated with BMI but not with body fat percentage (Tan, Tint, Kothandaraman, Yap, et al., 2022).	Tryptophan Metabolites-kynurenines
4	Kynurenine	In children, plasma kynurenine has been positively associated with BMI and with body fat percentage (Tan, Tint, Kothandaraman, Yap, et al., 2022).	Tryptophan Metabolites-kynurenines
5	Kynurenic acid	In children, plasma kynurenic acid has been positively associated with BMI and with body fat percentage (Tan, Tint, Kothandaraman, Yap, et al., 2022).	Tryptophan Metabolites-kynurenines

	Metabolite	What is? Indication(s)?	Pathway/ function
6	Picolinic acid (PIC)	To the best of our knowledge, there are no reports studying plasma/serum PIC in association with fat mass in children.	Tryptophan Metabolites-kynurenines
7	Quinolinic acid (QA)	In children, plasma QA has been positively associated with BMI and with body fat percentage (Tan, Tint, Kothandaraman, Yap, et al., 2022).	Tryptophan Metabolites-kynurenines
8	Xanthurenic acid (XA)	In children, plasma XA has been positively associated with BMI and with body fat percentage (Tan, Tint, Kothandaraman, Yap, et al., 2022).	Tryptophan Metabolites-kynurenines
9	N1-methylnicotinamide	Vitamin B3 catabolite. Product of the methylation of nicotinamide (NAM) by the nicotinamide N-methyltransferase (NNMT) that uses S-adenosyl methionine (SAM). In adults, serum concentrations of N1-methylnicotinamide were positively associated with obesity and diabetes (M. Liu et al., 2015). To the best of our knowledge, there are no reports studying plasma/serum N1-methylnicotinamide in association with adiposity in children.	Vitamin B3 status
10	Nicotinamide (NAM)	Vitamin B3 (niacin) vitamer (Kirkland, 2007). Nicotinamide is a stimulator of appetite. In children, excess niacin (nicotinamide and nicotinic acid) intake might cause insulin resistance at high doses (Li et al., 2010).	Vitamin B3 status
11	4-pyridoxic acid (PA)	A vitamin B6 catabolite formed in the liver from pyridoxal (Ueland et al., 2017). To the best of our knowledge, there are no reports studying this metabolite in association with fat mass in children. However, in obese children an inverse correlation was found between blood vitamin B6 and body weight (Kardaş et al., 2021).	Vitamin B6 status
12	Pyridoxal (PL)	Vitamer of vitamin B6 (Van den Eynde et al., 2021). In obese children an inverse correlation was found between blood vitamin B6 and body weight (Kardaş et al.,	Vitamin B6 status

	Metabolite	What is? Indication(s)?	Pathway/ function
		2021).	
13	Pyridoxal 5'-phosphate (PLP)	Biological active vitamer of vitamin B6 (Van den Eynde et al., 2021). It is the most commonly used indicator of vitamin B6 status (Ueland et al., 2015). In obese children an inverse correlation was found between blood vitamin B6 and body weight (Kardaş et al., 2021).	Vitamin B6 status
14	Arginine	Basic amino acid precursor of methylated arginines (ADMA and SDMA) and nitric oxide (Morris, 2016). Current evidence supports the use of arginine supplementation in cardiovascular disorders (Gambardella et al., 2020). However one study in children, reported a positive correlation between serum arginine and obesity (Moran-Ramos et al., 2017).	ADMA/ Arginine pathway
15	Asymmetric dimethylarginine (ADMA)	ADMA is an endogenous competitive inhibitor of nitric oxide (NO) associated with endothelial dysfunction (Böger et al., 1998). Higher concentrations of ADMA were reported in children with excess body weight compared to children with normal weight (Czumaj et al., 2019). In obese participants (8-21 years) serum ADMA was increased without any robust correlations to obesity related disorders. ADMA was correlated with alkaline phosphatase (marker of growth) in obese and normal weight, healthy juveniles (Gruber et al., 2008).	ADMA/ Arginine pathway
16	Symmetric dimethylarginine (SDMA)	SDMA is formed by methylation of protein l-arginine residues in vivo and is useful as a marker of renal function (Meyer, 2021a). In obese participants (8-21 years) serum SDMA was decreased compared to normal weight participants (Gruber et al., 2008).	ADMA/ Arginine pathway
17	Histidine	Essential amino acid with anti-inflammatory and antioxidant properties (Holeček, 2020). A negative	Essential amino acid

	Metabolite	What is? Indication(s)?	Pathway/ function
		correlation was found between serum histidine and BMI z-score in Australian children (mean age 11.9 years) (Saner et al., 2019).	
18	Neopterin	Reflects cellular immune activation. Increases in neopterin correlate with a substantial decline in key vitamins, including folate, B6 and B12. Neopterin in serum/plasma shows a positive relation to total Hcy and to the kynurenine/tryptophan ratio (Capuron et al., 2014). Serum neopterin was inversely correlated in overweight/obese males (10 ≤ 18 years) opposite of what is seen in adults with atherosclerosis where the levels are increased (Mangge et al., 2014).	Cellular immune response
19	Sarcosine	Generated from demethylation of DMG and from methylation of glycine (Lever et al., 2011). In children, blood sarcosine positively correlated with BMI (Hirschel et al., 2020).	1C status
20	Trigonelline	A phytohormone usually used as a marker of coffee consumption (Midttun et al., 2018). To the best of our knowledge, there are no reports studying plasma/serum trigonelline in association with fat mass in children.	Phytohormone

From the above list, eight are from the kynurenine pathway that is involved in tryptophan catabolism (Stone & Darlington, 2002). An overview of the pathway is illustrated in **Figure 12** and vitamins B3 and B6 participate in this pathway. Tryptophan is one of eight essential amino acids involved in protein synthesis and a precursor of the kynurenine pathway and serotonin synthesis. Other functions include the synthesis of tryptamine (neuromodulator of serotonin), melatonin (hormone that regulates diurnal rhythms and influences the reproductive and immune systems), NAD, NADP (these enzymes can be synthesised de novo from tryptophan or from ingestion of vitamin B3 (niacin)) and niacin (Richard et al., 2009).

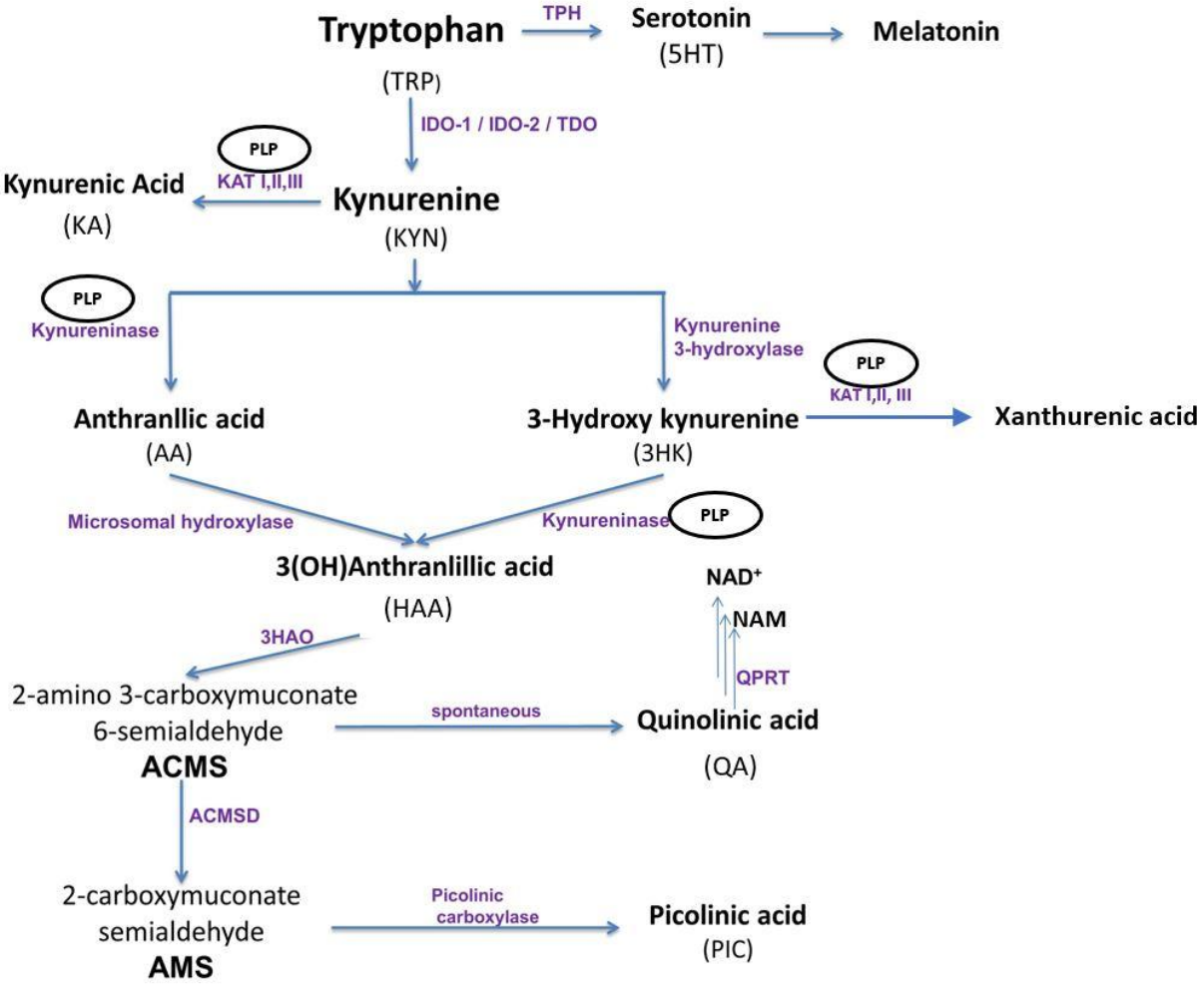


Figure 12. Kynurenine pathway.

Tryptophan is recruited into the pathway by activation of indolamine 2,3 dehydoxylase-1(IDO-1), indolamine 2,3 dehydoxylase-2 (IDO-2) or tryptophan 2,3 deogygenase-2 (TDO-2) and subsequent conversion into kynurenine (KYN). Kynurenine is metabolised along the pathway into a range of neuroactive and immunomodulatory metabolites. KYN can be metabolised by kynurenine aminotransferase (KAT) −1, 2 or 3 to kynurenic acid (KA) or by kynureninase to anthranillic acid (AA) or kynurenine 3-hydroxylase (KMO) to 3-hydroxykynurenine (3HK). Both 3HK and AA can be metabolised to 3-hydroxyanthranillic acid (HAA) by kynureninase and microsomal hydroxylase respectively. The latter can be metabolised by 3-Hydroxyanthranilic acid 3,4-dioxygenase (3HAO) to 2-amino 3-carboxymuconate 6-semialdehyde (ACMS) which spontaneously forms the nicotinamide adenine dinucleotide (NAD) precursor quinolinic acid (QA). ACMS can also actively be metabolised into 2-carboxymuconate semialdehyde (AMS) which can then be metabolised into picolinic acid (PIC) by picolinic carboxylase. KAT and kynureninase are pyridoxal 5´-phosphate (PLP)-dependent enzymes. Adapted from (de Bie et al., 2016) and from (Ueland et al., 2017).

There are numerous studies looking at tryptophan associated with behaviour and neurodegenerative diseases as it is the precursor to serotonin (Richard et al., 2009). More recently, tryptophan and kynurenine pathway have been associated with obesity. For

instance, before and after bariatric surgery, morbidly obese patients have chronic immune activation, which, in turn, activates the kynurenine pathway leading to tryptophan reduction. The authors propose that the persistent immune activation also causes serotonin reduction, generating satiety dysregulation and a reward-deficiency-syndrome (Brandacher et al., 2006). A marker of the activation of the immune system during inflammation processes is neopterin (Gieseg et al., 2018). Some studies have reported an inverse relationship between tryptophan and neopterin concentrations, which is probably also connected to a positive correlation observed between neopterin and the activity of the enzyme indolamine 2,3 dehydoxylase (IDO) (which converts tryptophan to kynurenine as shown in **Figure 12**) (Brandacher et al., 2007).

Other authors have reviewed additional data suggesting that one of the mechanisms by which chronic inflammation could contribute to the development of the metabolic syndrome is the upregulation of the kynurenine pathway (Oxenkrug, 2010). However, in children there are few studies investigating this pathway in association with metabolic syndrome in children, specially the kynurenine downstream metabolites (Barat et al., 2016; Butte et al., 2015; Lischka et al., 2022; Mangge et al., 2014; Tan, Tint, Kothandaraman, Yap, et al., 2022).

Regarding other plasma metabolites that we are going to explore in association with child adiposity, trigonelline (a type of betaine) is a phytohormone very abundant in coffee (Midttun et al., 2018) and in *Trigonella foenum-graecum L.* (fenugreek) (Choi et al., 2021). Also found in cereal flours (Servillo et al., 2018), alfalfa sprouts, peas, fruits, citruss juice (particularly mandarin juice), tomato, avocado and bananas (Servillo et al., 2015). Trigonelline can be produced by the methylation of vitamin B3 (niacin) and also trigonelline breaks down to niacin at high temperatures (Garg, 2016). In animal models it has been found that trigonelline decreased the concentration of trimethylamine N-oxide (TMAO) that is a pro-atherosclerotic metabolite product of choline, through the inhibition of a bacteria involved in choline metabolism (Anwar et al., 2018). There is evidence in animals of its effects especially to lower blood sugar, cholesterol and triglyceride, but also as neuroprotective and for the protection of liver and kidney (Mohamadi et al., 2018).

2. Hypothesis

Imbalances in one carbon metabolism during pregnancy and childhood are associated with offspring growth and metabolic outcomes from birth to 6-8 years of age.

3. Global aim

To investigate the transgenerational association between maternal one carbon metabolism, and growth and health in the child.

Specific aims

- To investigate the association between maternal pregnancy plasma tHcy, cobalamin status, folate and betaine with a metabolic score in children aged 6–7.5 years.
- To determine the association between maternal pregnancy plasma betaine, DMG/betaine ratio and the *BHMT* c.716G>A polymorphism with foetal growth (birthweight and small for gestational age).
- To describe the association between child plasma betaine and related 1C metabolites with adiposity and other anthropometric measures in the child during mid-childhood.
- To investigate differences between mid-childhood (7.5 y) plasma metabolite status according to maternal pregnancy plasma cobalamin and tHcy status and to identify child plasma markers of adiposity.

4. Methods

4.1. Participants

Pregnancy cobalamin status, homocysteine, folate and mid childhood outcomes.

Mother-child dyads from the PreC (Preconception) and RTBC (Reus-Tarragona Birth Cohort) studies, participated from preconception/early pregnancy (< 12 gestational weeks) over 7-9 years (**Figure 13**). The studies were approved by the Sant Joan Reus (SJR) and Joan XXIII Tarragona (JXXIII) University Hospitals' Ethics Committees and conducted in accordance with the Declaration of Helsinki guidelines (World Medical Association, 2013) with signed informed consent from participants. Signed consent was obtained from the parents/guardians and verbal assent from the children for the child phase.

Recruitment, described previously (PreC (Murphy et al., 2002, 2004, 2017) and RTBC (Fernàndez-Roig et al., 2013)), was by the Unit of Preventive Medicine and Public Health, Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili and the Units of Obstetrics and Gynaecology, SJR and JXXIII University Hospitals.

Non-pregnant women volunteered for the PreC study following local city hall and media advertisements. None took folic acid supplements periconceptionally because the study was before the introduction of current recommendations. Some took folic acid-containing supplements coinciding with iron supplementation in mid-late pregnancy and 35 women never took them throughout pregnancy.

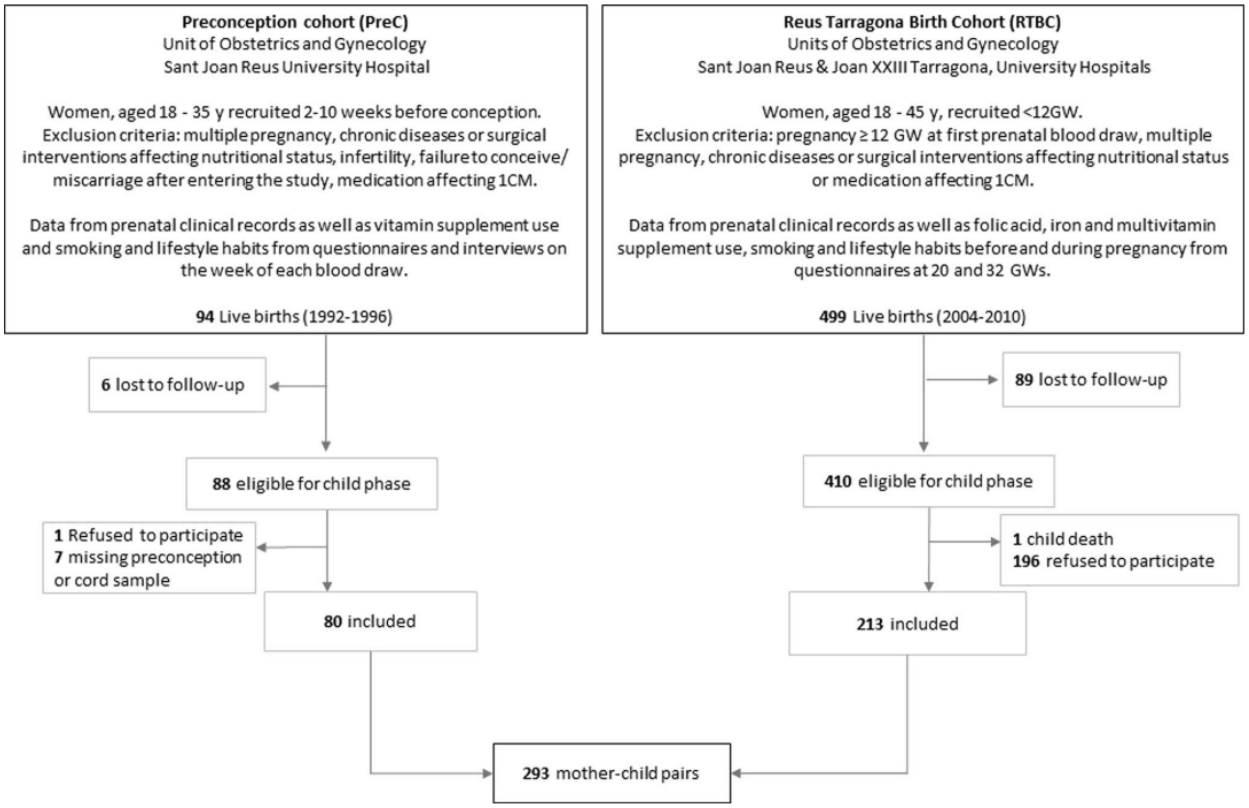


Figure 13. Participant recruitment and follow-up of the mother-child dyads used for the study of pregnancy cobalamin status, homocysteine, folate, betaine and mid childhood outcomes.
1CM: 1 carbon metabolism. Pregnancy betaine-mid childhood outcomes analysis only includes RTBC children.

For the RTBC, women with confirmed singleton pregnancies and providing their first prenatal blood draw at <12 GW were recruited from the high risk obstetrics units and University/Hospital staff. They were advised at their first prenatal check-up to take supplements containing 400 µg folic acid/d and 2 µg cyanocobalamin/d for the 1st trimester and 40 mg iron/d, after 12 GW. Women with anaemia were treated by their clinicians, and the iron doses recorded.

Betaine and the DMG pathway.

Figure 14 shows participant recruitment and follow up of the RTBC cohort included in the section studying the association between pregnancy betaine status, DMG/betaine ratio, the maternal and foetal *BHMT* c.716G>A polymorphism and foetal growth (n=748).

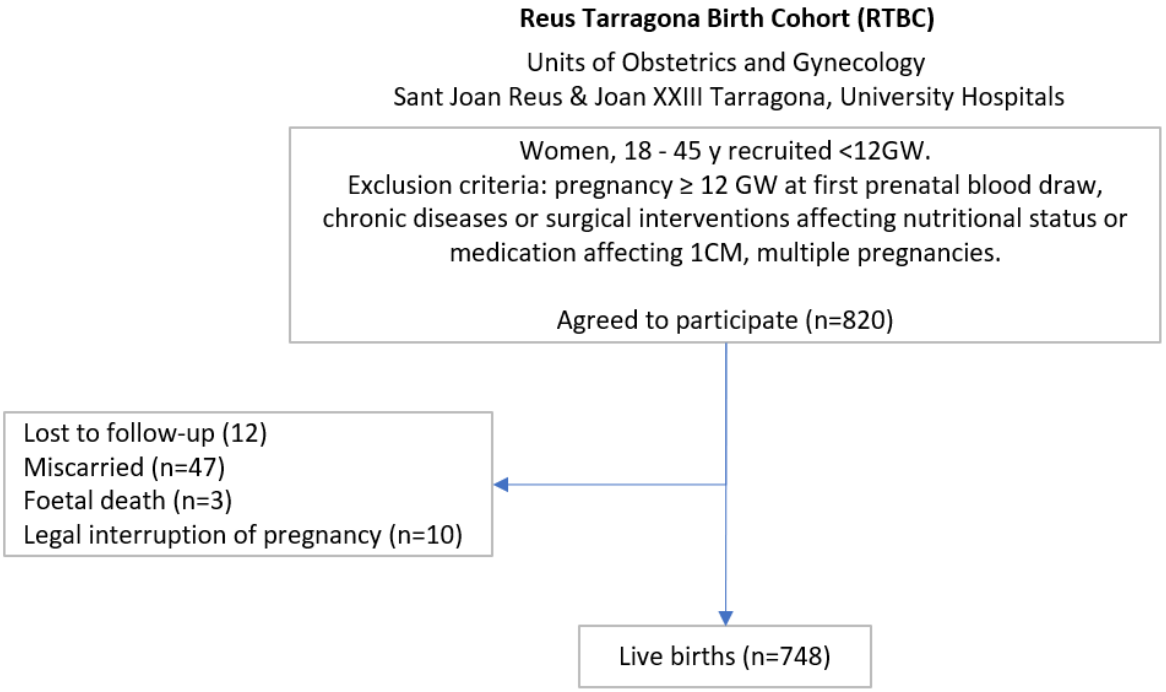


Figure 14. Participants recruitment and follow-up of the pregnancy phase of the mother-neonate dyads used for betaine and DMG pathway study in association with foetal growth.
1CM: 1 carbon metabolism.

For the association of maternal pregnancy plasma betaine with mid-childhood outcomes, we included the same 213 RTBC mother-child pairs shown in **Figure 13**.

To study the association between child plasma nutrient or metabolite status with anthropometric measurements at 7.5 years of age, we included 238 RTBC children (**Figure 15**).

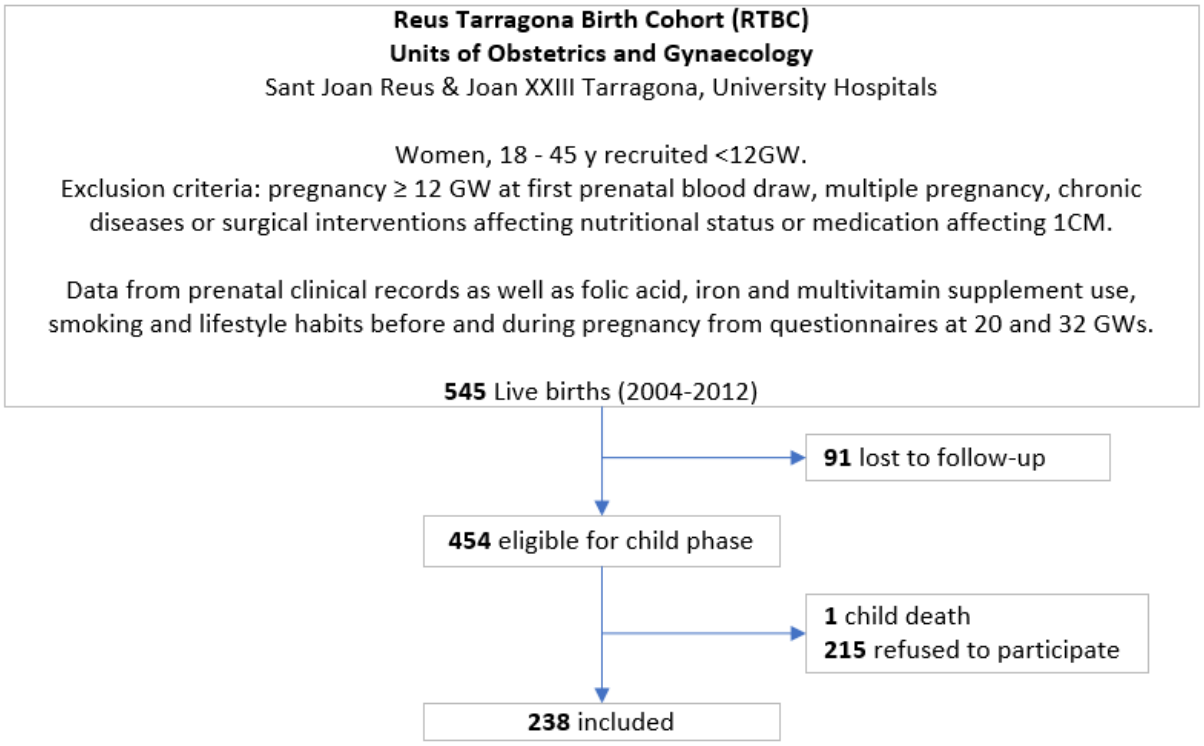


Figure 15. RTBC children including for the analysis of plasma nutrient/metabolite status with anthropometric measurements.

4.2. Processing and storage of blood samples

Fasting blood samples were collected from the mothers at <12 GW (both cohorts), 15 GW (RTBC), 20 GW (Prec), 24-27 GW (RTBC), 32 GW (PreC), 34 GW (RTBC), non-fasting samples on admission to the hospital with confirmed labour and from the cord for both cohorts (**Figure 16**). The samples were kept at 4°C, and plasma separated within 1-2 hours. Plasma samples were stored at -20 ºC (PreC) and -80°C (RTBC) until analysis of all samples from the same pregnancy in the same batch.

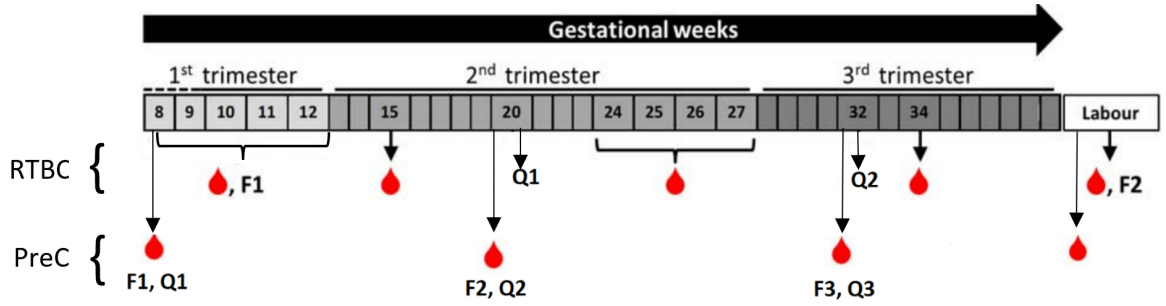


Figure 16. PreC and RTBC Study pregnancy phase.
Red drops indicate the timing of blood sample collection. F shows the food frequency questionnaires. Q shows the lifestyle questionnaires. Adapted from (Colomina, 2017).

Aliquots of 50µl of non-clotted (EDTA-K2 tube) whole blood for red blood cell folate (RBCF) determinations, are diluted with 450 µl freshly prepared ascorbic acid solution (1% *(w/v) Panreac)* and kept at room temperature for 30 minutes. The proportion of whole blood to ascorbic acid solution is 1:10. This procedure hemolyzed RBCF osmotically in the ascorbic acid hypotonic solution. Due to the hemolysis, gamma glutamyl hydrolase and cellular folates are released. The activity of the endogenous hydrolase is improved by the acidic pH, and it leads to the deconjugation of the polyglutamates to monoglutamates (Pfeiffer et al., 2009).

4.3. Medical and obstetrical history and lifestyle data collection

The following data was recorded from the prenatal clinical histories: 1st trimester weight, height, parity, age, blood pressure, haemoglobin and blood glucose at the routine prenatal checkups; gestational hypertension, preeclampsia and gestational diabetes.

Participants completed questionnaires on vitamin supplement use, toxic and lifestyle habits before and during pregnancy at 20 and 32 GW.

Similarly, for the PreC cohort, questionnaires about lifestyle and vitamin use were completed around the time of each blood sample (8 GW, 20 GW, 32 GW, birth).

Birthweight percentile was recorded according to the Spanish growth curves based on sex and gestational age at delivery (Santamaría et al., 1998). Babies below percentile 10 were considered small for gestational age (SGA) (World Health Organization, 1995).

4.4. Child follow-up

Children participated in the PreC study at 6 years and in the RTBC at 7.5-8 years of age.

They were called for a check-up in which clinical data was recorded. Parents were also asked to bring the children's health records, where further information was collected from. The parents were interviewed to complete questionnaires regarding the children's lifestyle habits.

Anthropometric measurements were also recorded at the check-ups. Height was measured by a stadiometer (0.1 cm accuracy). Children stood still, with their heels together and feet facing outwards at a 60º angle, head in the Frankfort plane and palms of their hands placed on their legs.

Weight was measured by mechanical beam scale with height rod (Pesperson model) (PreC) and electronic scale with a precision of 0.100 g (Tanita BC-420MA, Tanita Corporation, Tokyo, Japan) (RTBC).

BMI was calculated as weight (kg) divided by height squared (m^2). Age-and sex-specific BMI z-scores were calculated according to Spanish references (Carrascosa et al., 2008).

Skinfold thicknesses were recorded as indicators of body composition in both the Prec and RTBC child check-ups. The following circumferences were measured twice with a

non-stretchable measuring tape (Seca GmBH & Co Kg, Hamburg, Germany) with an accuracy of 0.1 cm: head, mid-upper-arm, chest, waist (according to WHO (World Health Organization, n.d.) and hip.

Means of triplicate triceps (halfway between the acromion and the olecranon process at the back of the arm) and subscapular (20 mm below the tip of the scapula, at an angle of 45° to the lateral side of the body) skinfold thicknesses were measured by Harpenden skinfold calliper (Holtain Ltd, Crymych, Wales), with an accuracy of 0.2 mm. Fat mass percentage (x) was determined from the sum of triceps (mm) and subscapular (mm) skinfold thicknesses (y) (Slaughter et al., 1988). Fat mass percentage was used to calculate fat mass index (Nagy et al., 2016).

For y ≤ 35 mm:

x (Boys)= $1.21y-0.008y^2-1.7$

x (Girls)= $1.33y-0.013y^2-2.5$

For y >35 mm:

x (Boys)=0.783y+1.6

x (Girls)= 0.546y+9.7

$$Fat\ mass\ index\ (FMI) = \frac{((weight\ in\ kg)\ X\ ((\frac{Sex\ specific\ x)}{100}))}{(Height\ in\ m)^2}$$

Only for RTBC children, body composition was also estimated using tetrapolar bioelectrical impedance analysis (Tanita BC-420MA, Tanita Corporation, Tokyo, Japan). This non-invasive technique is based on the concept that the electrical resistance to a current flow (50 kHz) in

the body is related to the amount of total body water and, therefore, to the fat-free mass (Davies, 1993). Fat-free mass and muscle mass were recorded.

Blood pressure was only measured at the RTBC health check-up. Children were asked to sit without speaking or crossing their legs. Three blood pressure measurements, at 1 minute intervals, were made with an automatic blood pressure monitor (Omron M6 AC, Omron Healthcare Co, Kyoto, Japan) with a precision of 1mm Hg.

Children's blood samples were collected at 6-7.5 years and processed and stored as described for the mothers, in the Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili until analysis.

4.5. Biochemical determinations of metabolites in fasting blood

Frozen plasma aliquots were sent to Trinity College Dublin (Prof John Scott's lab, Biochemistry Department) in the case of the PreC study. Plasma folate concentrations were determined by microbiological assay using *Lactobacillus casei (Molloy & Scott, 1997)* and plasma cobalamin using *Lactobacillus leichmannii* (Kelleher & Broin, 1991). Fasting plasma total homocysteine was determined by IMx homocysteine immunoassay (PreC) (Abbott Laboratories Diagnostics Division, Abbott Park, IL) (Murphy et al., 2002). The same determinations were carried out in the BeVital AS laboratory (Bergen, Norway) for the RTBC study, including folate erythrocyte using the microbiological assay mentioned above and using liquid-tandem mass spectrometry to determine tHcy (Ueland et al., 2007). Plasma MMA was determined by gas chromatography–mass spectrometry (GC-MS) with methylchloroformate derivatization (Ueland et al., 2007).

For the RTBC study, plasma status in other nutrients and metabolites including choline, betaine, dimethylglycine, cysteine and methionine were determined by liquid or gas chromatography - mass spectrometry.

The determination of a further 20 plasmatic nutrient/metabolites was carried out by liquid, gaseous chromatography or high performance liquid chromatography - mass spectrometry (LC-MS/MS, GC-MS/MS, HPLC-MS/MS). The metabolites studied were: 3-hydroxyanthranilic acid (HAA), 3-hydroxykynurenine (HK), 4-pyridoxic acid (PA), arginine, asymmetric dimethylarginine (ADMA), histidine, kynurenine, kynurenic acid, N1-methylnicotinamide, neopterin, nicotinamide (NAM), picolinic acid (PIC), pyridoxal (PL), pyridoxal 5'-phosphate (PLP), quinolinic acid (QA), sarcosine, symmetric dimethylarginine, trigonelline, tryptophan, xanthurenic acid (XA).

Plasma Insulin: was determined by Mercodia Iso-Insulin ELISA Kit (a solid-phase two-site enzyme immunoassay, Mercodia, Sweden).

Plasma glucose: was determined by the glucose oxidase (GOD) peroxidase (POD) method (Spinreact, Spain).

4.5.1. Homeostatic model assessment (HOMA)

Insulin resistance in children was calculated with the formula: HOMA-IR = (FPI × FPG)/22.5 where FPI is fasting plasma insulin concentration (mU/l) and FPG is fasting plasma glucose (mmol/l) (T. M. Wallace et al., 2004).

4.5.2. Lipid profile

All of the children lipid profile determinations were carried out on the COBAS MIRA autoanalyser (Roche Diagnostics SL, Basel, Switzerland).

Total cholesterol and high density lipoprotein cholesterol (HDLc) were determined in plasma by enzymatic colorimetric techniques (Spinreact, Spain).

Triglycerides were determined in plasma by glycerol phosphate oxidase (GPO) peroxidase (POD) technique (Spinreact, Spain).

Low-density lipoprotein cholesterol (LDL) was estimated by the Friedewald formula:

LDLc = Total cholesterol - HDL cholesterol - triglycerides/5 in mg/dL (Friedewald et al., 1972).

When plasma TG exceeds 400 mg/dL (4.52mmol/L) the formula cannot be used. In our cohort, the maximum concentration reached in the children was 1.6 mmol/L.

Plasma lipoprotein(a) (Lp(a)) was determined by quantitative turbidimetric test Lp(a)-turbilatex (Spinreact, Spain) .

Plasma apolipoprotein A1 (ApoA1) and B (ApoB): were determined by turbidimetry technique (ABX Pentra, France).

4.5.3. Metabolic score

The dichotomous nature of the definition of metabolic syndrome results in loss of information (Steinberger et al., 2009). For this reason, a metabolic score is studied. We chose a score proposed by Ahrens et al. (Ahrens et al., 2014) that combines the components used to define metabolic syndrome in adults, that is, waist circumference, blood pressure, dyslipidemia and hyperglycemia. Ahrens et al. metabolic score is defined as:

Metabolic score: zWC+(zSBP+zDBP)/2+(zTRG–zHDL)/2+zHOMA-IR

WC: waist circumference; SBP: systolic blood pressure; DBP: diastolic blood pressure; TRG: triglycerides; HDL: HDL cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; zvar: standardized residuals of the corresponding variable.

For the thesis, a modified version of the score (MetSco) is proposed in order to include children who do not have blood pressure measurement and waist circumference, but instead have skinfold measurements. We derived z-scores (standardised residuals) from a generalized linear model (GLM) of every component (FMI, HOMA-IR, Lipids) as the dependent variable, and age and sex as the predictors. A higher score indicates a less-favourable metabolic profile.

MetSco modified: zFMI + ((zTRG – zHDLc)/2) + zHOMA-IR

FMI: fat mass index from skinfold measurements. It was chosen as a surrogate for excess fat mass instead of waist circumference. TRG: triglycerides; HDLc: HDL cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; zvar: standardised residuals of the corresponding variable.

The results using the adapted version of Ahrens' metabolic score were compared with the same score as Ahrens in a subgroup of RTBC children in which all of the variables were available to calculate the score.

4.6. DNA extraction and genotyping from leukocytes

After separating the plasma, the cell pellets are hemolyzed and kept at room temperature in Cell Lysis solution (Qiagen, Germany) for a minimum of one month to initiate the process of DNA extraction from the leukocytes.

The Gentra Puregene Blood Kit (Qiagen, Germany) was subsequently used to extract DNA from the leukocytes. In this procedure, the proteins are precipitated in order to separate them from the DNA. Following DNA precipitation, the sample is kept for approximately one week between 2-8ºC before quantifying the DNA using the NanoDrop ND-1000

Genetic polymorphisms were studied by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (Bevital; www.bevital.no and Universidad de Santiago de Compostela). All samples from the same pregnancy are analysed in the same batch.

4.7. Statistical analysis

Pregnancy cobalamin status, homocysteine, folate and mid childhood outcomes.

Data from the Prec and RTBC cohorts were combined to ensure sufficient statistical power.

Variable distribution normality was tested by the Kolmogorov-Smirnov test and ln-transformation to approach normality applied as required for parametric tests. Quantitative variables were compared between categories by the Student's unpaired t-test, medians by the median test for K independent samples (SPSS) and proportions by the Chi-square test. Correlations between variables are reported as Spearman's rank-order correlation coefficients. Associations between pregnancy tHcy, cobalamin, holoTC and folate status and mid-childhood outcomes (MetSco and its components) were investigated by multiple linear regression analysis. Associations were determined for the highest maternal tertiles of plasma tHcy and MMA compared to the low-mid tertiles (combined) and lowest maternal tertiles of plasma cobalamin, holoTC and folate compared to the mid-high tertiles (combined).

Associations between pregnancy tHcy, cobalamin, holoTC, MMA and folate status and mid-childhood outcomes (MetSco and its components) were investigated by multiple linear regression analysis. Models were adjusted for maternal characteristics (preconception (PreC) and 1st trimester (RTBC) maternal age, body mass index (BMI), socioeconomic status, pregnancy smoking pattern ((never (reference group), 1st trimester only, throughout pregnancy) and child characteristics ((breastfeeding yes/no), BMI z-score (Carrascosa et al., 2008) as a substitute for energy intake for both cohorts because energy intake was not available for PreC, and tHcy). Mediation analysis was used to test whether the pregnancy tHcy-offspring outcomes associations were mediated by birthweight z-score (Spanish birthweight tables (Santamaría et al., 1998)).

Assumptions in linear regression (linearity, homogeneity of variance (homoscedasticity), normality of errors, independence of errors between the 2 cohorts, model specification and multicollinearity) were checked (UCLA: Statistical Consulting Group., n.d.).

Unusual and influential data were detected by inspecting scatterplots of the independent and dependent variables for potential atypical values. Those with a Cook's distance > 4/n and in the top 10 greatest Cook's distance were excluded (N=4).

SPSS version 27.0 for Windows, with the PROCESS macro (Hayes, 2017) for the mediation analysis, was used.

Status in RTBC children plasma nutrients/metabolites at 7.5 years according to pregnancy plasma cobalamin (lowest vs mid-high cobalamin tertile) and tHcy status (highest vs low-mid tertile) was compared by ANCOVA with post hoc Bonferroni correction, adjusting for child cobalamin or child tHcy respectively. Furthermore, descriptive results of child plasma metabolite status according to child plasma cobalamin and tHcy status is reported.

Betaine and the DMG pathway.

We performed the betaine and the DMG pathway analysis considering only the RTBC cohort. In PreC nutrients and metabolites of these pathways were not determined.

Betaine, DMG/betaine during pregnancy and in the cord, BHMT polymorphism and birthweight and SGA babies.

Descriptive data is reported as percentages (95% CIs) for categorical variables and arithmetic means (95% CIs) or geometric means (95% CIs) for continuous variables.

Linear and logistic regressions were used to explore the association between plasma betaine, DMG/betaine ratio (during pregnancy and in cord in both cases) and *BHMT* c.716G>A polymorphism with the outcomes of birthweight and SGA babies.

Assumptions in linear regression (linearity, homogeneity of variance (homoscedasticity), normality of errors) were checked. (UCLA: Statistical Consulting Group., n.d.).

Linear models of pregnancy and cord plasma betaine associations with birthweight were adjusted for maternal and baby characteristics: sex, gestational age at delivery, socioeconomic status (low versus mid-high), parity (primipara or multipara versus nullipara (meaning this is the first pregnancy)), maternal age at the beginning of pregnancy, maternal body mass index at the beginning of pregnancy, maternal height, smoking in the 1st trimester versus never, smoking throughout pregnancy versus never, gestational diabetes mellitus (yes/no), anaemia in the last trimester, maternal plasma folate at each point of pregnancy or in the cord and plasma cobalamin (low tertile vs. mid-high) in the 1st trimester, hypertension (participant with underlying hypertension, pregnancy induced hypertension and preeclampsia versus normotensive participants).

Linear models of DMG/betaine, as the independent variable and birthweight as an outcome, were adjusted for the same covariables as betaine models but without folate and cobalamin because they might affect the ratio.

When studying SGA as an outcome, sex and gestational age were not included as covariables because the SGA definition already includes them. Logistic models testing the association between pregnancy and cord plasma betaine with SGA were adjusted for socioeconomic status (low versus mid-high), parity (primipara or multipara versus nullipara (meaning this is the first pregnancy)), maternal age at the beginning of pregnancy, maternal BMI at the beginning of pregnancy, maternal height, smoking during pregnancy (yes/no), gestational diabetes mellitus (yes/no), anaemia in the last trimester, plasma folate in each point of pregnancy or cord plasma folate and 1st trimester plasma cobalamin (lowest tertile (≤ 315.5) vs. mid-high) hypertension (underlying hypertension, pregnancy induced hypertension and preeclampsia versus normotensive participants).

The Hardy–Weinberg equilibrium of the *BHMT* c.716 G>A polymorphism was tested using the Court Lab-HW calculator (Michael H. Court, 2005–2008) (*Website*, n.d.).

Logistic models testing the association between the maternal and child *BHMT c.716 G>A* polymorphism and SGA at birth were adjusted for socioeconomic status (low versus mid-high), parity (primipara or multipara versus nullipara (meaning this is the first pregnancy)), maternal age at the beginning of pregnancy, maternal body mass index (BMI) at the beginning of pregnancy, maternal height, smoking in the 1st trimester versus never, smoking throughout pregnancy versus never, hypertension (participant with underlying hypertension, pregnancy induced hypertension and preeclampsia versus normotensive participants), gestational diabetes mellitus (yes/no), anaemia in the last trimester, 1st trimester plasma cobalamin (low tertile (≤ 315.5 pmol/L) vs. mid-high), pregnancy or cord plasma folate. Plasma betaine during pregnancy was not included due to an interaction at 12 GW and in cord with the maternal polymorphism.

For analysing the relationship between the maternal and baby *BHMT* c.716 G>A polymorphism with SGA we tested the 4 main risk models: codominant (GA versus GG, AA versus GG), dominant (GA+AA versus GG), recessive (AA versus GG+GA) and additive that are explained in detail next (Iniesta et al., 2005): The codominant model tests whether each genotype provides a different and non-additive disease risk. Heterozygotes (GA) and variant homozygotes (AA) are compared separately to the homozygotes of the most frequent allele (GG). The dominant model tests whether a single copy of A is enough to modify the risk, compared to the GG homozygotes. The same magnitude of risk occurs for 2 copies: GA and AA carry the same risk. The recessive model tests whether 2 copies of A are necessary to modify the risk. In that case, the GA and GG genotypes would have the same risk and the AA variant genotype is compared with the combination of the GG and GA genotypes. The additive model assumes that each copy of A modifies the risk by an additive amount so that the AA genotype will have double the risk of the GA genotype. Weight 1 is given to GA heterozygotes and weight 2 to AA homozygotes.

We checked all of the logistic regressions analyses for outliers, outside 3 studentized residues and a sensitivity analysis was performed to see the effect of excluding these from the models, if any were found.

In all models adjusting for cobalamin status, 1st trimester status (lowest plasma tertile vs. mid-high) was always used because we considered it to be the best indicator of underlying impaired maternal status (not due to the normal course of pregnancy). Low plasma concentrations in late pregnancy may reflect placental uptake of the vitamins rather than impaired maternal status (Graber et al., 1971).

Betaine and DMG/betaine during pregnancy and in cord and mid childhood outcomes.

Linear regressions were used to explore the association between plasma betaine and DMG/betaine during pregnancy and in the cord with mid-childhood outcomes (MetSco and its components).

Unusual and influential data from the linear regressions were first detected by inspecting scatterplots of the independent and dependent variables to find atypical data. Those with a Cook's distance > 4/n and in the top 10 greatest Cook's distance were excluded (N=3). (UCLA: Statistical Consulting Group., n.d.).

To investigate the association between plasma betaine (1st trimester, 3rd trimester and cord) and mid-childhood outcomes, models included 1st trimester maternal age, body mass index (BMI), pregnancy smoking pattern ((never (reference group), 1st trimester only, throughout pregnancy), plasma folate at each time point (1st trimester, 3rd trimester and cord) and 1st trimester cobalamin (lowest tertile vs. mid-high). Child characteristics: BMI z-score (Carrascosa et al., 2008) as a substitute for energy intake for consistency with the analysis of the other section (association of pregnancy folate, tHcy, cobalamin and related markers with metabolic score) and child betaine.

Models testing the association between pregnancy DMG/betaine (1st trimester, 3rd trimester and cord) and mid-childhood outcomes adjusted for: same covariables as the plasma betaine model above but excluding plasma folate and cobalamin (because they might affect the ratio) and substituting child betaine with child DMG/betaine.

Models of both maternal plasma betaine and DMG/betaine were not adjusted for breastfeeding and for socioeconomic status so as not to exceed the recommended inclusion of excessive variables in the regression model (10% of the n included in the model). There were only 6 children in the low socioeconomic category. A sensitivity analysis showed that their inclusion/exclusion did not change the betaine or DMG/betaine associations with the studied outcomes.

Testing the associations between child plasma betaine and other 1C metabolites with adiposity and other anthropometric measures in the child.

The associations between child plasma betaine , other 1C nutrients and metabolites (plasma DMG, methionine, choline, folate, red blood cell folate, cobalamin, MMA, total homocysteine and total cysteine) with child adiposity were tested by Spearman's rank-order correlation tests. Five adiposity measures were tested (waist circumference according to WHO, waist circumference/height ratio, fat percentage from tetrapolar bioelectrical impedance, fat mass index from skinfold thickness (Nagy et al., 2016), body mass index).

The association between child plasma betaine, other 1C nutrients and metabolites (plasma DMG, methionine, choline, folate, red blood cell folate, cobalamin, MMA, total homocysteine and total cysteine) and fat free mass, muscle mass, height and body circumferences other than waist circumference (head, arm, trunk) were tested by Spearman's rank-order correlation tests.

Identification of metabolites associated with adiposity in 7.5 year-old children.

We performed these analyses considering only the RTBC cohort. In PreC nutrients and metabolites of **Table 7** were not determined.

In order to identify metabolites potentially associated with adiposity that may be relevant for the study and prevention of metabolic syndrome in children, we performed a bivariate Spearman's rank-order correlation of 20 nutrients/metabolites (**Table 7**) with measures of fat mass (waist circumference according to WHO, waist circumference/height ratio, fat

percentage from tetrapolar bioelectrical impedance, fat mass index from skinfold thickness (Nagy et al., 2016), body mass index). Nutrients/metabolites that had correlations approaching significance ($p<0.1$) with at least 3 measurements of fat mass (looking at girls and boys together) were reported.

5. Results

5.1. Pregnancy cobalamin status, homocysteine, folate and mid childhood outcomes

The results of this section are part of the publication:

Alejandra Rojas-Gómez, Pol Solé-Navais, Pere Cavallé-Busquets, Gemma Ornosa-Martin, Carme Grifoll, Carla Ramos-Rodriguez, Joan Fernandez-Ballarta, Luis Masana, Mónica Ballesteros, Per Magne Ueland, Michelle M Murphy. *Pregnancy Homocysteine and Cobalamin Status Predict Childhood Metabolic Health in the Offspring.* Pediatrics Research 2022. DOI: 10.1038/s41390-022-02117-5.

Participant characteristics according to pregnancy tHcy status are reported in **Table 8**. Maternal (including age, BMI, parity, smoking habits and socioeconomic status) and child (including male sex prevalence, birthweight z-score, low birthweight (<P10) and breastfeeding regime) characteristics were similar between the pregnancy tHcy categories.

The prevalence of overweight-obesity according to Spanish tables (Carrascosa et al., 2008) was higher in children born to mothers in the highest tHcy tertile in the 1st trimester of pregnancy compared to the low-mid tertiles but there was no difference among the 3rd trimester tHcy tertiles.

Table 8. Participant characteristics according to pregnancy tHcy status.

		1st trimester		3rd trimester	
	All	**tHcy low-mid tertiles[a]**	**tHcy highest tertile**	**tHcy low-mid tertiles[b]**	**tHcy highest tertile**
Mothers during pregnancy					
Age (years)[c,d]	32.0 (27.0, 37.0) [289]	32.0 (27.9, 37.0) [178]	30.5 (26.0, 36.1) [98]	32.0 (27.0, 37.0) [173]	31.0 (26.7, 36.0) [86]
BMI (kg/m^2)[c,d]	23.0 (19.9, 27.3) [285]	22.8 (20.1, 27.1) [178]	23.3 (19.7, 28.1) [95]	22.7 (20.1, 27.0) [171]	22.9 (19.2, 28.7) [85]
Parity (nulliparous)[e]	52.6 (46.8, 58.2) [152/289]	48.9 (41.6, 56.2) [87/178]	56.1 (46.3, 65.5) [55/98]	51.4 (44.0, 58.8) [89/173]	52.3 (41.9, 62.6) [45/86]
Smoking during pregnancy[e]					
Never	78.8 (73.7, 83.1) [227/288]	79.8 (73.3, 85.0) [142/178]	77.6 (68.3, 84.7) [76/98]	79.8 (73.2, 85.1) [138/173]	79.1 (69.3, 86.3) [68/86]
Periconception/ 1st trimester only	6.9 (4.5, 10.5) [20/288]	6.2 (3.5, 10.7) [11/178]	7.1 (3.5, 14.0) [7/98]	7.5 (4.4, 14.4) [13/173]	5.8 (2.5, 12.9) [5/86]
Throughout	14.2 (10.7, 18.7) [41/288]	14.0 (9.7, 19.9) [25/178]	15.3 (9.5, 23.7) [15/98]	12.7 (8.5, 18.5) [22/173]	15.1 (9.1, 24.2) [13/86]

		1st trimester		3rd trimester	
	All	**tHcy low-mid tertiles[a]**	**tHcy highest tertile**	**tHcy low-mid tertiles[b]**	**tHcy highest tertile**
Socioeconomic status[e]					
Low	10.4 (7.4, 14.5) [30/288]	7.3 (4.3, 12.1) [13/178]	14.3 (8.7, 22.6) [14/98]	9.8 (6.2, 15.2) [17/173]	10.5 (5.6, 18.7) [9/86]
Middle	42.0 (36.5, 47.8) [121/288]	42.1 (35.1, 49.5) [75/178]	41.8 (32.6, 51.7) [41/98]	39.9 (32.9, 47.3) [69/173]	41.9 (32.0, 52.4) [36/86]
High	47.6 (41.9, 53.3) [137/288]	50.6 (43.3, 57.8) [90/178]	43.9 (34.5, 53.7) [43/98]	50.3 (42.9, 57.7) [87/173]	47.7 (37.4, 58.1) [41/86]
Children at birth and infancy					
Boys[e]	48.4 (42.7, 54.2) [140/289]	46.6 (39.4, 54.0) [83/178]	52.0 (42.3, 61.7) (51/98)	49.1 (41.8, 56.5) (85/173)	46.5 (36.3, 57.0) (40/86)
Birthweight z-score[d,f]	-0.073 (-1.055, 1.224) [286]	-0.042 (-0.992, 1.279) [178]	-0.097 (-1.178, 1.185) [97]	-0.097 (-1.015, 1.190) [173]	-0.002 (-0.996, 1.385) [86]
Birthweight < P10[e,f]	6.6 (4.3, 10.1) [19/286]	5.6 (3.1, 10.0) [10/178]	7.2 (3.5, 14.2) [7/97]	5.8 (3.2, 10.3) [10/173]	5.8 (2.5, 12.9) [5/86]

		1st trimester		3rd trimester	
	All	**tHcy low-mid tertiles[a]**	**tHcy highest tertile**	**tHcy low-mid tertiles[b]**	**tHcy highest tertile**
Breastfed: Yes (min 1 month)[e]	72.4 (66.9, 77.3) [202/279]	69.7 (62.5, 76.0) [122/175]	74.5 (64.8, 82.2) [70/94]	75.1 (68.1, 81.1) [127/169]	67.5 (56.8, 76.6) [56/83]
Mid-childhood check-up					
Age (months)[d]	89.0 (72.0, 91.0) [289]	89.0 (72.0, 91.0) [178]	88.0 (72, 92) [98]	89.0 (72.0, 91.6) [173]	88.0 (72.0, 92.0) [86]
BMI (kg/m^2)[d]	16.3 (14.1, 19.7) [287]	16.2 (14.1, 19.8) [177]	16.4 (14.2, 20.2) [97]	16.3 (14.2, 19.3) [171]	16.4 (14.1, 21.2) [86]
Z-score BMI[d,g]	-0.409 (-2,449, 2.042) [287]	-0.559 (-2.430, 1.866) [177]	-0.277 (-2.404, 2,733) [97]	-0.435 (-2.442, 1.742) [171]	-0.523 (-2.375, 3.458) [86]
Overweight-obesity[e,g]	20.2 (16.0, 25.2) [58/287]	16.4 (11.7, 22.5) [29/177]	27.8 (19.9, 37.5)* [27/97]	19.3 (14.1, 25.9) [33/171]	23.3 (15.6, 33.2) [20/86]

Abbreviations: BMI, body mass index; tHcy, fasting plasma total homocysteine.
[a]RTBC <5.7 µmol/L, PreC <7.1 µmol/L
[b]<5.7 µmol/L both cohorts
[c]at the beginning of pregnancy
[d]P50 (P10, P90) [N],
[e]% (95% CI) [N]
[f]Based on Spanish tables (Santamaría et al., 1998).
[g]Based on Spanish tables (Carrascosa et al., 2008).
Ns vary between the data reported for all participants and the stratified analysis and also between each trimester due to not turning up for blood draws or unreturned questionnaires.

Comparison between low-mid tertile vs. highest tertile in each trimester: Proportions were compared using Chi-square test, continuous variables were compared using median test for K independent samples (SPSS).
*P <0.05

Detailed maternal and child characteristics of both cohorts are reported in **Table 9**. Compared to the PreC cohort, RTBC mothers were slightly older, less of them smoked but more of the smokers continued smoking throughout pregnancy, and they had higher socioeconomic status. Generally, the RTBC had better 1st trimester 1-CM status, based on the biomarkers determined, except for plasma MMA which did not differ between the 2 cohorts. The same was true for 3rd trimester indicators except for plasma folate concentration which was lower in RTBC mothers. The prevalence of low birthweight was lower in the RTBC and less of the babies had been breastfed for at least 1 month. Child plasma tHcy and triglycerides were lower in the RTBC, and HDLc and glucose were higher. None of the other metabolic or biochemical parameters differed between the 2 cohorts.

Table 9. Comparison of lifestyle, nutritional and biochemical characteristics between the PreC and RTBC cohorts.

	PreC	**RTBC**
Mothers during pregnancy		
Age (years)[a,b]	29.5 (28.9, 30.2) [79]	32.0 (31.4, 32.5) [210]***
BMI (kg/m^2)[a,b]	22.9 (22.4, 23.5) [78]	23.4 (23.0, 23.9) [207]
Parity (nulliparous)[c]	60.8 (49.7,70.8) [48/79]	49.5 (42.8, 56.2) [104/210]
Never smoked[c]	69.2 (58.3, 78.4) [54/78]	82.4 (76.7, 86.9 [173/210]***
Smoked during periconception - 1st trimester only[c]	23.1 (15.1, 33.6) [18/78]	1.0 (0.3, 3.4) [2/210]***
Smoked throughout pregnancy[c]	7.7 (3.6, 15.8) [6/78]	16.7 (12.2, 22.3) [35/210]***
Socioeconomic status[c]		
Low	27.8 (19.2, 38.6) [22/79]	3.8 (2.0, 7.4) [8/209]***
Middle	32.9 (23.6, 43.9) [26/79]	45.5 (38.8, 52.2) [95/209]***
High	39.2 (29.2, 50.3) [31/79]	50.7 (44.0, 57.4) [106/209]***
1st trimester		
Fasting plasma total homocysteine (µmol/L)[b]	6.5 (6.1, 7.0) [70]	5.5 (5.1, 5.3) [206]***
Fasting plasma cobalamin (pmol/L)[b]	259.0 (234.3, 286.4) [68]	358.9 (343.6, 374.9) [206]***
Fasting plasma HoloTC (pmol/L)[b]	47.0 (42.3, 52.3) [46]	71.9 (66.4, 77.8) [174]***
Fasting plasma MMA (µmol/L)[b]	0.101 (0.107, 0.101) [69]	0.115 (0.111, 0.120) [205]
Fasting plasma folate (nmol/L)[b]	11.1 (9.1, 13.5) [64]	25.3 (23.1, 27.8) [206]***

	PreC	RTBC
3rd trimester		
Fasting plasma total homocysteine (µmol/L)[b]	4.8 (4.5, 5.2) [72]	5.3 (5.1, 5.5) [187]*
Fasting plasma cobalamin (pmol/L)[b]	195.9 (176.4, 217.5) [70]	245.1 (232.0, 258.9) [187]***
Fasting plasma HoloTC (pmol/L)[b]	48.7 (42.3, 56.0) [50]	64.6 (59.5, 70.1) [160]**
Fasting plasma MMA (µmol/L)[b]	0.139 (0.129, 0.150) [72]	0.148 (0.141, 0.156) [187]
Fasting plasma Folate (nmol/L)[b]	16.8 (12.8, 22.1) [59]	11.3 (10.1, 12.6) [187]**
Birth and infancy		
Boys[c]	50.6 (39.8, 61.4) [40/79]	47.6 (41.0, 54.4) [100/210]
Birthweight z-score[d,e]	-0.002 (-0.229, 0.225) [78]	0.023 (-0.098, 0.144) [208]
Birthweight <P10[c,e]	14.1 (8.1, 23.5) [11/78]	3.8, (2.0, 7.4) [8/208]**
Breastfed: Yes (1 month minimum)[c]	85.7 (75.7, 92.1) [60/70]	67.9 (61.3, 73.9) [142/209]**
Mid-childhood check-up		
Age (months)[b]	72.9, (72.4, 73.5) [79]	89.6 (89.3, 89.9) [210]***
BMI (kg/m^2)[b]	17.1 (16.7, 17.6) [79]	16.2 (16.0, 16.5) [208]**
Z-score BMI[b,f]	0.431 (0.157, 0.722) [79]	-1.242 (-1.514, -0.941) [208]***
Fasting plasma total homocysteine (µmol/L)[d]	6.0 (5.7, 6.3) [77]	5.5 (5.3, 5.7) [145]**

	PreC	RTBC
Plasma HDLc, mmol/L[d]	1.5 (1.4, 1.6) [79]	1.7 (1.6, 1,7) [156]***
Plasma triglycerides, mmol/L[b]	0.72 (0.66, 0.77) [79]	0.57 (0.54, 0.60) [156]***
Plasma insulin, mU/L[b]	5.8 (5.7, 6.0) [79]	5.6 (5.5, 5.8) [155]
Plasma glucose mmol/L[b]	4.8 (4.7, 4.9) [79]	5.2 (5.1, 5.2) [155]***
HOMA-IR[b]	1.25 (1.20, 1.30) [79]	1.29 (1.25, 1.33) [155]
Fat Mass Index[b]	2.6 (2.4, 2.9) [71]	2.4 (2.3, 2.6) [203]
Metabolic score[b]	-0.450 (-0.752, -0.105) [71]	-0.756 (-1.024, -0.451) [152]
Plasma total cholesterol, mmol/L[d]	4.3 (4.1, 4.4) [79]	4.4 (4.3, 4.5) [156]
Plasma Lipoprotein (a) mg/dl[b]	4.8 (3.6, 6.4) [79]	6.6 (5.4, 8.1) [156]
Plasma ApoA1 mg/dl[d]	136.8 (132.0, 141.6) [70]	140.6 (137.3, 143.8) [153]
Plasma ApoB mg/dl[b]	73.6 (69.9, 77.5) [77]	73.6 (71.6, 75.7) [154]
Plasma LDLc-Friedewald mmol/L[d]	2.4 (2.3, 2.6) [79]	2.5 (2.4, 2.6) [156]

Abbreviations: ApoA1 – apolipoprotein A1, ApoB – apolipoprotein B, BMI – body mass index, HDLc – high density lipoprotein cholesterol HoloTC – holotranscobalamin, LDLc - low-density lipoprotein cholesterol, MMA – methylmalonic acid,
[a]At the beginning of pregnancy
[b]Geometric mean (95% Confidence interval) [N].
[c]% (95% Confidence interval) [N]
[d]Arithmetic mean (95% Confidence interval) [N].
[e]Based on Spanish tables (Santamaría et al., 1998)
[f]Based on Spanish tables (Carrascosa et al., 2008).
Comparison between PreC and RTBC: Proportions were compared using Chi-square test, arithmetic or geometric means were compared using Student's unpaired T test. *P <0.05, **P <0.01, ***P <0.001.

Plasma holoTC concentrations in both trimesters were strongly correlated with cord holoTC. 1st trimester tHcy and folate were weakly correlated with the corresponding variables in cord and the correlations were stronger in the case of the 3rd trimester determinations. Pregnancy plasma cobalamin and MMA in the 1st and 3rd trimesters were moderately correlated with the corresponding variables in cord. Pregnancy tHcy, plasma cobalamin and holoTC were weakly correlated with the corresponding variables in the children. Maternal plasma holoTC was relatively strongly correlated with plasma cobalamin and tHcy, compared to plasma MMA. Plasma holoTC, cobalamin and tHcy were all only weakly correlated with plasma MMA. Cord plasma cobalamin was very weakly correlated with the corresponding variable in the children. Cord MMA and tHcy were each weakly correlated with the corresponding variables in the children. The child holoTC–cobalamin correlation was relatively strong, and stronger than any of the other correlations among child nutrients or tHcy. Child plasma folate, holoTC and cobalamin were inversely correlated with tHcy in that decreasing order of strength of correlation (**Table 10**).

Table 10. Correlation matrix biochemical markers in plasma in each trimester of pregnancy, in cord and children.

	Mothers									
	Folate		B12		HoloTC		MMA		tHcy	
	1st	3rd	1st	3rd	1st	3rd	1st	3rd	1st	3rd
Mothers										
Folate 1st	1 [275]									
Folate 3rd	0.315[a] [245]***	1 [252]								
B12 1st	0.299[a] [268]***	0.110[a] [241]†	1 [274]							
B12 3rd	0.140[a] [247]*	0.124[a] [248]#	0.672[a] [250]***	1 [257]						
HoloTC 1st	0.262[a] [213]***	0.136[a] [192]¶	0.664[a] [216]***	0.493[a] [199]***	1 [220]					
HoloTC 3rd	0.192[a] [204]**	0.215[a] [202]**	0.528[a] [203]***	0.577[a] [209]***	0.743[a] [188]***	1 [210]				
MMA 1st	0.049[a] [267]	0.076[a] [240]	-0.081[a] [272]	-0.013[a] [249]	-0.143[a] [218]*	-0.060[a] [203]	1 [274]			
MMA 3rd	0.059[a] [248]	0.037[a] [248]	-0.172[a] [250]**	-0.102[a] [257]	-0.199[a] [201]**	-0.174[a] [210]*	0.620[a] [251]***	1 [259]		
tHcy 1st	-0.429[a] [269]***	-0.204[a] [241]**	-0.194[a] [274]**	-0.053[a] [250]	-0.295[a] [218]***	-0.226[a] [204]**	0.192[a] [274]**	0.135[a] [252]*	1 [276]	
tHcy 3rd	-0.088[a] [248]	-0.512[a] [248]***	-0.111[a] [250]‡	-0.108[a] [257]†	-0.201[a] [201]**	-0.237[a] [210]***	0.074[a] [251]	0.180[a] [259]**	0.445[a] [252]***	1 [259]
Cord[b]										
Folate	0.263[a] [170]***	0.609[a] [158]***	0.021[a] [170]	0.220[a] [158]**	0.039[a] [144]	0.227[a] [134]**	0.107[a] [170]	0.121[a] [158]	-0.138[a] [170]§	-0.262[a] [158]***
B12	-0.015[a] [170]	0.308[a] [158]***	0.452[a] [170]***	0.561[a] [158]***	0.491[a] [144]***	0.571[a] [134]***	0.055[a] [170]	-0.108[a] [158]	0.008[a] [170]	-0.285[a] [158]***
HoloTC	0.131[a] [144]	0.353[a] [134]***	0.498[a] [144]***	0.551[a] [134]***	0.673[a] [144]***	0.722[a] [134]***	-0.076[a] [144]	-0.047[a] [134]	-0.147[a] [144]‡	-0.278[a] [134]**
MMA	0.035[a] [170]	0.044[a] [158]	-0.206[a] [170]**	-0.140[a] [158]‡	-0.228[a] [144]**	-0.172[a] [134]*	0.481[a] [170]***	0.560[a] [158]***	0.104[a] [170]	0.152[a] [158]¶
tHcy	-0.136[a] [170]‡	-0.452[a] [158]***	-0.212[a] [170]**	-0.245[a] [158]**	-0.286[a] [144]***	-0.273[a] [134]**	0.016[a] [170]	0.079[a] [158]	0.351[a] [170]***	0.684[a] [158]***
Children										
Folate	0.013[a] [205]	0.073[a] [185]	0.056[a] [205]	0.037[a] [190]	0.088[a] [170]	0.021[a] [159]	-0.015[a] [205]	0.039[a] [192]	-0.010[a] [207]	-0.109[a] [192]
B12	0.053[a] [209]	-0.004[a] [189]	0.234[a] [208]***	0.215[a] [193]**	0.268[a] [171]***	0.240[a] [161]**	-0.040[a] [208]	-0.042[a] [195]	-0.090[a] [210]	0.127[a] [195]‡
HoloTC[c]	-0.060[a] [60]	-0.013[a] [57]	0.090[a] [60]	0.268[a] [62]*	0.398[a] [41]**	0.334[a] [43]*	-0.158[a] [61]	-0.224[a] [64]‡	-0.230[a] [62]§	-0.058[a] [64]
MMA[c]	-0.028[a] [142]	-0.040[a] [125]	-0.006[a] [142]	-0.008[a] [125]	-0.136[a] [126]	-0.051[a] [113]	0.361[a] [141]***	0.286[a] [125]**	0.006[a] [142]	-0.030[a] [125]
tHcy	-0.218[a] [210]**	-0.068[a] [190]	-0.123[a] [209]‡	-0.146[a] [194]*	-0.204[a] [172]**	-0.146[a] [162]¶	0.160[a] [209]*	0.153[a] [196]*	0.234[a] [211]***	0.184[a] [196]**

Cord					Children				
Folate	B12	HoloTC	MMA	tHcy	Folate	B12	HoloTC	MMA	tHcy
1 [171]									
0.259[a] [171][***]	1 [171]								
0.224[a] [145][**]	0.602[a] [145][***]	1 [145]							
0.110[a] [171]	-0.161[a] [171][*]	-0.167[a] [145][*]	1 [171]						
-0.251[a] [171][***]	-0.321[a] [171][***]	-0.362[a] [145][***]	0.278[a] [171][***]	1 [171]					
0.043[a] [119]	0.017[a] [119]	0.003[a] [107]	-0.018[a] [119]	-0.161[a] [119][‡]	1 [217]				
-0.048 [118]	0.191 [118][*]	0.128 [106]	-0.126 [118]	-0.043[a] [118]	0.222[a] [216][**]	1 [221]			
0	0	0	0	0	0.145[a] [69]	0.652[a] [69][***]	1 [69]		
-0.097[a] [119]	-0.097[a] [119]	-0.171[a] [107][‡]	0.383[a] [119][***]	0.008[a] [119]	-0.059[a] [145]	-0.200[a] [144][*]	0	1 [145]	
-0.133[a] [119]	-0.008[a] [119]	0.029[a] [107]	0.093[a] [119]	0.248[a] [119][**]	-0.433[a] [217][***]	-0.259[a] [221][***]	-0.322[a] [69][**]	0.146 [145][‡]	1 [222]

Abbreviations: Holotranscobalamin, MMA methylmalonic acid, tHcy fasting plasma total homocysteine.
[a] Spearman's rank-order correlation coefficient, [N]. *P<0.05, **P<0.01, ***P<0.001, †P=0.09, ‡P=0.08, §P=0.07, ¶P=0.06, $^{\#}$P=0.051.
[b] Cord metabolites are only available for RTBC.
[c] HoloTC is only available for the PreC children and MMA only for the RTBC children.

Pregnancy and child 1-CM status according to pregnancy tHcy status (tertiles) is reported in **Table 11**. Only maternal folate status differed significantly between the corresponding tHcy categories in both trimesters. Folate status was lower and deficiency more prevalent in mothers in the highest versus low-mid tHcy tertiles. More mothers had cobalamin and folate deficiency in the 3rd trimester compared to the 1st. None of the children had cobalamin deficiency (data not shown) but 4.6% had folate deficiency. Offspring tHcy and plasma glucose concentration were higher when pregnancy tHcy was in the highest versus low-mid tertiles and this was also true for HOMA-IR when mothers had the 3rd trimester tHcy in the highest tertile. No differences were observed in any of the child lipid parameters (total cholesterol, plasma lipoprotein (a), ApoA1 and ApoB, LDL cholesterol) according to pregnancy tHcy status.

Table 11. Nutritional and metabolic status in the mothers and children from fasting plasma samples, according to pregnancy tHcy status.

	1st trimester			3rd trimester		
	All	**tHcy low-mid tertiles[a]**	**tHcy highest tertile**	**All**	**tHcy low-mid tertiles[b]**	**tHcy highest tertile**
Mothers during pregnancy						
Cobalamin (pmol/L)[c]	348.1 (201.2, 496.4) [274]	361.8 (213.4, 521.2) [177]	324.2 (189.2, 482.7) [97]	233.3 (142.7, 375.9) [257]	238.2 (147.1, 384.6) [172]	230.9 (140.1, 348.8) [85]
Cobalamin deficiency[d,e]	2.6 (1.2, 5.2) [7/274]	2.3 (0.9, 5.7) [4/177]	3.1 (1.1, 8.7) [3/97]	12.1 (8.6, 16.6) [31/257]	11.0 (7.2, 16.6) [19/172]	14.1 (8.3, 23.1) [12/85]
HoloTC (pmol/L)[c]	65.5 (36.9, 106.9 [220]	67.8 (42.4, 110.2) [145]	58.7 (28.1, 96.0) [73]	61.4, (32.6, 103.2) [210]	63.8 (37.3, 104.4) [139]	54.9 (27.5, 90.7) [71]
MMA (µmol/L)[c]	0.110 (0.080, 0.159) [274]	0.108 (0.080, 0.148) [177]	0.113 (0.084, 0.175) [97]	0.140 (0.100, 0.211) [259]	0.140 (0.100, 0.200) [173]	0.141 (0.103, 0.228) [86]
Folate (nmol/L)[c,f]	22.5 (8.2, 50.7) [270]	25.9 (10.6, 51.7) [175]	15.3 (5.7, 47.8) [89]***	10.2 (4.6, 42.3) [246]	15.8 (5.5, 50.3) [164]	6.5 (4.0, 15.9) [78]***
Folate deficiency[d,f,g]	7.4 (4.8, 11.2) [20/270]	3.4 (1.6, 7.3) [6/175]	13.5 (7.9, 22.1) [12/89]**	32.1 (26.6, 38,2) [79/246]	20.1 (14.7, 26.9) [33/164]	55.1 (44.1, 65.7) [43/78]***

	1st trimester			3rd trimester		
	All	**tHcy low-mid tertiles[a]**	**tHcy highest tertile**	**All**	**tHcy low-mid tertiles[b]**	**tHcy highest tertile**
Children at check-up						
tHcy (µmol/L)[c]	5.6 (4.2, 7.3) [222]	5.4 (4.2, 7.1) [126]	5.8 (4.3, 7.4) [85] *	5.6 (4.2, 7.3) [222]	5.4 (4.1, 7.2) [126]	5.9 (4.5, 7.4) [70]*
Cobalamin (nmol/L)[c]	579.2 (393.0, 870.8) [221]	583.3 (393.1, 891.9) [125]	570.6 (392.5, 875.0) [85]	579.2 (393.0, 870.8) [221]	576.1 (386.0, 816.2) [125]	594.4 (398.9,943.3) [70]
Folate (pmol/L)[c]	17.1 (9.3, 32.5) [217]	17.1 (9.6, 32.7) [126]	17.1 (9.2, 32.3) [81]	17.1 (9.3, 32.5) [217]	17.9 (9.3, 33.1) [125]	15.6 (8.6, 27.9) [67]
Folate deficiency[d,g]	4.6 (2.5, 8.3) [10/217]	4.8 (2.2, 10.0) [6/126]	4.9 (1.9, 12.0) [4/81]	4.6 (2.5, 8.3) [10/217]	4.8 (2.2, 10.1) [6/125]	4.5 (1.5, 12.4) [3/67]
HDLc (mmol/L)[c]	1.6 (1.2, 2.0) [235]	1.7 (1.2, 2.0) [135]	1.5 (1.2, 1.9) [87]	1.6 (1.2, 2.0) [235]	1.6 (1.2, 2.0) [133]	1.6 (1.3, 2.0) [74]
Triglycerides (mmol/L)[c]	0.6 (0.4, 1.0) [235]	0.6 (0.4, 1.0) [135]	0.6 (0.4, 1.1) [88]	0.6 (0.4, 1.0) [235]	0.6 (0.4, 1.0) [133]	0.6 (0.4, 1.0) [74]
Insulin (mU/L)[c]	5.5 (4.9, 6.9) [234]	5.5 (4.9, 6.9) [135]	5.6 (4.8, 7.4) [87]	5.5 (4.9, 6.9) [234]	5.5 (4.9, 6.9) [133]	5.6 (4.9, 7.6) [74]

	1st trimester			3rd trimester		
	All	**tHcy low-mid tertiles[a]**	**tHcy highest tertile**	**All**	**tHcy low-mid tertiles[b]**	**tHcy highest tertile**
Glucose (mmol/L)[c]	5.1 (4.6, 5.5) [234]	5.1 (4.6, 5.5) [135]	5.2 (4.7, 5.6) [88]**	5.1 (4.6, 5.5) [234]	5.0 (4.4, 5,4) [133]	5.2 (4.6, 5.6) [74]*
HOMA-IR[c]	1.2 (1.0, 1.6) [234]	1.2 (1.0, 1.6) [135]	1.3 (1.0, 1.7) [87]	1.2 (1.0, 1.6) [234]	1.2 (1.0, 1.5) [133]	1.3 (1.1, 1.7) [74]*
Fat mass index[c]	2. 3 (1.5, 4.7) [274]	2.3 (1.5, 4.3) [171]	2.4 (1.5, 5.0) [95]	2.3 (1.5, 4.7) [274]	2.3 (1.6, 4.7) [167]	2.4 (1.4, 5.6) [82]
Metabolic score[c,h]	-0.530 (-2.018, 2.168) [223]	-0.683 (-2.181, 1.532) [130]	-0.224 (-1.776, 2.934) [86]	-0.530 (-2.018, 2.168) [223]	-0.693 (-2.144, 1.524) [131]	-0.233 (-1.676, 3.203) [70]
Total cholesterol (mmol/L)[c]	4.3 (3.5, 5.3) [235]	4.3 (3.5, 5.3) [135]	4.4 (3.5, 5.3) [87]	4.3 (3.5, 5.3) [235]	4.3 (3.5, 5.3) [133]	4.4 (3.5, 5.4) [74]
Lipoprotein (a) (mg/dl)[c]	5.5 (1.0, 33.2) [235]	6.4 (0.9, 30.7) [135]	5.0 (1.0, 33.3) [87]	5.5 (1.0, 33.2) [235]	5.1 (0.8, 30.3) [133]	5.5 (1.3, 33.3) [74]
ApoA1 (mg/dl)[c]	139.0 (116.0, 164.8) [223]	141.0 (115.0, 163.0) [129]	135.5 (117.5, 169.5) [84]	139.0 (116.0, 164.8) [223]	138.0 (114.7, 161.3) [126]	140.0 (116.2, 168.9) [70]

	1st trimester			3rd trimester		
	All	**tHcy low-mid tertiles[a]**	**tHcy highest tertile**	**All**	**tHcy low-mid tertiles[b]**	**tHcy highest tertile**
ApoB (mg/dl)[c]	74.0 (57.2, 93.8) [231]	72.5 (56.5, 94.0) [134]	75.5 (57.7, 89.3) [86]	74.0 (57.2, 93.8) [231]	73.0 (56.2, 93.0) [131]	76.0 (58.4, 98.6) [73]
LDLc-Friedewald (mmol/L)[c]	2.4 (1.8, 3.2) [235]	2.3 (1.8, 3.3) [135]	2.5 (1.8, 3.1) [87]	2.4 (1.8, 3.2) [235]	2.3 (1.7, 3.3) [133]	2.5 (1.9, 3.3) [74]
Systolic blood pressure, mmHg[c, i]	104.0 (93.7, 112.3 [181]	103.3 (93.1, 112.1) [117]	104.7 (96.4, 116.0) [60]	104.0 (93.7, 112.3 [181]	103.0 (94.3, 112.6) [106]	104.7 (91.0, 112.0) [54]
Diastolic blood pressure, mmHg[c, i]	63.3 (55.0, 72.3) [181]	63.9 (54.3, 72.4) [117]	64.3 (56.8, 72.0) [60]	63.3 (55.0, 72.3) [181]	63.0 (54.0, 72.3) [106]	64.7 (55.7, 73.3) [54]

Abbreviations: ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; HDLc, high density lipoprotein cholesterol; HoloTC, holotranscobalamin; LDLc, low-density lipoprotein cholesterol; MMA, methylmalonic acid; tHcy, fasting plasma total homocysteine.

[a]RTBC: low mid <5.7 µmol/L, high ≥ 5.7 µmol/L; PreC low mid <7.1 µmol/L, high ≥ 7.1 µmol/L. 1st trimester tHcy tertile values differed between the cohorts due to different folic acid supplementation patterns.

[b]Both cohorts: low mid <5.7 µmol/L, high ≥5.7 µmol/L.

[c]P50 (P10, P90) [N]

[d]% (95% CI) [N]

[e]<148 pmol/L

[f]Not including mothers with plasma folate below the limit of detection (2nmol/L, 1st trimester N=5, 3rd trimester N=6).

[g]<7.0 nmol/L
[h]Metabolic score: zFMI + ((zTG–zHDLc)/2) + zHOMA-IR
[i]Blood pressure information is only available for RTBC children
Numbers vary between the data reported in the "All" column and the stratified analysis by tHcy tertiles because tHcy measurements in each trimester were not available for all women.
Comparison between low-mid tertile vs highest tertile in each trimester: proportions using Chi-square test and continuous variables using median test for K independent samples (SPSS). $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001.

Determinants of 1st trimester maternal tHcy, are reported in **Table 12**. Plasma folate concentration was the strongest determinant.

Table 12. Factors associated with maternal fasting plasma total homocysteine (Ln) in the 1st trimester by multiple linear regression analysis.

Independent variables	Standardised Beta coefficients
Plasma folate (nmol/L)	-0.241***
Plasma cobalamin (pmol/L)	-0.094
Plasma creatinine (μmol/L)	0.194**
Age (years)	-0.078
Body mass index (kg/m^2)	-0.004
Low versus mid-high socioeconomic status	0.086
Pregnancy smoking periconceptionally versus never	0.062
Pregnancy smoking during pregnancy versus never	0.006

N=259, Adjusted R^2= 0.140***, **P <0.01, ***P <0.001.

Associations between pregnancy tHcy status, child MetSco and its components are reported in **Table 13**. Offspring of mothers with highest versus low-mid tHcy tertiles during pregnancy had higher MetSco and zFMI. Stratifying by sex, the associations were only significant in boys. Furthermore, in boys only, zHOMA-IR was higher when mothers had 3rd trimester tHcy in the highest versus the low-mid tertiles.

Table 13. Association between pregnancy maternal tHcy highest tertile vs low-mid (reference)[a], and child metabolic outcomes at 6-8 years by multiple linear regression analysis.

		All		Girls		Boys	
		Adjusted R^2	**B coefficients[b] (SE)[c]**	**Adjusted R^2**	**B coefficients[b] (SE)[c]**	**Adjusted R^2**	**B coefficients[b] (SE)[c]**
1st trimester[d]	Metabolic score	0.437***	0.418 (0.189)*	0.399***	0.325 (0.315)	0.514***	0.462 (0.224)*
	zFat Mass Index	0.680***	0.211 (0.073)**	0.749***	0.150 (0.099)	0.637***	0.276 (0.108)*
	zHOMA-IR	0.152***	0.081 (0.091)	0.127**	0.044 (0.156)	0.155**	0.109 (0.104)
	(zTG–zHDLc)/2	0.014	0.252 (0.226)	-0.036	0.263 (0.370)	0.065	0.154 (0.286)
3rd trimester[e]	Metabolic score	0.521***	0.435 (0.183)*	0.545***	0.446 (0.280)	0.519***	0.511 (0.236)*
	zFat Mass Index	0.664***	0.190 (0.081)*	0.727***	0.111 (0.113)	0.632***	0.312 (0.113)**
	zHOMA-IR	0.202***	0.157 (0.087)†	0.205***	0.129 (0.138)	0.140*	0.238 (0.114)*
	(zTG–zHDLc)/2	0.053*	0.176 (0.227)	0.076	0.413 (0.339)	0.051	-0.077 (0.308)

Abbreviations: HDLc, high density lipoprotein cholesterol HOMA-IR, homeostasis model assessment of insulin resistance; TG, triglycerides.

[a]1st trimester RTBC: low-mid <5.7 µmol/L, highest ≥ 5.7 µmol/L; PreC low mid <7.1 µmol/L, highest ≥ 7.1 µmol/L; 1st trimester tHcy tertile values differed between the cohorts due to different folic acid supplementation patterns. 3rd trimester (both cohorts): low mid <5.7 µmol/L, highest ≥ 5.7 µmol/L.

[b]Unstandardized B coefficients of maternal tHcy highest vs low-mid tertiles (reference).

[c]Standard errors.

[d]All (n=197), girls (n=103), boys (n= 94).

[e]All (n=182), girls (n=95), boys (n= 87).

Models adjusted for: maternal age, maternal body mass index, socioeconomic status, pregnancy smoking periconceptionally versus never, pregnancy smoking during pregnancy versus never, breastfeeding (yes/no), zBMI at childhood check-up, child tHcy at check-up. *P <0.05, **P < 0.01, ***P < 0.001, †P =0.07.

Associations between pregnancy indicators of cobalamin status and childhood outcomes are reported in **Table 14**. 1st trimester plasma cobalamin was not associated with any child outcomes but boys born to mothers with low 3rd trimester plasma cobalamin status had lower mid-childhood FMI. On the other hand, 1st trimester holoTC in the lowest versus mid-high tertiles, was associated with higher MetSco, FMI and insulin resistance, in boys. Highest 1st trimester MMA tertile versus low-mid tertiles was associated with increased child metabolic score and dyslipidemia ((zTG–zHDLc)/2)) in boys. Associations between pregnancy folate status and mid-childhood outcomes are reported in **Table 15**. Children of mothers with 1st trimester plasma folate concentration in the lowest (<14.6 nmol/L) versus mid-high tertiles had higher insulin resistance and stratifying by sex, this was limited to girls. Boys born to mothers with 3rd trimester plasma folate in the lowest compared to mid-high tertiles had lower dyslipidemia (negative coefficient in the linear regression).

Table 14. Association between indicators of pregnancy plasma cobalamin status (plasma cobalamin, holoTC, MMA) and child outcomes by multiple linear regression analysis.

			All		Girls		Boys	
			Adjusted R^2	**B coefficients[b] (SE)[c]**	**Adjusted R^2**	**B coefficients[b] (SE)[c]**	**Adjusted R^2**	**B coefficients[b] (SE)[c]**
1st trimester	*Cobalamin*[a]	Metabolic score	0.420***	0.097 (0.210)	0.397***	-0.236 (0.321)	0.486***	0.464 (0.274)
		zFat Mass Index	0.664***	-0.008 (0.083)	0.736***	-0.122 (0.105)	0.592***	0.113 (0.137)
		zHOMA-IR	0.128***	0.009 (0.101)	0.101*	-0.030 (0.162)	0.154**	0.063 (0.124)
		(zTG–zHDLc)/2	0.020	0.191 (0.244)	-0.035	-0.166 (0.371)	0.093*	0.574 (0.337)
	HoloTC[d]	Metabolic score	0.432***	0.332 (0.241)	0.376***	-0.172 (0.373)	0.542***	0.897 (0.328)**
		zFat Mass Index	0.695***	0.115 (0.089)	0.767***	-0.098 (0.108)	0.645***	0.323 (0.157)*
		zHOMA-IR	0.144***	0.087 (0.117)	0.117*	-0.059 (0.188)	0.171**	0.306 (0.152)*
		(zTG–zHDLc)/2	0.016	0.260 (0.279)	-0.071	-0.028 (0.421)	0.075	0.536 (0.421)
	MMA[e]	Metabolic score	0.422***	0.122 (0.206)	0.398***	-0.229 (0.329)	0.497***	0.529 (0.241)*
		zFat Mass Index	0.679***	0.049 (0.079)	0.753***	-0.061 (0.104)	0.597***	0.175 (0.120)
		zHOMA-IR	0.130***	-0.107 (0.099)	0.114*	-0.224 (0.165)	0.148**	0.004 (0.110)
		(zTG–zHDLc)/2	0.028	0.359 (0.239)	-0.036	0.111 (0.380)	0.118*	0.699 (0.293)*

			All		Girls		Boys	
			Adjusted R^2	**B coefficients[b] (SE)[c]**	**Adjusted R^2**	**B coefficients[b] (SE)[c]**	**Adjusted R^2**	**B coefficients[b] (SE)[c]**
3rd trimester	*Cobalamin[f]*	Metabolic score	0.509***	-0.082 (0.184)	0.513***	-0.036 (0.280)	0.513***	-0.213 (0.265)
		zFat Mass Index	0.667***	-0.133 (0.080)	0.720***	-0.021 (0.112)	0.615***	-0.269 (0.127)*
		zHOMA-IR	0.176***	-0.064 (0.088)	0.162**	-0.092 (0.137)	0.218**	-0.100 (0.122)
		(zTG–zHDLc)/2	0.066*	0.230 (0.220)	0.028	0.154 (0.330)	0.083	0.312 (0.335)
	HoloTC[g]	Metabolic score	0.514***	0.060 (0.208)	0.508***	-0.030 (0.293)	0.527***	0.173 (0.318)
		zFat Mass Index	0.686***	0.002 (0.087)	0.720***	0.014 (0.114)	0.647***	-0.038 (0.144)
		zHOMA-IR	0.173***	0.036 (0.102)	0.180**	-0.034 (0.147)	0.159*	0.139 (0.151)
		(zTG–zHDLc)/2	0.063*	0.043 (0.250)	0.028	-0.020 (0.340)	0.068	0.145 (0.419)
	MMA[h]	Metabolic score	0.514***	-0.263 (0.189)	0.522***	-0.367 (0.284)	0.509***	0.007 (0.257)
		zFat Mass Index	0.662***	-0.049 (0.084)	0.720***	0.018 (0.114)	0.592***	-0.029 (0.126)
		zHOMA-IR	0.180***	-0.101 (0.091)	0.177**	-0.199 (0.139)	0.212**	0.040 (0.118)
		(zTG–zHDLc)/2	0.065*	-0.225 (0.227)	0.039	-0.372 (0.335)	0.072	-0.008 (0.325)

Abbreviations: HDLc high density lipoprotein cholesterol, HOMA-IR homeostatic model assessment of insulin resistance, TG triglycerides.

[a]Lowest (<286.8 pmol/L) versus mid-high tertiles (reference). All (n=196), girls (n=105), boys (n= 91).

[b]Unstandardized B coefficient

[c]Standard error.

[d]Lowest (<53.3 pmol/L) versus mid-high tertiles (reference). All (n=159), girls (n=82), boys (n= 77);

[e]Highest (≥0.12 µmol/L) versus low-mid tertiles (reference). All (n=195), girls (n=105), boys (n= 90);

[f]Lowest (<194.6 pmol/L) versus mid-high tertile (reference). All (n=180), girls (n=97), boys (n= 83);
[g]Lowest (<47.6 pmol/L) versus mid-high tertile (reference). All (n=150), girls (n=81), boys (n= 69);
[h]Highest (≥0.16 µmol/L) versus low-mid tertile (reference). All (n=180), girls (n=97), boys (n= 83).

Models adjusted for: maternal age, maternal body mass index, maternal plasma folate in the corresponding trimester, pregnancy smoking periconceptionally versus never, pregnancy smoking during pregnancy versus never, socioeconomic status, zBMI at childhood check-up, child tHcy. Models were not adjusted for breastfeeding due to the limited sample size. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Table 15. Association between pregnancy maternal plasma folate in the lowest versus mid-high tertile (reference)[a] and child outcomes.

		All		Girls		Boys	
		Adjusted R^2	**B coefficients[b] (SE)[c]**	**Adjusted R^2**	**B coefficients[b] (SE)[c]**	**Adjusted R^2**	**B coefficients[b] (SE)[c]**
1st trimester[d]	Metabolic score	0.423***	0.018 (0.204)	0.402***	0.028 (0.335)	0.504***	-0.041 (0.237)
	zFat Mass Index	0.663***	-0.120 (0.081)	0.738***	-0.166 (0.109)	0.593***	-0.105 (0.123)
	zHOMA-IR	0.152***	0.213(0.097)*	0.148**	0.338 (0.165)*	0.166**	0.110 (0.106)
	(zTG–zHDLc)/2	0.022	-0.149 (0.239)	-0.024	-0.288 (0.386)	0.097*	-0.092 (0.298)
3rd trimester[e]	Metabolic score	0.507***	-0.156 (0.192)	0.519***	-0.076 (0.282)	0.496***	-0.244 (0.268)
	zFat Mass Index	0.667***	-0.036 (0.083)	0.713***	-0.014 (0.114)	0.609***	-0.004 (0.126)
	zHOMA-IR	0.170***	0.128 (0.092)	0.178**	0.138 (0.137)	0.118*	0.151 (0.128)
	(zTG–zHDLc)/2	0.098**	-0.497 (0.228)*	0.052	-0.402 (0.329)	0.132*	-0.781 (0.338)*

Abbreviations: HDLc high density lipoprotein cholesterol, HOMA-IR homeostatic model assessment of insulin resistance, TG triglyceride.

[a]Plasma folate lowest tertile (1st trimester <14.6 nmol/L, 3rd trimester <6.9 nmol/L).

[b]Unstandardized B coefficients of maternal plasma folate lowest vs mid-high tertiles (reference).

[c]Standard error.

[d]All (n=191), girls (n=103), boys (n= 88).

[e]All (n=174), girls (n=96), boys (n= 78).

Models adjusted for: maternal age, maternal body mass index, maternal plasma cobalamin in the 1st or 3rd trimester, pregnancy smoking periconceptionally versus never, pregnancy smoking during pregnancy versus never, socioeconomic status, zBMI at childhood check-up, child tHcy. Models were not adjusted for breastfeeding due to the limited sample size. Women with plasma folate below the limit of detection (< 2nmol/L, 1st trimester N=5, 3rd trimester N=6) were not included in the models. *P<0.05, **P<0.01, ***P<0.001.

Mediation analysis was used to explore whether the associations between 1st trimester tHcy and zFMI are partially mediated via foetal growth (birthweight z-score) (**Figure 17**). The direct effect (tHcy-outcome, coefficient *c'*), indirect effect ((tHcy-birthweight z-score-outcome, coefficient *a* (tHcy-birthweight) and coefficient *b* (birthweight z-score-outcome)) and total effect (tHcy-outcome, coefficient *c*, unadjusted for birthweight z-score) are illustrated. The indirect effect (*a* X *b*) represents the association between tHcy and child zFMI via the sequence tHcy-birthweight-outcome. Monte Carlo confidence interval for the indirect effect including 0 indicates that birthweight does not play a role in the association between early pregnancy tHcy and fat mass index in the offspring.

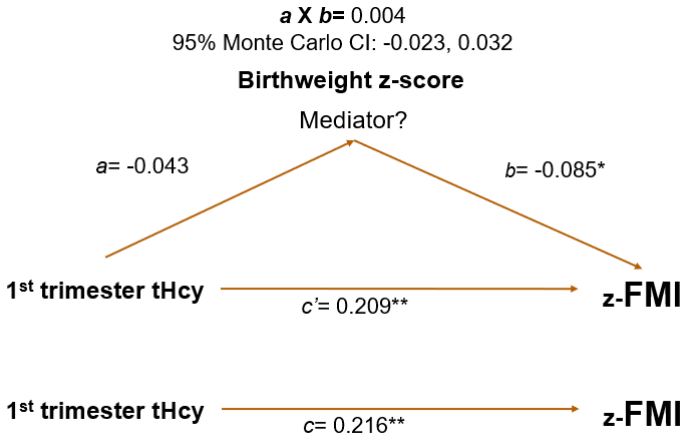


Figure 17. Mediation analysis (Hayes, 2017): association between 1st trimester tHcy-child z-fat mass index via birthweight z-score.

a b, *c*' and *c* ((linear regression analysis B-coefficients adjusting for maternal age, BMI, socioeconomic status, smoking (*a*) and birthweight z-score, breastfeeding, and child zBMI, tHcy *(b* and *c*') and excluding birthweight (*c)*). N = 196 mother-child dyads. *a* X *b*= indirect effect of tHcy on zFMI via birthweight z-score. $^{*}P<0.05$, $^{**}P<0.01$.

Differences between mid-childhood (7.5 y) plasma metabolite status according to maternal pregnancy plasma cobalamin and tHcy status.

Plasma nutrients/metabolites status in RTBC children aged 7.5 years according to pregnancy plasma cobalamin and tHcy status is reported in **Table 16**. After Bonferroni corrections for multiple comparisons, we observed that when maternal cobalamin was in the lowest tertile at 34 GW, child plasma betaine and sarcosine were higher compared to the mid-high cobalamin tertile. These results were confirmed when we tested them according to maternal MMA status (highest vs. low-mid tertile, data not shown). When maternal 1st trimester tHcy was in the highest vs low-mid tertile, plasma tryptophan was lower in the children (**Table 16**).

Table 16. Child plasma metabolite concentrations according to pregnancy cobalamin and tHcy status.

	Maternal plasma B12 tertile				**Maternal tHcy tertile**	
	1st trimester		**34 gestational weeks**		**1st trimester**	
	Lowest[1]	**Mid-high**	**Lowest[2]**	**Mid-high**	**Highest[3]**	**Low-mid**
Child metabolite						
Betaine[4] (µmol/L)			39.2 (37.1; 41.5)	36.2 (34.8; 37.6)*		
DMG[4] (µmol/L)			4.6 (4.2; 5.1)	4.1 (3.8; 4.4)		
Total cysteine[5] (µmol/L)	235 (229; 240)	224 (220; 229)	233 (227; 239)	225 (220; 230)		
Sarcosine[5] (µmol/L)	5.3 (4.7; 5.9)	4.5 (4.0; 4.9)	5.4 (4.9; 6.0)	4.3 (3.8; 4.9)**		
Tryptophan[5] (µmol/L)					65.4 (62.9; 68.0)	70.2 (67.9; 72.5)*

Abbreviations: DMG dimethylglycine. tHcy: fasting plasma total homocysteine.
[1]Plasma B12 ≤ 315 pmol/L at <12 GW or ≤ 288 pmol/L at 15 GW.
[2]Plasma B12 ≤ 216 pmol/L at 34 GW.
[3]Plasma tHcy ≥ 5.75 µmol/L at <12 GW or ≥ 4.87 µmol/L at 15 GW.
[4]Geometric means (95% CI) or [5]Arithmetric means (95% CI) are shown.
In red: ANCOVA adjusting for child B12 or child tHcy respectively and Bonferroni correction.
*p<0.05, **p<0.01

Child/nutrient metabolite status were also compared according to child status in plasma cobalamin (lowest vs mid-high tertile) and tHcy (highest vs low-mid tertile) (**Table 17**). After Bonferoni correction for multiple comparisons, we observed that plasma total cysteine and SDMA were lower in the lowest child cobalamin tertile compared to the mid-high tertiles. In the case of serine, it was higher in the lowest child cobalamin tertile compared to the mid-high tertile. Additionally, child plasma total cysteine was higher in the highest tHcy tertile vs low-mid tertiles, while betaine was lower in the highest tHcy tertile vs the low-mid tertiles.

Table 17. Child plasma metabolite concentrations according to child cobalamin and tHcy status.

	Child plasma B12 tertile		**Child tHcy tertile**	
	Lowest[1]	**Mid-high**	**Highest[2]**	**Low-mid**
Total cysteine[3] (µmol/L)	223 (216; 229)*	231 (226; 235)	237 (232; 243)***	223 (219; 227)
Serine[3] (µmol/L)	133 (129; 138)***	123 (120; 127)		
SDMA[3] (µmol/L)	0.49 (0.45; 0.52)*	0.52 (0.50; 0.53)		
Betaine[4] (µmol/L)			35.9 (34.1; 37.8)*	38.7 (37.1; 40.2)

Abbreviations SDMA: Symmetric dimethylarginine
[1]Plasma B12 ≤ 538 pmol/L .
[2]Plasma tHcy ≥ 5.87 µmol/L .
[5]Arithmetic means (95% CI) are shown.
[3]Arithmetic means (95% CI) or [4]Geometric means (95% CI) are shown.
ANOVA with posthoc Bonferoni correction for multiple comparisons: *p<0.05, **p<0.01, ***p<0.001

5.2. Betaine and the DMG pathway

5.2.1. Pregnancy and cord betaine and DMG/betaine and birthweight

The analysis of the betaine and DMG pathway during pregnancy were determined in the RTBC study only. The RTBC participant characteristics for the 748 pregnancies with available biochemical data are reported in **Table 18**. Women had a mean age of 32.1 and mean BMI of 24.3. Mean birthweight was 3233 g and 6.7% of the newborns were SGA (birthweight below the 10th percentile according to Spanish birthweight curves). The maternal *BHMT* c.716 G>A polymorphism AA frequency was 11.1% and for the baby 9.3%. All the observed genotype frequencies were in Hardy-Weinberg equilibrium ($p>0.05$).

Plasma 1C nutrient and metabolite status during pregnancy and in the cord are reported in **Table 19**.

Table 18. Descriptive characteristics of the participants.[a]

Maternal age (years)[b]	32.1 (31.8; 32.4)	748[c]
Maternal BMI (kg/m^2)[b]	24.3 (23.9; 24.6)	741
Parity (nulliparous), %	48.1 (44.4; 51.7)	720
Smoking during pregnancy, %		720
Never	73.3 (70.0; 76.4)	528
1st trimester only	9.9 (7.9; 12.3)	71
Throughout	16.8 (14.3; 19.7)	121
Socioeconomic status, %		721
Low	11.7 (9.5; 14.2)	84
Middle	46.0 (42.4; 49.7)	332
High	42.3 (38.7; 45.9)	305
Mother *BHMT c.716 G>A,* %		677
GG	47.3 (43.5; 51.0)	320
GA	41.7 (38.0; 45.4)	282
AA	11.1 (8.9; 13.7)	75
Pregnancy outcomes		
Sex (boys), %	50.1 (46.6; 53.7)	748
Birthweight, grams	3233 (3198; 3268)	747
Birthweight <P10, %	6.7 (5.1; 8.7)	747
Completed gestational weeks at delivery	39.0 (38.8; 39.1)	748
Hypertension, %		543
Normotensive	85.1 (81.8; 87.8)	462
Pregnancy induced hypertension	11.0 (8.7; 14.0)	60
Underlying hypertension	1.3 (0.6; 2.6)	7
Preeclampsia	2.6 (1,5; 4,3)	14
Gestational diabetes, %	6.5 (5.0; 8,6)	734
Preterm delivery, (<37GW) %	5.6 (4.2; 7.5)	748
Baby *BHMT c.716 G>A,* %		559
GG	43.1 (39.1; 47.3)	241
GA	47.6 (43.5; 51.7)	266
AA	9.3 (7.2; 12.0)	52

Abbreviations: GW: gestational weeks.
[a]Values are means or percentages (95% confidence interval).
[b]At the beginning of pregnancy. [c]N.

Table 19. One carbon nutrient and metabolite status[a] of the participants.

Betaine µmol/L		
<12GW	20.4 (19.9, 20.9)[b]	702[c]
15 GW	14.9 (14.6, 15.2)	444
24-27 GW	12.6 (12.4, 12.8)	654
34 GW	12.7 (12.5, 12.9)	630
Labour	13.1 (12.8, 13.3)	611
Cord	24.1 (23.6, 24.6)	574
DMG µmol/L		
<12GW	2.5 (2.4, 2.6)	702
15 GW	2.2 (2.1, 2.3)	429
24-27 GW	2.2 (2.1, 2.2)	653
34 GW	2.4 (2.3, 2.5)	630
Labour	2.8 (2.7, 2.9)	611
Cord	3.6 (3.5, 3.8)	574
DMG/Betaine		
<12GW	0.122 (0.119, 0.126)	702
15 GW	0.147 (0.142, 0.152)	429
24-27 GW	0.172 (0.167, 0.177)	653
34 GW	0.188 (0.182, 0.194)	630
Labour	0.218 (0.210, 0.225)	611
Cord	0.151 (0.146, 0.156)	574
Choline µmol/L		
<12GW	7.6 (7.4, 7.7)	702
15 GW	7.7 (7.6, 7.9)	444
24-27 GW	9.1 (8.9, 9.2)	654
34 GW	10.2 (10.0, 10.3)	630
Labour	11.6 (11.4, 11.8)	611
Cord	29.7 (28.6, 30.7)	573
Methionine µmol/L		
<12GW	23.2 (23.0, 23.5)	703
15 GW	22.2 (21.9, 22.4)	444
24-27 GW	21.9 (21.7, 22.2)	654
34 GW	22.2 (22.0, 22.5)	630
Labour	22.5 (22.1, 23.0)	611
Cord	30.1 (29.5, 30.8)	577
Folate nmol/L		
<12GW	27.3 (25.9, 28.7)	703
15 GW	24.6 (23.1, 26.2)	444
24-27 GW	15.1 (14.2, 16.0)	654
34 GW	12.8 (12.0, 13.6)	630
Labour	12.1 (11.3, 12.9)	610
Cord	26.0 (24.8, 27.3)	577
RBCF nmol/L		
<12GW	951 (912, 990)	690
15 GW	1232 (1176, 1291)	442
24-27 GW	1144 (1100, 1190)	642

34 GW	973 (930, 1019)	612
Cobalamin pmol/L		
<12GW	360 (351, 369)	703
15 GW	324 (314, 334)	444
24-27 GW	274 (266, 281)	654
34 GW	250 (243, 257)	630
Labour	231 (224, 238)	605
Cord	316 (299, 333)	550
HoloTC pmol/L		
<12GW	72.2 (68.7, 76.0)	414
15 GW	63.8 (60.6, 67.3)	372
24-27 GW	59.0 (56.1, 61.9)	396
34 GW	61.9 (58.6, 65.3)	384
Birth	58.1 (54.8, 61.7)	357
Cord	159.1 (145.6, 173.9)	339
MMA µmol/L		
<12GW	0.12 (0.11, 0.12)	704
15 GW	0.12 (0.11, 0.12)	444
24-27 GW	0.13 (0.12, 0.13)	654
34 GW	0.15 (0.15, 0.15)	630
Labour	0.16 (0.16, 0.17)	611
Cord	0.27 (0.26, 0.28)	577

Abbreviations: DMG: dimethylglycine HoloTC: holotranscobalamin. MMA: methylmalonic acid. RBCF: red blood cell folate.

[a]All measurements are plasma concentrations except red blood cell folate.

[b]Values are geometric means (95% confidence interval).

[c]N.

The Spearman's rank-order correlation coefficients of 1C metabolites during pregnancy and in cord with birthweight and birthweight z-score (adjusted for sex and gestational age according to Spanish references (Santamaría et al., 1998)) are reported in **Table 20**.

All reported correlations are very weak (coefficients ≤ |0.2|). The highest correlations, in boys and girls together and in girls alone, were between cord DMG/betaine and birthweight and birthweight z-score (positively correlated). In boys alone, it was in the case of cord betaine (negatively correlated with birthweight z-score) followed by cord DMG/betaine (positively correlated with birthweight z-score). Most of the associations between the nutrients or metabolites with birthweight z-score were observed in the cord (tHcy, B12, MMA, betaine, DMG, DMG/betaine). The metabolite/nutrients at ≤12 GW were less frequently correlated with birthweight z-score (only plasma folate and betaine were negatively correlated with birthweight z-score) compared to the other points of pregnancy.

Table 20. Spearman's rank-order correlation between pregnancy and cord 1C nutrients/metabolites and birthweight and z-birthweight.

Metabolite	Spearman correlation	Birthweight (grams)			Birthweight z-score[a]		
		All	Girls	Boys	All	Girls	Boys
tHcy µmol/L	coefficient	0.014	-0.005	-0.007	0.018	0.041	-0.008
<12 GW	N	703	352	351	703	352	351
tHcy µmol/L	coefficient	-0.026	0.005	-0.087	-0.015	0.062	-0.093
15 GW	N	445	229	216	445	229	216
tHcy µmol/L	coefficient	0.015	0.041	-0.023	0.054	0.090	0.015
24-27 GW	N	654	334	320	654	334	320
tHcy µmol/L	coefficient	0.049	0.108	-0.046	**0.091***	**0.168****	0.010
34 GW	N	630	318	312	630	318	312
tHcy µmol/L	coefficient	0.061	**0.125***	-0.019	0.055	**0.116***	-0.007
Labour	N	612	306	306	612	306	306
tHcy µmol/L	coefficient	0.045	**0.144***	-0.021	0.081	**0.159****	0.010
Cord	N	578	289	289	578	289	289
Folate nmol/L	coefficient	-0.065	-0.013	**-0.126***	**-0.079***	-0.067	-0.093
<12 GW	N	702	352	350	702	352	350
Folate nmol/L	coefficient	-0.024	-0.024	-0.050	-0.016	-0.045	0.009
15 GW	N	444	229	215	444	229	215
Folate nmol/L	coefficient	0.071	0.069	0.047	0.063	0.043	0.067
24-27 GW	N	653	334	319	653	334	319
Folate nmol/L	coefficient	0.031	-0.024	0.051	0.010	-0.043	0.055
34 GW	N	629	318	311	629	318	311
Folate nmol/L	coefficient	0.042	-0.016	0.073	0.034	-0.022	0.074
Labour	N	610	306	304	610	306	304
Folate nmol/L	coefficient	0.035	-0.027	0.072	0.026	-0.041	0.077
Cord	N	577	289	288	577	289	288
RBCF nmol/L	coefficient	-0.022	0.041	-0.087	-0.041	-0.029	-0.051
<12 GW	N	689	346	343	689	346	343
RBCF nmol/L	coefficient	-0.031	0.024	-0.076	-0.009	0.024	-0.031
15 GW	N	442	228	214	442	228	214
RBCF nmol/L	coefficient	0.057	0.104	0.004	0.054	0.075	0.027
24-27 GW	N	641	330	311	641	330	311
RBCF nmol/L	coefficient	0.060	0.043	0.053	0.053	0.041	0.056
34 GW	N	611	311	300	611	311	300
B12 pmol/L	coefficient	-0.020	-0.019	-0.022	-0.061	-0.075	-0.046
<12 GW	N	702	352	350	702	352	350
B12 pmol/L	coefficient	0.005	-0.059	0.057	-0.026	-0.066	0.013
15 GW	N	444	229	215	444	229	215
B12 pmol/L	coefficient	-0.033	-0.069	-0.015	**-0.098***	**-0.118***	-0.078
24-27 GW	N	653	334	319	653	334	319
B12 pmol/L	coefficient	-0.041	-0.093	-0.021	**-0.088***	**-0.124***	-0.059
34 GW	N	629	318	311	629	318	311
B12 pmol/L	coefficient	**-0.100***	-0.107	**-0.121***	**-0.146*****	**-0.151****	**-0.148***
Labour	N	605	304	301	605	304	301

Metabolite	Spearman correlation	Birthweight (grams)			Birthweight z-score[a]		
		All	Girls	Boys	All	Girls	Boys
B12 pmol/L	coefficient	0.025	-0.017	-0.006	**-0.112****	**-0.140***	-0.108
Cord	N	550	273	277	550	273	277
HoloTC pmol/L	coefficient	0.004	-0.069	0.060	-0.006	-0.071	0.067
<12 GW,	N	414	221	193	414	221	193
HoloTC pmol/L	coefficient	0.011	-0.077	0.057	-0.002	-0.062	0.054
15 GW	N	372	198	174	372	198	174
HoloTC pmol/L	coefficient	0.024	-0.068	0.072	0.002	-0.057	0.067
24-27 GW	N	396	213	183	396	213	183
HoloTC pmol/L	coefficient	0.038	-0.033	0.041	0.024	-0.007	0.049
34 GW	N	384	206	178	384	206	178
HoloTC pmol/L	coefficient	0.016	-0.025	-0.002	0.003	-0.013	0.010
Labour	N	357	188	169	357	188	169
HoloTC pmol/L	coefficient	0.007	-0.039	-0.066	-0.077	-0.103	-0.070
Cord	N	339	180	159	339	180	159
MMA µmol/L	coefficient	-0.020	-0.061	0.006	-0.007	0.009	-0.025
<12 GW	N	703	353	350	703	353	350
MMA µmol/L	coefficient	-0.048	-0.065	0.004	0.016	0.014	0.031
15 GW	N	444	229	215	444	229	215
MMA µmol/L	coefficient	-0.012	-0.019	-0.012	0.006	0.013	-0.002
24-27 GW	N	653	334	319	653	334	319
MMA µmol/L	coefficient	0.027	0.066	-0.023	0.048	0.102	-0.007
34 GW	N	629	318	311	629	318	311
MMA µmol/L	coefficient	**0.099***	0.103	0.077	**0.102***	**0.126***	0.076
Labour	N	611	306	305	611	306	305
MMA µmol/L	coefficient	0.066	0.074	0.080	**0.139****	**0.149***	**0.133***
Cord	N	577	289	288	577	289	288
Choline µmol/L	coefficient	0.002	0.011	-0.010	0.010	0.002	0.017
<12 GW	N	701	351	350	701	351	350
Choline µmol/L	coefficient	-0.008	0.006	-0.043	0.019	0.045	-0.012
15 GW	N	444	229	215	444	229	215
Choline µmol/L	coefficient	0.004	0.092	-0.083	0.030	**0.128***	-0.062
24-27 GW	N	653	334	319	653	334	319
Choline µmol/L	coefficient	-0.009	-0.002	-0.045	0.026	0.038	0.012
34 GW	N	629	318	311	629	318	311
Choline µmol/L	coefficient	**0.098***	**0.125***	0.037	**0.088***	**0.121***	0.043
Labour	N	611	306	305	611	306	305
Choline µmol/L	coefficient	-0.049	-0.019	-0.091	-0.041	-0.031	-0.060
Cord	N	573	288	285	573	288	285
Betaine µmol/L	coefficient	-0.059	-0.100	-0.003	**-0.082***	**-0.126***	-0.025
<12 GW	N	701	351	350	701	351	350
Betaine µmol/L	coefficient	-0.075	-0.072	-0.055	-0.079	-0.060	-0.084
15 GW	N	444	229	215	444	229	215
Betaine µmol/L	coefficient	-0.073	-0.063	-0.082	**-0.090***	-0.045	**-0.131***
24-27 GW	N	653	334	319	653	334	319
Betaine µmol/L	coefficient	**-0.102***	**-0.141***	-0.097	**-0.121****	**-0.128***	**-0.123***
34 GW	N	629	318	311	629	318	311

Metabolite	Spearman correlation	Birthweight (grams)			Birthweight z-score[a]		
		All	Girls	Boys	All	Girls	Boys
Betaine µmol/L	coefficient	0.005	-0.027	0.024	-0.028	-0.012	-0.044
Labour	N	611	306	305	611	306	305
Betaine µmol/L	coefficient	**-0.117****	**-0.139***	**-0.160****	**-0.122****	-0.089	**-0.176****
Cord	N	574	288	286	574	288	286
DMG µmol/L	coefficient	-0.047	-0.050	-0.031	-0.021	-0.049	0.009
<12 GW	N	701	351	350	701	351	350
DMG µmol/L	coefficient	-0.024	0.056	-0.048	-0.009	0.026	-0.025
15 GW	N	429	224	205	429	224	205
DMG µmol/L	coefficient	-0.037	0.011	-0.061	-0.004	0.007	-0.008
24-27 GW	N	652	333	319	652	333	319
DMG µmol/L	coefficient	-0.023	-0.020	-0.032	0.018	0.010	0.029
34 GW	N	629	318	311	629	318	311
DMGµmol/L	coefficient	0.019	0.009	0.032	0.047	0.016	0.078
Labour	N	611	306	305	611	306	305
DMG µmol/L	coefficient	0.046	0.053	0.034	**0.090***	0.089	0.093
Cord	N	574	288	286	574	288	286
DMG/Betaine	coefficient	0.026	0.067	-0.009	0.072	0.085	0.057
<12 GW	N	701	351	350	701	351	350
DMG/Betaine	coefficient	0.023	0.101	-0.013	0.038	0.071	0.018
15 GW	N	429	224	205	429	224	205
DMG/Betaine	coefficient	0.000	0.063	-0.036	0.043	0.044	0.045
24-27 GW	N	652	333	319	652	333	319
DMG/Betaine	coefficient	0.026	0.057	0.002	0.073	0.075	0.078
34 GW	N	629	318	311	629	318	311
DMG/Betaine	coefficient	0.018	0.035	0.007	0.055	0.026	0.086
Labour	N	611	306	305	611	306	305
DMG/Betaine	coefficient	**0.119****	**0.160****	0.107	**0.167*****	**0.169****	**0.176****
Cord	N	574	288	286	574	288	286
Methionine µmol/L	coefficient	0.070	0.086	0.038	0.066	0.087	0.043
<12 GW	N	702	352	350	702	352	350
Methionine µmol/L	coefficient	**0.098***	0.116	0.081	**0.117***	**0.168***	0.055
15 GW	N	444	229	215	444	229	215
Methionine µmol/L	coefficient	0.061	**0.112***	-0.013	0.075	**0.149****	-0.005
24-27 GW	N	653	334	319	653	334	319
Methionine µmol/L	coefficient	0.009	0.006	-0.023	0.020	0.079	-0.044
34 GW	N	629	318	311	629	318	311
Methionine µmol/L	coefficient	0.060	0.010	0.093	0.050	0.010	0.090
Labour	N	611	306	305	611	306	305
Methionine µmol/L	coefficient	0.077	0.004	0.065	0.028	0.001	0.030
Cord	N	577	289	288	577	289	288

Abbreviation: DMG: dimethylglycine, MMA: methylmalonic acid, RBCF: red blood cell folate, tHcy: fasting plasma total homocysteine.
Bold values highlight p <0.05.
[a]According to Spanish references (Santamaría et al., 1998).

Scatterplots showing the relationship between maternal plasma betaine, during pregnancy and in the cord, with birthweight are shown in **Figure 18**. As found in the scatterplots, the multiple linear regression models reported in **Table 21** generally showed negative coefficients. The crude models are unadjusted, model 1 adjusts for sex, gestational age at birth and maternal variables (body mass index, age etc.), plasma folate and plasma cobalamin status. Model 2 is the same as model 1 but also excluding preterm babies (<37 GW), model 3 is the model 1 that additionally includes hypertension (yes/no). The addition of hypertension causes that the mother-baby pairs included in the regression decrease by 141-168 pairs depending on the point of pregnancy.

At ≤ 12 GW there were no significant associations between pregnancy plasma betaine and birthweight. Plasma betaine status at 34 GW was inversely associated with birthweight in the crude model. With further adjustments, the association was no longer significant.

Cord plasma betaine was inversely associated with birthweight in all models for girls and boys together and separated by sex, only in boys, except for the final model that included hypertension and had less statistical power. In girls, betaine was inversely associated in the crude model and when preterm babies were excluded.

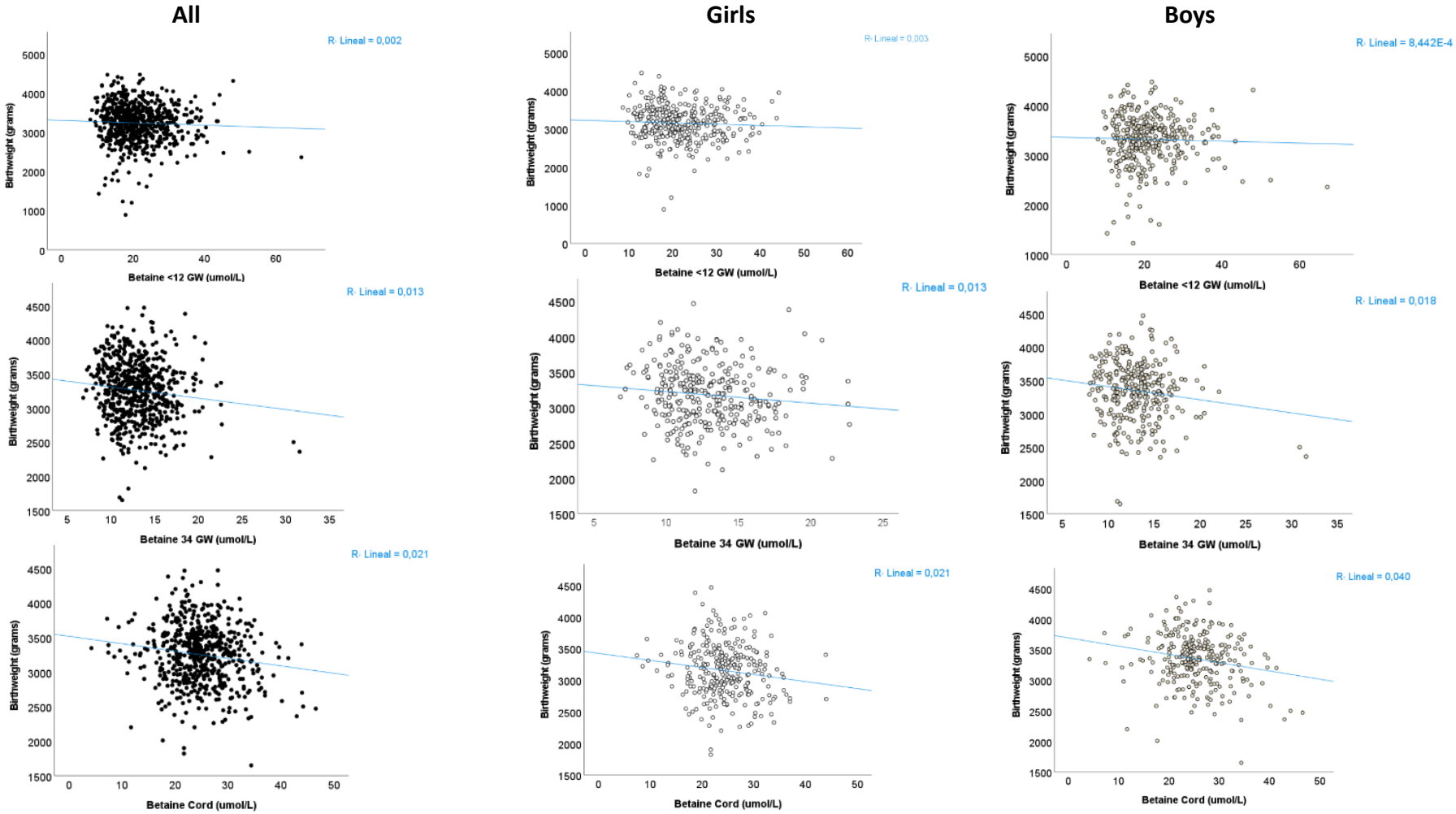


Figure 18. Scatterplots between betaine and birthweight.
Linear regression line and R^2 value.

Table 21. Association of maternal plasma betaine during pregnancy and in cord with birthweight by multiple linear regression analysis.

	All			Girls			Boys		
	N, Adjusted R^2	**B (SE)**	***P***	**N, Adjusted R^2**	**B (SE)**	***P***	**N, Adjusted R^2**	**B (SE)**	***P***
≤ 12 GW									
Crude	654, 0.001	-3.024 (2.470)	0.221	334, 0.002	-4.275 (3.320)	0.199	320, -0.003	-0.964 (3.560)	0.787
Model 1	654, 0.366***	-1.168 (2.033)	0.566	334, 0.339***	-4.345 (2.781)	0.119	320, 0.349***	2.426 (3.000)	0.419
Model 2	629, 0.282***	-0.927 (2.019)	0.646	319, 0.253***	-3.173 (2.784)	0.255	310, 0.244***	1.669 (2.978)	0.576
Model 3	486, 0.307***	-0.066 (2.508)	0.979	248, 0.288***	-4.763 (3.431)	0.166	238, 0.309***	6.272 (3.638)	0.086
34 GW									
Crude	596, 0.006*	-13.192 (6.202)	**0.034**	304, 0.011*	-17.236 (8.365)	**0.040**	292, 0.003	-11.837 (8.897)	0.184
Model 1	596, 0.339***	-4.972 (5.222)	0.341	304, 0.287***	-4.626 (7.431)	0.534	292, 0.332***	-6.800 (7.590)	0.371
Model 2	579, 0.296***	-5.909 (5.202)	0.256	295, 0.261***	-3.367 (7.513)	0.654	284, 0.252***	-9.267 (7.425)	0.213
Model 3	445, 0.279***	-4.907 (6.704)	0.465	228, 0.222***	-9.434 (9.508)	0.322	217, 0.303***	-1.464 (9.751)	0.881
Cord									
Crude	549, 0.017**	-9.868 (3.060)	**0.001**	276, 0.018*	-11.232 (4.547)	**0.014**	273, 0.030**	-12.025 (3.904)	**0.002**
Model 1	549, 0.345***	-7.763 (2.598)	**0.003**	276, 0.344***	-7.576 (3.872)	0.051	273, 0.249***	-8.210 (3.576)	**0.022**
Model 2	535, 0.298***	-8.208 (2.637)	**0.002**	266, 0.278***	-8.294 (3.947)	**0.037**	269, 0.227***	-8.569 (3.614)	**0.018**
Model 3	408, 0.287***	-5.810 (3.171)	0.068	206, 0.276***	-7.631 (4.767)	0.111	202, 0.196***	-4.152 (4.374)	0.344

B coefficient. SE Standard error.

Crude model was unadjusted. Model 1: Adjusted for sex, gestational age at delivery, socioeconomic status (low versus mid-high), parity (primipara or multipara versus nullipara), maternal age at the beginning of pregnancy, maternal body mass index at the beginning of pregnancy, maternal height, smoking in the 1st trimester versus never, smoking throughout pregnancy versus never, gestational diabetes mellitus (yes/no), anaemia in the last trimester, plasma folate in each point of pregnancy or in the cord and plasma cobalamin (low tertile (≤ 315.5 pmol/L) vs. mid-high) in the 1st trimester. Model 2: model 1 excluding preterm deliveries (<37 GW.) Model 3: model 1 adjusting for hypertension (yes/no). All models excluded unusual/influential cases (1 individual at each point of pregnancy and 1 in cord).

*p=<0.05. **p=<0.01. ***p=<0.001.

Bold values highlight p <0.05.

From the above table, we chose the completely adjusted model with more participants included (models 1) and we studied whether there was an interaction between maternal plasma betaine or cord betaine and folate concentrations in the relationship with birthweight. We found an interaction at 12 GW (for all P=0.019 and for girls P=0.040) and in cord (for all P=0.024 and for girls P=0.012).

Therefore, we stratified by pregnancy folate status (above and below median plasma folate at 12 GW and in cord). The results are reported in **Table 22**. Cord plasma betaine is inversely associated with birthweight when plasma folate is above the median in the cord (all and girls only).

Table 22. Association of maternal plasma betaine during pregnancy and in the cord with birthweight stratifying by median plasma folate by multiple linear regression analysis.

	All			Girls			Boys		
	N, Adjusted R^2	**B (SE)**	***P***	**N, Adjusted R^2**	**B (SE)**	***P***	**N, Adjusted R^2**	**B (SE)**	***P***
≤ 12 GW									
Folate >P50	327, 0.385***	-2.682 (2.872)	0.351	166, 0.293***	-4.164 (3.853)	0.281	161, 0.447***	1.158 (4.391)	0.792
Folate ≤P50	327, 0.331***	0.022 (2.952)	0.994	168, 0.384***	-4.002 (4.073)	0.327	159, 0.234***	4.396 (4.362)	0.315
Cord									
Folate >P50	274, 0.376***	-10.023 (3.764)	**0.008**	137, 0.416***	-13.882 (5.920)	**0.021**	137, 0.255***	-6.162 (4.868)	0.208
Folate ≤P50	275, 0.319***	-5.774 (3.751)	0.125	139, 0.402***	-1.924 (5.163)	0.710	136, 0.232***	-9.653 (5.538)	0.084

Abbreviations: B coefficient. SE: Standard error. P50: percentile 50.
Models adjusted for sex, gestational age at delivery, socioeconomic status (low versus mid-high), parity (primipara or multipara versus nullipara), maternal age at the beginning of pregnancy, maternal body mass index at the beginning of pregnancy, maternal height, smoking in the 1st trimester versus never, smoking throughout pregnancy versus never, gestational diabetes mellitus (yes/no), anaemia in the last trimester, plasma cobalamin (low tertile (≤ 315.5 pmol/L) vs. mid-high) in the 1st trimester. All models removed unusual/influential cases (1 individual at 12 GW and 1 in cord).
Folate P50 at 12 GW: 28.9 nmol/L; in cord: 26.3 nmol/L.
*p=<0.05. **p=<0.01. ***p=<0.001
Bold values highlight p <0.05.

Considering the association between betaine and birthweight described previously, we want to understand how this association is related with betaine's function as 1C donor or as an osmolyte. With this objective, we investigated the association between pregnancy and cord plasma DMG/betaine ratio with birthweight considering that DMG is only produced during the methylation of Hcy to methionine by BHMT enzyme using betaine as carbon donor (Lever, Slow, and Ueland 2011). Scatterplots between DMG/betaine and birthweight are shown in **Figure 19**. In those scatterplots were identified a few individuals with a very high ratio. To better understand the association for most of the population that are below those values, in **Figure 20** is shown the same association removing those with highest DMG/betaine ratio. The individuals removed were approximately above percentile 98 of the DMG/betaine ratio.

The association between pregnancy and cord plasma DMG/betaine with birthweight was not significant when including all the population (data not shown).

The association between pregnancy and cord plasma DMG/betaine with birthweight is reported in **Table 23** excluding participants with the highest ratio. DMG/betaine at 12 GW was positively associated with birthweight looking at girls and boys together ("All") but the association was lost when adjusted for hypertension and had less statistical power. There was no association between pregnancy plasma DMG/betaine at 34 GW with birthweight. Cord plasma DMG/betaine ratio was positively associated with birthweight in the models of girls and boys together and in boys only. The association was not significant in girls. A sensitivity analysis was performed removing preterm deliveries (<37 GW) and above results remain the same (data not shown).

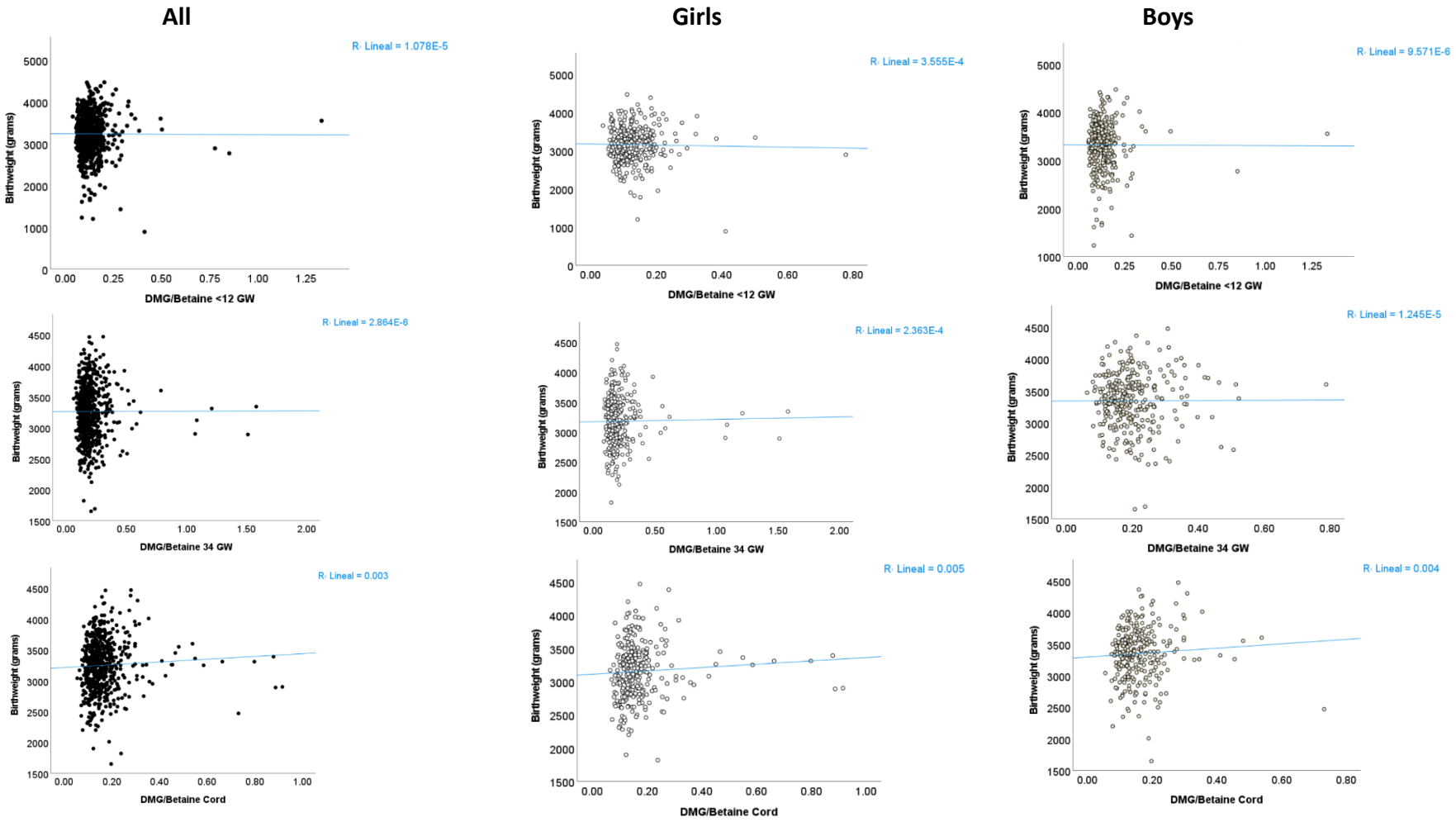


Figure 19. Scatterplots between DMG/betaine and birthweight.

Linear regression line and R^2 value.

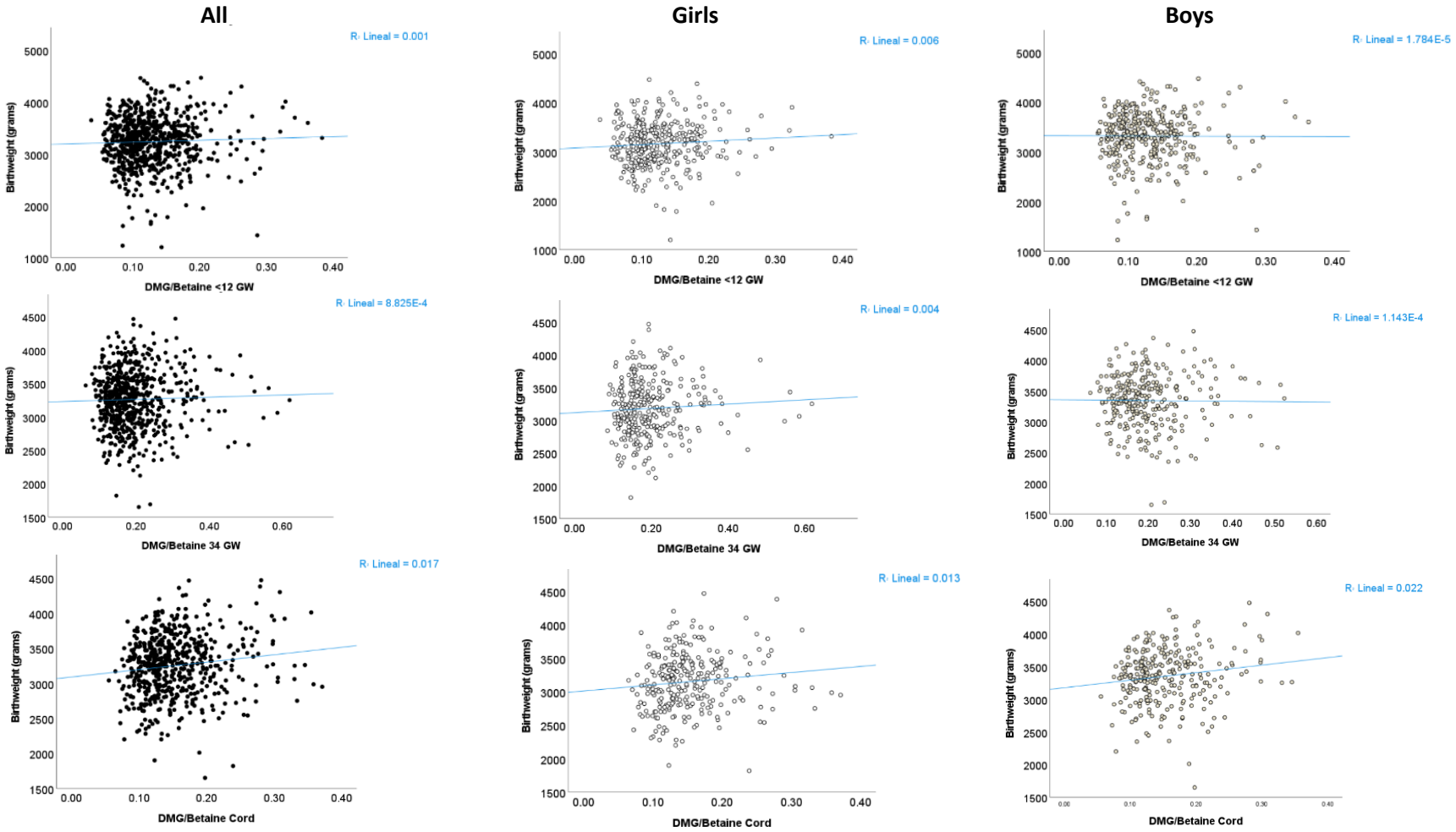


Figure 20. Scatterplots between DMG/betaine and birthweight without the highest ratios.
Linear regression line and the R^2 value. The highest ratios values are above 98th percentile (12 GW DMG/betaine>0.40, 34 GW DMG/betaine>0.78, Cord DMG/betaine> 0.40).

Table 23. Association of plasma DMG/betaine during pregnancy and in cord with birthweight by multiple linear regression analysis.

	All			Girls			Boys		
	N, Adjusted R^2	**B (SE)**	***P***	**N, Adjusted R^2**	**B (SE)**	***P***	**N, Adjusted R^2**	**B (SE)**	***P***
≤ 12 GW									
Crude	651, 0.002	530.6 (360.9)	0.142	332, 0.006	846.2 (494.8)	0.088	319, -0.003	134.5 (510.7)	0.792
Model 1	651, 0.367***	656.5 (292.2)	**0.025**	332, 0.346***	793.1 (410.5)	0.054	319, 0.336***	587.9 (425.3)	0.168
Model 2	483, 0.309***	609.4 (339.5)	0.073	246, 0.296***	727.2 (467.4)	0.121	237, 0.269***	404.4 (507.9)	0.427
34 GW									
Crude	604, -0.001	154.8 (223.8)	0.489	307, 0.001	341.4 (300.1)	0.256	297, -0.003	-80.6 (323.1)	0.803
Model 1	604, 0.341***	236.7 (184.0)	0.199	307, 0.296***	24.6 (258.4)	0.924	297, 0.334***	497.8 (271.8)	0.068
Model 2	451, 0.285***	136.6 (217.2)	0.530	231, 0.232***	-68.3 (303.8)	0.822	220, 0.305***	294.3 (319.1)	0.357
Cord									
Crude	544, 0.017***	1096.6 (343.3)	**0.001**	272, 0.010	932.7 (483.7)	0.055	272, 0.020*	1189.5 (462.0)	**0.011**
Model 1	544, 0,362***	1153.5 (281.2)	**<0.001**	272, 0.354***	781.4 (404.6)	0.054	272, 0.290***	1443.8 (399.8)	**<0.001**
Model 2	404, 0.313***	1051.3 (327.0)	**0.001**	201, 0.293***	854.4 (484.1)	0.079	203, 0.249***	1150.9 (461.0)	**0.013**

Abbreviations: B coefficient. SE Standard error.

Unusual/influential cases were identified in a scatterplot of the DMG/betaine ratio versus birthweight. Those above next cut-offs were removed in all models. Cut-offs for exclusion are above 98th percentile of DMG/betaine:

12 GW DMG/betaine>0.40. Number of participants excluded: 4.

34 GW DMG/betaine>0.78. Number of participants excluded: 5.

Cord DMG/betaine>0.40. Number of participants excluded: 14.

Crude model was unadjusted. Model 1: Adjusted for sex, gestational age at delivery, socioeconomic status (low versus mid-high), parity (primipara or multipara versus nullipara), maternal age at the beginning of pregnancy, maternal body mass index at the beginning of pregnancy, maternal height, smoking in the 1st trimester versus never, smoking throughout pregnancy versus never, gestational diabetes mellitus (yes/no), anaemia in the last trimester. Model 2: model 1 adjusting for hypertension (yes/no).

*p=<0.05. **p=<0.01. ***p=<0.001

Bold values highlight p <0.05.

5.2.2. *BHMT* c.716G>A polymorphism and birthweight

Plasma 1C nutrient and metabolite status during pregnancy and in the cord according to maternal *BHMT* c.716 G>A genotype is reported in **Table 24**. Plasma betaine, choline, folate, MMA and tHcy did not differ between genotypes. Plasma DMG was lowest in the AA compared to GA and GG genotypes throughout pregnancy. In general, similar to DMG results, the DMG/betaine ratio was lowest in AA genotypes.

Plasma 1C nutrient and metabolite status in the cord according to the baby's *BHMT* c.716 G>A genotype is reported in **Table 25**. There were differences in cord betaine (in GA higher than GG) and in DMG/betaine cord (in GA lower than GG).

Table 24. 1C metabolites during pregnancy and in cord according to maternal *BHMT* c.716 G>A polymorphism.[a]

	Maternal *BHMT* c.716 G>A polymorphism			
	GG	**GA**	**AA**	***P*[b]**
Betaine µmol/L				
≤12 GW	20.4 (19.7, 21.1) [301]	21.0 (20.2, 21.9) [274]	19.4 (17.9, 21.0) [70]	0.137
15 GW	14.6 (14.2, 15.0) [210]	15.3 (14.9, 15.8) [184]	14.7 (13.7, 15.7) [46]	0.074
24-27 GW	12.6 (12.2, 12.9) [286]	12.9 (12.5, 13.2) [255]	12.3 (11.7, 12.9) [65]	0.278
34 GW	12.7 (12.4, 13.0) [276]	12.9 (12.6, 13.3) [251]	12.6 (11.9, 13.3) [56]	0.464
Labour	13.0 (12.7, 13.4) [263]	13.2 (12.8, 13.6) [245]	12.9 (12.2, 13.6) [60]	0.654
Cord[c]	24.6 (23.8, 25.3) [251]	25.0 (24.3, 25.7) [230]	25.6 (23.9, 27.2) [53]	0.470
DMG µmol/L				
≤12 GW	2.6 (2.5, 2.7) [301][d]	2.4 (2.3, 2.5) [274]	2.4 (2.2, 2.6) [70]	**0.041**
15 GW	2.3 (2.2, 2.4) [205]	2.2 (2.1, 2.3) [180]	2.0 (1.8, 2.3) [42]	0.130
24-27 GW	2.2 (2.1, 2.3) [286]	2.1 (2.0, 2.2) [254]	2.0 (1.8, 2.2) [65]	0.058
34 GW	2.5 (2.4, 2.6) [276][e]	2.4 (2.3, 2.5) [251][f]	2.0 (1.8, 2.1) [56]	**<0.001**
Labour	3.0 (2.9, 3.2) [263][g,h]	2.7 (2.6, 2.9) [245]	2.5 (2.2, 2.9) [60]	**0.010**
Cord	3.7 (3.5, 3.9) [251]	3.5 (3.4, 3.7) [230]	3.4 (3.0, 3.8) [53]	0.366
DMG/Betaine				
≤12 GW	0.126 (0.121, 0.132) [301][i]	0.113 (0.109, 0.118) [274]	0.124 (0.110, 0.139) [70]	**0.004**
15 GW	0.155 (0.147, 0.162) [205][j]	0.141 (0.135, 0.148) [180]	0.137 (0.123, 0.153) [42]	**0.012**
24-27 GW	0.178 (0.170, 0.187) [286][k]	0.166 (0.158, 0.173) [254]	0.161 (0.147, 0.177) [65]	**0.039**
34 GW	0.197 (0.187, 0.207) [276][l]	0.184 (0.175, 0.193) [251][m]	0.156 (0.144, 0.168) [56]	**<0.001**
Labour	0.230 (0.218, 0.243) [263][n]	0.207 (0.196, 0.218) [245][o]	0.197 (0.174, 0.223) [60]	**0.005**
Cord	0.155 (0.147, 0.163) [251][p]	0.145 (0.139, 0.152) [230]	0.137 (0.123, 0.152) [53]	**0.044**
Methionine µmol/L				
≤12 GW	22.9 (22.5, 23.3) [302]	23.4 (23.0, 23.7) [274]	23.2 (22.6, 23.9) [70]	0.228
15 GW	22.1 (21.7, 22.4) [210]	22.1 (21.7, 22.6) [184]	22.7 (21.7, 23.8) [46]	0.395
24-27 GW	21.5 (21.2, 21.9) [286][q]	22.0 (21.6, 22.4) [255]	22.7 (21.8, 23.6) [65]	**0.019**
34 GW	21.8 (21.4, 22.2) [276]	22.4 (22.0, 22.8) [251]	22.3 (21.5, 23.1) [56]	0.095
Labour	22.3 (21.7, 22.9) [263]	22.2 (21.5, 22.9) [245]	23.6 (22.0, 25.4) [60]	0.211
Cord[c]	30.4 (29.5, 31.2) [251]	30.7 (29.9, 31.5) [233]	32.1 (30.0, 34.1) [53]	0.232
Choline µmol/L				
≤12 GW	7.5 (7.3, 7.7) [301]	7.6 (7.4, 7.8) [274]	7.6 (7.3, 8.0) [70]	0.855
15 GW	7.7 (7.4, 7.9) [210]	7.7 (7.5, 8.0) [184]	8.0 (7.6, 8.5) [46]	0.384
24-27 GW	9.0 (8.8, 9.2) [286]	9.1 (8.8, 9.3) [255]	9.4 (9.0, 9.8) [65]	0.306
34 GW	10.2 (10.0, 10.5) [276]	10.1 (9.8, 10.4) [251]	10.3 (9.9, 10.8) [56]	0.566[r]

	Maternal *BHMT* c.716 G>A polymorphism			
	GG	**GA**	**AA**	***P*[b]**
Labour	11.6 (11.3, 12.0) [263]	11.6 (11.2, 12.0) [245]	12.0 (11.2, 12.8) [60]	0.677
Cord	28.2 (26.7, 29.7) [250]	29.5 (28.1, 30.9) [230]	32.2 (28.0, 37.0) [53]	0.093
Folate nmol/L				
≤12 GW	27.5 (25.4, 29.8) [302]	26.4 (24.3, 28.6) [274]	25.2 (21.1, 30.1) [70]	0.574
15 GW	25.1 (22.9, 27.5) [210]	24.4 (22.2, 26.9) [184]	24.7 (19.8, 30.8) [46]	0.925
24-27 GW	14.4 (13.2, 15.7) [286]	14.1 (12.9, 15.4) [255]	14.9 (12.1, 18.2) [65]	0.862
34 GW	11.8 (10.8, 12.9) [276]	12.0 (10.9, 13.2) [251]	13.4 (10.9, 16.6) [56]	0.529
Labour	11.4 (10.3, 12.5) [263]	11.9 (10.7, 13.1) [244]	11.2 (9.0, 13.9) [60]	0.795
Cord	24.3 (22.6, 26.2) [251]	25.5 (23.8, 27.4) [233]	24.5 (21.0, 28.5) [53]	0.634
Cobalamin pmol/L				
≤12 GW	360 (347, 373) [302]	360 (346, 375) [274]	365 (328, 405) [70]	0.954
15 GW	316 (303, 330) [210]	330 (315, 346) [184]	337 (297, 383) [46]	0.309
24-27 GW	273 (263, 284) [286]	278 (265, 291) [255]	258 (239, 279) [65]	0.324
34 GW	242 (232, 252) [276]	259 (247, 271) [251]	239 (218, 262) [56]	0.060
Labour	225 (215, 236) [262][s]	244 (231, 257) [241][t]	210 (194, 227) [60]	**0.005**[r]
Cord	297 (273, 323) [246]	337 (310, 368) [223]	302 (255, 356) [51]	0.094
HoloTC pmol/L				
≤12 GW	69.6 (64.8, 74.7) [203]	75.5 (69.9, 81.5) [169]	72.5 (58.9, 89.1) [42]	0.331
15 GW	61.8 (57.5, 66.4) [179]	65.7 (60.8, 71.0) [156]	66.0 (51.5, 84.6) [37]	0.514
24-27 GW	58.8 (54.7, 63.3) [193]	61.0 (56.6, 65.9) [164]	51.4 (45.3, 58.4) [39]	0.154
34 GW	60.1 (55.7, 64.9) [191]	65.1 (59.9, 70.8) [158]	57.3 (46.3, 70.9) [35]	0.269
Labour	55.8 (51.4, 60.5) [170]	63.1 (57.2, 69.6)[u] [153]	49.5 (41.4, 59.2) [34]	**0.034**
Cord	157.8 (138.8, 179.4) [160]	170.8 (148.7, 196.2) [149][v]	116.8 (89.7, 152.1) [30]	**0.041**[r]
MMA µmol/L				
≤12 GW	0.119 (0.112, 0.127) [303]	0.115 (0.111, 0.120) [274]	0.119 (0.112, 0.127) [70]	0.490
15 GW	0.119 (0.114, 0.124) [210]	0.117 (0.112, 0.123) [184]	0.114 (0.106, 0.122) [46]	0.605
24-27 GW	0.130 (0.125, 0.135) [286]	0.127 (0.122, 0.132) [255]	0.125 (0.117, 0.134) [65]	0.580
34 GW	0.152 (0.146, 0.158) [276]	0.149 (0.143, 0.156) [251]	0.143 (0.132, 0.155) [56]	0.489
Labour	0.168 (0.161, 0.175) [263]	0.161 (0.154, 0.169) [245]	0.164 (0.150, 0.181) [60]	0.471
Cord	0.267 (0.255, 0.280) [251]	0.270 (0.259, 0.282) [233]	0.279 (0.257, 0.304) [53]	0.701
tHcy µmol/L				
≤12 GW	5.3 (5.2, 5.4) [302]	5.3 (5.1, 5.4) [275]	5.4 (5.1, 5.8) [70]	0.731
15 GW	4.6 (4.4, 4.7) [210]	4.5 (4.4, 4.6) [185]	4.5 (4.2, 4.8) [46]	0.780
24-27 GW	4.7 (4.6, 4.8) [286]	4.7 (4.5, 4.8) [256]	4.7 (4.4, 5.0) [65]	0.967
34 GW	5.3 (5.1, 5.4) [276]	5.2 (5.1, 5.4) [252]	5.3 (4.9, 5.6) [56]	0.988
Labour	6.2 (6.0, 6.5) [263]	6.1 (5.9, 6.3) [246]	6.3 (5.8, 6.7) [60]	0.620
Cord	4.8 (4.6, 5.0) [251]	4.9 (4.7, 5.1) [234]	4.9 (4.5, 5.3) [53]	0.706

Abbreviation: *BHMT:* betaine-homocysteine methyltransferase. DMG: dimethylglycine. GW: gestational weeks. HoloTC: holotranscobalamin. MMA: methylmalonic acid. tHcy: fasting plasma total homocysteine.

[a]All data shown are geometric means (95% confidence interval) [N], unless otherwise indicated.

[b]*P* value of the comparison of metabolite status between different genotypes at each time point was performed with ANOVA with post hoc Bonferroni correction for multiple comparisons, unless otherwise indicated.

[c]Mean (95% confidence interval) [N].

[d]Compared to GA p=0.049. [e]Compared to AA p=<0.001 [f]Compared to AA p=0.006.

[g]Compared to GA p=0.060. [h]Compared to AA p=0.033. [i]Compared to GA p=0.003.

[j]Compared to GA p=0.025. [k]Compared to GA p=0.086. [l]Compared to AA p=<0.001

[m]Compared to AA p=0.015 [n]Compared to GA p=0.017 [o]Compared to AA p=0.042

[p]Compared to AA p=0.094 [q]Compared to AA p=0.023

[r]Welch's ANOVA (for not homogeneous variance) with post hoc Games-Howell.

[s]Compared to GA p=0.071 [t]Compared to AA p=0.005 [u]Compared to AA p=0.075

[v]Compared to AA p=0.033

Bold values highlight p <0.05.

Table 25. Plasma 1C nutrient and metabolite concentrations in the cord according to baby's *BHMT* c.716 G>A genotype.[a]

Cord metabolite	Baby *BHMT* c.716 G>A			
	GG	GA	AA	*P*[b]
Betaine[c] µmol/L	24.3 (23.6, 25.1) [231][d]	25.5 (24.8, 26.2) [247]	24.1 (22.5, 25.6) [49]	**0.050**
DMG µmol/L	3.7 (3.5, 3.9) [231]	3.5 (3.4, 3.7) [247]	3.4 (3.0, 3.8) [49]	0.294
DMG/ Betaine	0.157 (0.148, 0.165) [231][e]	0.143 (0.137, 0.149) [247]	0.145 (0.131, 0.160) [49]	**0.026**
Choline µmol/L	29.7 (28.1, 31.3) [230]	28.4 (27.1, 29.8) [247]	31.6 (27.3, 36.5) [49]	0.247[f]
Methionine[c] µmol/L	30.7 (29.8, 31.5) [231]	30.9 (30.0, 31.7) [248]	31.5 (29.8, 33.2) [50]	0.704
Folate nmol/L	25.7 (23.8, 27.7) [231]	25.4 (23.7, 27.3) [248]	26.3 (22.7, 30.4) [50]	0.928
Cobalamin pmol/L	313 (288, 340) [222]	322 (297, 350) [242]	322 (269, 386) [48]	0.881
HoloTC pmol/L	158.6 (138.5, 181.6) [143]	165.6 (144.1, 190.1) [144]	159.8 (118.4, 215.6) [32]	0.905
MMA µmol/L	0.274 (0.261, 0.287) [231]	0.268 (0.256, 0.280) [248]	0.257 (0.235, 0.280) [50]	0.476
tHcy µmol/L	4.8 (4.6, 5.0) [231]	4.7 (4.5, 5.0) [249]	5.0 (4.6, 5.3) [50]	0.664

Abbreviation: DMG: dimethylglycine. HoloTC: holotranscobalamin. tHcy: fasting plasma total homocysteine. Bold values highlight p <0.05.

[a]All metabolites are geometric mean (95% confidence interval) [N], unless otherwise indicated.

[b]P-value of metabolite comparison between genotypes through ANOVA with post hoc Bonferroni correction for multiple comparisons, unless otherwise indicated.

[c]Mean (95% confidence interval) [N]

[d]Compared to GA p=0.077. [e]Compared to GA p=0.024.

[f]Welch's ANOVA (for not homogeneous variance).

There was no difference (ANOVA>0.05) in mean birthweight between *BHMT* c.716 G>A maternal or baby genotypes. In **Figure 21** and **Figure 22** birthweight according to maternal and baby genotypes respectively is illustrated. The results were similar stratifying by sex (not shown).

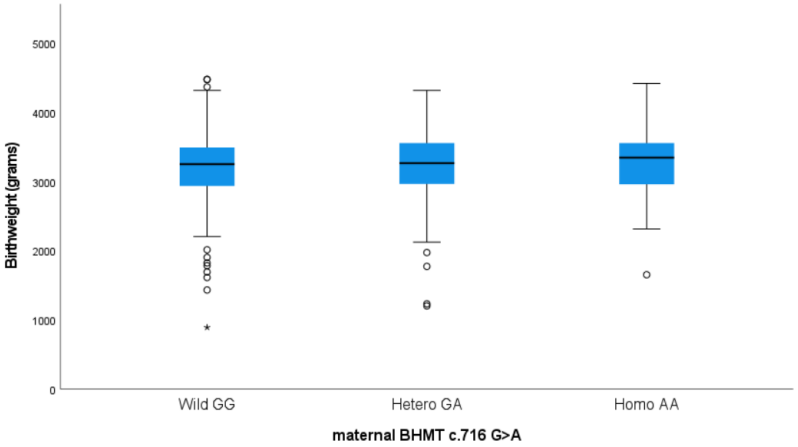


Figure 21. Boxplot of birthweight according to maternal *BHMT* c.716 G>A genotype.

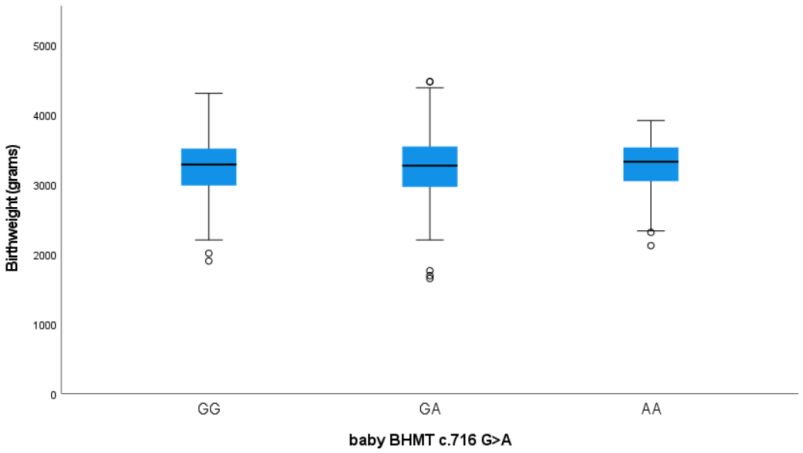


Figure 22. Boxplot of birthweight according to baby *BHMT* c.716 G>A genotype.

5.2.3. Pregnancy and cord betaine and SGA babies

Maternal and baby characteristics according to SGA babies are shown in **Table 26**. Babies SGA are born to mothers with lower BMI and shorter, higher frequency of nulliparous pregnancies, higher proportion of smoking, lower proportion of women with plasma cobalamin in the lowest tertile in the 1st trimester, higher plasma betaine at 34 GW and in cord and higher plasma folate at 12 GW.

Table 26. Maternal and baby characteristics according to SGA babies.[a]

	Baby small for gestational age		
	No	**Yes**	***P*[b]**
Age (years)[c]	32.1 (31.7, 32.4) [697]	32.1 (30.8, 33.5) [50]	0.937
BMI (kg/m^2)	24.1 (23.7;24.4) [690]	22.0 (21.2; 23.0) [50]	**0.001**
Height (cm)	163.0 (162.5; 163.4)	160.2 (158.5; 161.9)	**0.001**
Parity, nulliparous, %	46.9 (43.2, 50.7) [314/669]	62.0 (48.2, 74.1) [31/50]	**0.040**
Smoking during pregnancy, %			
No	74.3 (70.8; 77.5) [497]	60.0 (46.2; 72.4) [30]	**0.028**
Yes	25.7 (22.5; 29.2) [172]	40.0 (27.6; 53.8) [20]	
Socioeconomic status, %			
Low	11.8 (9.6; 14.5) [79]	10.0 (4.3; 21.4) [5]	0.212
Middle	45.2 (41.5; 49,0) [303]	58.0 (44.2; 70.6) [29]	
High	43.0 (39.3; 46.8) [288]	32.0 (20.8; 45.8) [16]	
Mother *BHMT* c.716 G>A, %			
GG+GA	89.5 (86.8; 91.7) [562]	81.6 (68.6; 90.0) [40]	0.091
AA	10.5 (8.3; 13.2) [66]	18.4 (10.0; 31.4) [9]	
Mother *MTHFR* 677C > T, %			
CC+CT	83.3 (80.2; 85.9) [532]	85.7 (73.3; 92.9) [42]	0.655
TT	16.7 (14.1; 19.8) [107]	14.3 (7.1; 26.7) [7]	
Gestational weeks at the first blood sample	9.4 (9.2; 9.5) [686]	10.2 (9.7; 10.7) [48]	**0.002**
Betaine			
≤12 GW	20.4 (19.9; 20.9) [654]	21.2 (19.1; 23.6) [47]	0.390
15 GW	14.8 (14.5; 15.1) [414]	16.1 (14.4; 18.1) [30]	0.147
24-27 GW	12.6 (12.3; 12.8) [609]	13.0 (12.0; 14.1) [44]	0.329
34 GW	12.6 (12.4; 12.9) [588]	13.9 (12.9; 14.9) [41]	**0.006**
Labour	13.0 (12.8; 13.3) [570]	13.4 (12.2; 14.6) [41]	0.495
Cord[c]	24.6 (24.2; 25.1) [537]	27.4 (25.0; 29.9) [37]	**0.029**
DMG			
≤12 GW	2.5 (2.4; 2.6) [654]	2.6 (2.3; 2.9) [47]	0.399
15 GW	2.2 (2.1; 2.3) [401]	2.4 (2.1; 2.9) [28]	0.087
24-27 GW	2.2 (2.02; 2.2) [609]	2.3 (2.0; 2.7) [43]	0.267
34 GW	2.4 (2.3; 2.5) [588]	2.5 (2.2; 2.8) [41]	0.578

	Baby small for gestational age		
	No	**Yes**	***P*[b]**
Labour	2.8 (2.7; 2.9) [570]	2.9 (2.4; 3.6) [41]	0.717
Cord	3.6 (3.5; 3.7) [537]	3.9 (3.2; 4.7) [37]	0.331
DMG/Betaine			
≤12 GW	0.122 (0.118; 0.126) [654]	0.123 (0.111; 0.136) [47]	0.937
15 GW	0.147 (0.142; 0.152) [401]	0.154 (0.134; 0.177) [28]	0.487
24-27 GW	0.171 (0.166; 0.177) [609]	0.177 (0.157; 0.200) [43]	0.580
34 GW	0.188 (0.182; 0.195) [588]	0.178 (0.161; 0.197) [41]	0.379
Labour	0.218 (0.210; 0.225) [570]	0.218 (0.181; 0.263) [41]	0.984
Cord	0.151 (0.146; 0.156) [537]	0.147 (0.127; 0.169) [37]	0.633
Folate			
≤12 GW	26.8 (25.4; 28.3) [655]	33.8 (27.4; 41.6) [47]	**0.030**
15 GW	24.4 (22.9; 26.0) [414]	26.9 (21.0; 34.5) [30]	0.442
24-27 GW	15.0 (14.1; 15.9) [609]	15.9 (12.6; 20.0) [44]	0.634
34 GW	12.7 (11.9; 13.6) [588]	13.5 (10.5; 17.4) [41}	0.655
Labour	12.1 (11.3; 12.9) [569]	11.6 (8.5; 15.9) [41]	0.765
Cord	26.1 (24.8; 27.4) [540]	24.9 (19.4; 31.9) [37]	0.630
RBCF			
≤12 GW	943 (904; 984) [642]	1052 (886; 1249) [47]	0.191
15 GW	1233 (1175; 1293) [412]	1221 (980; 1521) [30]	0.918
24-27 GW	1138 (1093; 1185) [598]	1195 (1101; 1412) [43]	0.544
34 GW	968 (923; 1015) [571]	1043 (868; 1253) [40]	0.430
Low tertile of 1st trimester maternal plasma cobalamin (≤ 315.5 pmol/L), %	34.4 (30.8; 38.1) [225/655]	19.1 (10.4; 32.5) [9/47]	**0.033**
Pregnancy outcomes			
Sex (boys), %	49.6 (45.9; 53.3) [346/697]	56.0 (42.3; 68.8) [28/50]	0.385
Birthweight, grams[c]	3288 (3254; 3321) [697]	2465 (2363; 2566) [50]	**<0.001**
Completed gestational weeks at delivery	39.0 (38.8; 39.1) [697]	38.7 (38.1; 39.2) [50]	0.223
Gestational hypertension, %	14.6 (11.8; 17.9) [74/508]	20.0 (10.0; 35.9) [7/35]	0.383
Gestational diabetes, %	6.6 (5.0; 8.7) [45/683]	6.0 (2.1; 16.2) [3/50]	1.000
Anaemia last trimester %	31.0 (27.5; 34.7) [197/635]	17.0 (8.9; 30.1) [8/47]	**0.043**
Preterm delivery (<37GW), %	5.2 (3.8; 7.1) [36/697]	10.0 (4.3; 21.4) [5/50]	0.259
Baby *BHMT c.716 G>A*, %			
GG+GA	90.8 (88.1; 93.0) [476]	88.6 (74.0; 95.5) [31]	0.883
AA	9.2 (7.0; 11.9) [48]	11.4 (4.5; 26.0) [4]	
Baby MTHFR 677 C > T, %			
CC+CT	81.0 (76.7,84.6) [307]	90.9 (72.2; 97.5) [20]	0.378
TT	19.0 (15.4; 23.3) [72]	9.1 (2.5;27.8) [2]	

Abbreviations: bet: Betaine. DMG: dimethylglycine. GW: gestational weeks. RBCF: red blood cell folate.

[a]All values are geometric means or percentage (95% confidence interval) [N], unless otherwise indicated.

[b]Proportions were compared using Chi-square test, arithmetic or geometric means were compared using Student's unpaired T test.
[*]P <0.05, [**]P <0.01, [***]P <0.001.
[c]Arithmetic means (95% confidence interval).
All 1C determination were done in plasma except for RBCF.

The association between maternal plasma betaine during pregnancy and cord betaine with SGA is shown in **Table 27**.

Plasma betaine concentration during pregnancy was not associated with risk of SGA except at 34 GW but only the crude model.

Cord plasma betaine concentration was associated with increased risk of SGA and stratifying by sex, showed that the association occurred in boys only.

Interaction between plasma betaine and folate was observed at 12 GW (in all the children P interaction: 0.030 and in boys P interaction=0.007) and in cord (in boys only P interaction: 0.038). Due to the limited number of babies with SGA, further stratification by folate was not possible.

Table 27. Association of maternal plasma betaine during pregnancy and cord betaine with small for gestational age (SGA) babies by multiple logistic regression analysis.

	All				Girls				Boys			
	N, R^2	**OR**	**CI**	***P***	**N, R^2**	**OR**	**CI**	***P***	**N, R^2**	**OR**	**CI**	***P***
≤12 GW												
Crude	655, 0.008	1.03	0.99; 1.07	0.152	334, 0.003	1.02	0.96; 1.08	0.532	321, 0.013	1.03	0.99; 1.08	0.171
Model 1	655, 0.145***	1.02	0.98; 1.06	0.376	334, 0.158*	1.01	0.95; 1.08	0.764	321, 0.249***	1.02	0.97; 1.08	0.464
Model 2	630, 0.154***	1.02	0.98; 1.07	0.273	319, 0.155	1.01	0.95; 1.08	0.747	311, 0.276***	1.03	0.97; 1.09	0.341
Model 3	486, 0.167**	1.01	0.96; 1.07	0.633	248, 0.162	1.03	0.96; 1.11	0.376	238, 0.317**	0.96	0.87; 1.06	0.428
34 GW												
Crude	597, 0.023*	**1.12**	**1.02; 1.23**	**0.017**	304, 0.032	1.16	1.00; 1.35	0.053	293, 0.016	1.09	0.97; 1.24	0.152
Model 1	597, 0.168***	1.09	0.98; 1.21	0.115	304, 0.252**	1.08	0.90; 1.30	0.415	293, 0.237**	1.06	0.92; 1.23	0.409
Model 2	580, 0.192***	1.10	0.99; 1.23	0.077	295, 0.252**	1.08	0.90; 1.29	0.432	285, 0.286***	1.08	0.94; 1.25	0.284
Model 3	445, 0.182**	1.04	0.90; 1.21	0.581	228, 0.255*	1.09	0.86; 1.37	0.476	217, 0.293*	0.94	0.74; 1.19	0.582
Cord												
Crude	550, 0.042**	**1.09**	**1.03; 1.15**	**0.003**	276, 0.029	1.08	0.99; 1.17	0.074	274, 0.061*	**1.11**	**1.02; 1.19**	**0.011**
Model 1	550, 0.147***	**1.08**	**1.02; 1.15**	**0.013**	276, 0.195*	1.07	0.97, 1.19	0.157	274, 0.205*	**1.09**	**1.00; 1.19**	**0.044**
Model 2	535, 0.150***	**1.08**	**1.01; 1.15**	**0.023**	266, 0.198*	1.08	0.97; 1.19	0.143	269, 0.206*	1.08	0.99; 1.18	0.091
Model 3	409; 0.155*	**1.08**	**1.00; 1.17**	**0.039**	206, 0.181	1.07	0.95; 1.20	0.253	203, 0.282	1.12	1.00; 1.25	0.057

Abbreviations: Nagelkerke R^2. OR: Odds ratio. CI: 95% confidence interval.
Crude model was unadjusted. Model 1: Adjusted for socioeconomic status (low versus mid-high), parity (primipara or multipara versus nullipara), maternal age at the beginning of pregnancy, maternal body mass index (BMI) at the beginning of pregnancy, maternal height, smoking during pregnancy (yes/no), gestational diabetes mellitus (yes/no), anaemia in the last trimester, plasma folate in each point of pregnancy or in the cord and plasma cobalamin (low tertile (≤ 315.5) vs. mid-high) in the 1st trimester. Model 2: model 1 removing preterm deliveries. Model 3: Model 1 including hypertension (yes/no).

5.2.4. *BHMT* c.716G>A polymorphism and SGA babies

The 4 main forms of inheritance testing the association between the maternal *BHMT* c.716G>A polymorphism and SGA are reported in **Table 28**.

The 4 main forms of inheritance were studied in 4 models:

- a crude model (including only the maternal polymorphism as the exposure and the SGA outcome).
- model 1 to 3, the same as the crude model but adjusted for several maternal covariables and plasma folate concentration associated with each blood sample (12 GW, 34 GW, cord).

In general, the AA genotype in the mothers was associated with increased risk of SGA in the codominant (AA vs. GG) and recessive (AA vs. GG+GA) models. Also each copy of A increased the risk of SGA by an additive amount (additive model). The dominant (GG vs. GA+AA) was never significant.

When both maternal *BHMT* c.716G>A genotype and betaine are included in the same regressions, the overall results from the models are similar. We observed an interaction between maternal *BHMT* c.716G>A genotype and plasma betaine at 12 GW (P for interaction term: codominant model P=0.009, dominant model P=0.002, additive model P=0.008) and with plasma betaine in cord (P for interaction term dominant model P=0.020). Further analysis by stratification is not possible due to the small number of babies born to mothers with AA genotype.

We also tested the interaction between maternal *BHMT* c.716G>A polymorphism and plasma folate status. It was significant at 34 gestational weeks for the 4 forms of inheritance (P for interaction terms: 0.003 in codominant model, 0.031 in dominant model, 0.006 in recessive model, <0.001 in additive model). The maternal *BHMT* c.716G>A polymorphism also interacted with cord plasma folate concentration in the additive model (P for interaction term: 0.038) and repeating the model with or without folate, the message is the same.

Further analysis by stratification is not possible due to the small number of babies born to mothers with AA genotype.

Table 28. *BHMT* c.716 G>A maternal polymorphism association with babies small for gestational age (SGA) by logistic regression analysis.

	Genotype	**No SGA**		**SGA**		**Model**	**OR**	**CI 95%**	**p**
		N	%	N	%	**N, $R^{2,}$, p**			
							Crude Model		
Codominant	GG	190	45.9	13	39.4	447, 0.018, 0.187	1.00		
	GA	183	44.2	13	39.4		1.04	0.47; 2.30	0.926
	AA	41	9.9	7	21.2		2.50	0.94; 6.64	0.064
Dominant	GG	190	45.9	13	39.4	447, 0.003, 0.468	1.00		
	GA+AA	224	54.1	20	60.6		1.30	0.63; 2.69	0.472
Recessive	GG+GA	373	90.1	26	78.8	447, 0.018, 0.067	1.00		
	AA	41	9.9	7	21.2		**2.45**	**1.00; 5.99**	**0.050**
Additive						447, 0.012, 0.145	1.47	0.88; 2.46	0.141

	Genotype	**No SGA**		**SGA**		**Model**	**OR**	**CI 95%**	**p**
		N	%	N	%	**N, $R^{2,}$, p**			
							Model 1		
Codominant	GG	190	45.9	13	39.4	447, 0.220, <0.001	1.00		
	GA	183	44.2	13	39.4		1.28	0.55; 2.98	0.572
	AA	41	9.9	7	21.2		**3.29**	**1.11; 9.77**	**0.032**
Dominant	GG	190	45.9	13	39.4	447, 0.206, <0.001	1.00		
	GA+AA	224	54.1	20	60.6		1.62	0.74; 3.52	0.227
Recessive	GG+GA	373	90.1	26	78.8	447, 0.218, <0.001	1.00		
	AA	41	9.9	7	21.2		**2.92**	**1.08; 7.88**	**0.035**
Additive						447, 0.216, <0.001	1.70	0.98; 2.95	0.059

	Genotype	No SGA		SGA		Model	OR	CI 95%	p
		N	%	N	%	N, $R^{2,}$, p			
							Model 2		
Codominant	GG	172	45.1	12	42.9	409, 0.223, <0.001	1.00		
	GA	174	45.7	10	35.7		0.92	0.37; 2.28	0.856
	AA	35	9.2	6	21.4		2.59	0.79; 8.47	0.116
Dominant	GG	172	45.1	12	42.9	409, 0.208, <0.001	1.00		
	GA+AA	209	54.9	16	57.1		1.18	0.52; 2.71	0.690
Recessive	GG+GA	346	90.8	22	78.6	409, 0.222, <0.001	1.00		
	AA	35	9.2	6	21.4		2.70	0.89; 8.13	0.079
Additive						409, 0.214, <0.001	1.42	0.77; 2.60	0.258

	Genotype	No SGA		SGA		Model	OR	CI 95%	p
		N	%	N	%	N, $R^{2,}$, p			
						Model 3			
Codominant	GG	163	46.4	9	34.6	377, 0.196, 0.007	1.00		
	GA	157	44.7	11	42.3		1.31	0.51; 3.40	0.577
	AA	31	8.8	6	23.1		**4.02**	**1.20; 13.52**	**0.024**
Dominant	GG	163	46.4	9	34.6	377, 0.176, 0.012	1.00		
	GA+AA	188	53.6	17	65.4		1.72	0.71; 4.15	0.227
Recessive	GG+GA	320	91.2	20	76.9	377, 0.194, 0.005	1.00		
	AA	31	8.8	6	23.1		**3.50**	**1.17; 10.49**	**0.025**
Additive						377, 0.191, 0.006	**1.87**	**1.00; 3.49**	**0.050**

Abbreviation: OR: odds ratio. CI: confidence interval. Nagelkerke R^2

Crude model was unadjusted. *Model 1:* adjusted for socioeconomic status (low versus mid-high), parity (primipara or multipara versus nullipara), maternal age at the beginning of pregnancy, maternal body mass index (BMI) at the beginning of pregnancy, maternal height, smoking in the 1st trimester versus never, smoking throughout pregnancy versus never, hypertension (yes/no), gestational diabetes mellitus (yes/no), anaemia in the last trimester, plasma folate and cobalamin (low tertile (≤ 315.5 pmol/L) vs. mid-high) in the 1st trimester. *Model 2:* model 1 changing folate in the 1st trimester for folate at 34 GW. *Model 3:* model 1 changing folate in the 1st trimester for plasma folate in cord. Plasma betaine during pregnancy was not included due to an interaction at 12 GW and in cord with the maternal polymorphism.

Table 29 presents the 4 main forms of inheritance (Iniesta, Guinó, and Moreno 2005) for the study of the association of baby *BHMT* c.716G>A polymorphism with SGA. We present a crude model (including only the baby polymorphism and the SGA outcome); and a model adjusted for maternal covariables, maternal plasma folate and cobalamin at 12 GW. The association was not significant. We studied it by adjusting for folate in the other points of pregnancy; however, they are not shown because they all give the same message as the 12 GW model.

Table 29. *BHMT* c.716 G>A baby polymorphism association with babies small for gestational age (SGA) by logistic regression analysis.

	Genotype	**No SGA**		**SGA**		**Model**	**OR**	**CI 95%**	**p**
		N	%	N	%	**N, $R^{2,}$, p**			
							Crude Model		
Codominant	GG	163	44.5	10	40.0	391, 0.002, 0.885	1.00		
	GA	167	45.6	12	48.0		1.17	0.49; 2.79	0.721
	AA	36	9.8	3	12.0		1.36	0.36; 5.19	0.654
Dominant	GG	163	44.5	10	40.0	391,0.001, 0.657	1.00		
	GA+AA	203	55.5	15	60.0		1.20	0.53; 2.75	0.659
Recessive	GG+GA	330	90.2	22	88.0	391, 0.001, 0.734	1.00		
	AA	36	9.8	3	12.0		1.25	0.36; 4.38	0.727
Additive						391, 0.002, 0.621	1.17	0.63; 2.15	0.619

	Genotype	No SGA		SGA		Model	OR	CI 95%	p
		N	%	N	%	N, $R^{2,}$, p			
							Model 1		
Codominant	GG	163	44.5	10	40.0	391, 0.161, 0.039	1.00		
	GA	167	45.6	12	48.0		1.03	0.41; 2.56	0.954
	AA	36	9.8	3	12.0		1.17	0.28; 4.78	0.832
Dominant	GG	163	44.5	10	40.0	391, 0.161, 0.027	1.00		
	GA+AA	203	55.5	15	60.0		1.05	0.44; 2.51	0.908
Recessive	GG+GA	330	90.2	22	88.0	391, 0.161, 0.027	1.00		
	AA	36	9.8	3	12.0		1.15	0.31; 4.33	0.837
Additive						391, 0.161, 0.027	1.06	0.56; 2.03	0.854

Abbreviation: OR: odds ratio. CI: confidence interval. Nagelkerke R^2

Crude model was unadjusted. *Model 1:* adjusted for socioeconomic status (low versus mid-high), parity (primipara or multipara versus nullipara), maternal age at the beginning of pregnancy, maternal body mass index (BMI) at the beginning of pregnancy, maternal height, smoking in the 1st trimester versus never, smoking throughout pregnancy versus never, hypertension (yes/no), gestational diabetes mellitus (yes/no), anaemia in the last trimester, plasma folate and cobalamin (low tertile (≤ 315.5 pmol/L) vs. mid-high) in the 1st trimester.

5.2.5. Betaine and DMG/betaine during pregnancy and in cord and mid childhood outcomes

Associations between pregnancy and cord plasma betaine, DMG/betaine and childhood outcomes are reported in **Table 30**. Plasma betaine and DMG/betaine were not associated with any childhood outcome. When the p values were <0.1, the coefficients between pregnancy betaine or DMG/betaine and the metabolic score or some of its components were positive.

Table 30. Association between plasma betaine, and DMG/betaine during pregnancy and in cord and child outcomes by multiple linear regression analysis.

		All		Girls		Boys	
		Adjusted R^2	**B[b] (SE)[c]**	**Adjusted R^2**	**B[b] (SE)[c]**	**Adjusted R^2**	**B[b] (SE)[c]**
Betaine 1st trimester[d]	Metabolic score	0.490***	0.007 (0.015)	0.477***	0.005 (0.023)	0.569***	-0.007 (0.018)
	zFat Mass Index	0.800***	**0.009 (0.005)**[p]	0.844***	**0.010 (0.006)**[m]	0.714***	0.003 (0.008)
	zHOMA-IR	0.157***	-0.001 (0.007)	0.169**	0.002 (0.012)	0.181*	-0.009 (0.008)
	(zTG–zHDLc)/2	0.028	-0.003 (0.018)	-0.022	-0.014 (0.027)	0.168*	-0.002 (0.024)
DMG/ Betaine 1st trimester[e]	Metabolic score	0.492***	0.461 (1.396)	0.478***	1.390 (3.615)	0.549***	1.158 (1.227)
	zFat Mass Index	0.797***	-0.695 (0.437)	0.843***	-1.380 (0.967)	0.715***	-0.242 (0.502)
	zHOMA-IR	0.180***	1.080 (0.687)	0.204***	2.162 (1.848)	0.164*	**0.960 (0.508)**[o]
	(zTG–zHDLc)/2	0.038	0.152 (1.682)	-0.009	1.215 (4.257)	0.186*	0.878 (1.561)
Betaine 3rd trimester[f]	Metabolic score	0.632***	-0.062 (0.039)	0.668***	-0.068 (0.052)	0.579***	-0.033 (0.062)
	zFat Mass Index	0.802***	-0.011 (0.015)	0.841***	-0.016 (0.019)	0.740***	-0.004 (0.025)
	zHOMA-IR	0.241***	-0.019 (0.020)	0.278***	-0.024 (0.028)	0.180*	-0.009 (0.026)
	(zTG–zHDLc)/2	0.124**	-0.066 (0.050)	0.145*	-0.055 (0.065)	0.135	-0.039 (0.083)
DMG/ Betaine 3rd trimester[g]	Metabolic score	0.622***	0.506 (0.715)	0.636***	0.701 (0.848)	0.580***	-0.497 (2.074)
	zFat Mass Index	0.798***	0.012 (0.273)	0.838***	-0.026 (0.293)	0.752***	0.138 (0.850)
	zHOMA-IR	0.239***	**0.611 (0.349)**[n]	0.267***	0.468 (0.432)	0.102[j]	0.914 (0.932)
	(zTG–zHDLc)/2	0.123**	-0.235 (0.903)	0.129*	0.516 (1.022)	0.198*	-3.098 (2.687)

		All		Girls		Boys	
		Adjusted R^2	**B[b] (SE)[c]**	**Adjusted R^2**	**B[b] (SE)[c]**	**Adjusted R^2**	**B[b] (SE)[c]**
Betaine in cord[h]	Metabolic score	0.598***	0.023 (0.020)	0.623***	0.000 (0.034)	0.589***	0.033 (0.029)
	zFat Mass Index	0.780***	0.005 (0.008)	0.830***	-0.014 (0.012)	0.704***	0.019 (0.012)
	zHOMA-IR	0.231***	-0.002 (0.010)	0.244**	0.000 (0.017)	0.106	-0.003 (0.012)
	(zTG–zHDLc)/2	0.106*	0.038 (0.026)	0.089[k]	0.026 (0.041)	0.206*	0.061 (0.039)
DMG/ Betaine in cord[i]	Metabolic score	0.592***	1.100 (1.182)	0.609***	1.062 (1.918)	0.533***	1.143 (1.601)
	zFat Mass Index	0.781***	0.731 (0.441)	0.825***	0.382 (0.659)	0.705***	1.029 (0.642)
	zHOMA-IR	0.242***	0.833 (0.547)	0.259***	0.640 (0.945)	0.122[l]	0.232 (0.629)
	(zTG–zHDLc)/2	0.108**	-0.929 (1.465)	0.106*	0.081 (2.255)	0.129[ñ]	-0.235 (2.150)

Abbreviations: DMG dimethylglycine, HDLc high density lipoprotein cholesterol, HOMA-IR homeostatic model assessment of insulin resistance, TG triglycerides.

[b]Unstandardized B coefficient

[c]Standard error.

[d]All (n=143), girls (n=82), boys (n=61).

[e]All (n= 143), girls (n=82), boys (n=61).

[f]All (n= 126), girls (n=72), boys (n=54).

[g]All (n=130), girls (n=75), boys (n= 55).

[h]All (n=117), girls (n=71), boys (n=46).

[i]All (n=121), girls (n=73), boys (n=48).

Models of betaine adjusted for: maternal age, maternal body mass index (BMI) at the beginning of pregnancy,, maternal plasma folate at each point (1st trimester, 3rd trimester and cord), maternal plasma cobalamin (low tertile vs. mid-high) in the 1st trimester, pregnancy smoking in the 1st trimester versus never, pregnancy smoking during pregnancy versus never, zBMI at childhood check-up, child betaine. Models of DMG/betaine adjusted for: same covariables than betaine models without folate and cobalamin and changing child betaine for child DMG/betaine. Models were not adjusted for breastfeeding due to the limited sample size. Models were not adjusted by socioeconomic status due to the limited sample size (there were only 6 children in the low category and the inclusion/exclusion of them does not change the betaine or DMG/betaine association with the outcomes).

Bold values denote B coefficients with p < 0.1. [j]P=0.096 [k]P=0.094, [l]P=0.089, [m]P=0.085, [n]P=0.083, [ñ]P=0.081 [o]P=0.064, [p]P=0.054.

*P<0.05, **P<0.01, ***P<0.001.

5.2.6. Testing the associations between child plasma betaine and other 1C metabolites with adiposity and other anthropometric measures in the child

In a previous section we found that RTBC child plasma betaine at 7.5 years was highest in the lowest vs mid-high maternal pregnancy plasma cobalamin tertile. Now, the association between RTBC child plasma betaine and other 1C nutrients or metabolites with adiposity measures estimated in 5 different ways is reported in **Table 31**. Child plasma betaine was associated with 5 adiposity measures looking at girls and boys together and stratifying by sex the same pattern was found. On the contrary, plasma DMG and the DMG/betaine ratio were associated only with one adiposity measure, as was the case with plasma choline. Total cysteine was associated with 3 adiposity measures and with the other 2 the trend was similar ($p<0.1$). The other plasma nutrients and metabolites (methionine, folate, red blood cell folate, cobalamin, MMA and tHcy) were not correlated with any adiposity measures.

Table 31. Spearman's rank-order correlation between child plasma 1C metabolism components and adiposity measures.

Child Metabolite			Waist circ. (cm)	Waist/ height	Fat mass (%)[a]	Fat mass index[b]	BMI (kg/m^2)
Betaine µmol/L	All	Coefficient	0.309	0.220	0.306	0.257	0.285
		P	**<0.001**	**0.003**	**<0.001**	**<0.001**	**<0.001**
		N	177	177	174	174	177
	Girls	Coefficient	0.311	0.177	0.261	0.213	0.255
		P	**0.002**	0.081	**0.010**	**0.037**	**0.011**
		N	98	98	97	96	98
	Boys	Coefficient	0.340	0.311	0.354	0.280	0.379
		P	**0.002**	**0.005**	**0.002**	**0.013**	**<0.001**
		N	79	79	77	78	79
DMG µmol/L	All	Coefficient	0.088	0.058	0.130	0.077	0.097
		P	0.244	0.440	0.087	0.313	0.199
		N	177	177	174	174	177
	Girls	Coefficient	0.062	0.017	0.064	0.010	0.042
		P	0.544	0.866	0.530	0.921	0.680
		N	98	98	97	96	98
	Boys	Coefficient	0.174	0.159	0.192	0.131	0.240
		P	0.125	0.161	0.094	0.253	**0.033**
		N	79	79	77	78	79
DMG/ Betaine	All	Coefficient	-0.149	-0.131	-0.101	-0.121	-0.126
		P	**0.047**	0.082	0.184	0.112	0.096
		N	177	177	174	174	177
	Girls	Coefficient	-0.204	-0.161	-0.128	-0.161	-0.181
		P	**0.044**	0.113	0.213	0.117	0.075
		N	98	98	97	96	98
	Boys	Coefficient	-0.070	-0.101	-0.075	-0.082	-0.030
		P	0.538	0.375	0.518	0.478	0.794
		N	79	79	77	78	79
Methionine µmol/L	All	Coefficient	0.057	0.033	0.095	0.125	0.099
		P	0.455	0.667	0.212	0.100	0.189
		N	177	177	174	174	177
	Girls	Coefficient	0.117	0.101	0.137	0.184	0.154
		P	0.251	0.324	0.180	0.073	0.131
		N	98	98	97	96	98
	Boys	Coefficient	0.015	-0.042	0.002	0.014	0.056
		P	0.893	0.712	0.987	0.904	0.624
		N	79	79	77	78	79

Child Metabolite			Waist circ. (cm)	Waist/ height	Fat mass (%)[a]	Fat mass index[b]	BMI (kg/m^2)
Choline µmol/L	All	Coefficient	0.166	0.095	0.143	0.116	0.145
		P	**0.027**	0.209	0.059	0.128	0.055
		N	177	177	174	174	177
	Girls	Coefficient	0.219	0.111	0.192	0.155	0.178
		P	**0.030**	0.279	0.059	0.131	0.079
		N	98	98	97	96	98
	Boys	Coefficient	0.061	0.016	0.141	0.105	0.102
		P	0.591	0.887	0.222	0.358	0.372
		N	79	79	77	78	79
Folate nmol/L	All	Coefficient	0.021	0.061	0.010	-0.003	0.040
		P	0.780	0.422	0.898	0.972	0.593
		N	177	177	174	174	177
	Girls	Coefficient	0.076	0.099	0.150	0.185	0.168
		P	0.457	0.331	0.143	0.072	0.098
		N	98	98	97	96	98
	Boys	Coefficient	-0.072	0.020	-0.147	-0.194	-0.160
		P	0.526	0.860	0.204	0.089	0.159
		N	79	79	77	78	79
Red blood cell folate nmol/L	All	Coefficient	-0.041	-0.033	-0.015	0.035	0.000
		P	0.589	0.667	0.843	0.648	0.998
		N	175	175	172	172	175
	Girls	Coefficient	-0.045	-0.087	0.013	0.135	0.079
		P	0.664	0.397	0.901	0.193	0.441
		N	97	97	96	95	97
	Boys	Coefficient	-0.047	0.041	-0.021	-0.045	-0.119
		P	0.682	0.719	0.859	0.694	0.301
		N	78	78	76	77	78
Cobalamin pmol/L	All	Coefficient	-0.072	-0.089	-0.096	-0.114	-0.055
		P	0.344	0.239	0.208	0.135	0.466
		N	176	176	173	173	176
	Girls	Coefficient	-0.011	-0.090	-0.020	-0.047	-0.027
		P	0.918	0.378	0.845	0.651	0.788
		N	98	98	97	96	98
	Boys	Coefficient	-0.169	-0.103	-0.170	-0.158	-0.143
		P	0.139	0.371	0.142	0.169	0.212
		N	78	78	76	77	78

Child Metabolite			Waist circ. (cm)	Waist/ height	Fat mass (%)[a]	Fat mass index[b]	BMI (kg/m^2)
MMA µmol/L	All	Coefficient	-0.030	-0.064	0.000	-0.029	-0.062
		P	0.696	0.396	0.996	0.706	0.412
		N	177	177	174	174	177
	Girls	Coefficient	0.084	0.027	0.066	-0.017	-0.002
		P	0.412	0.793	0.523	0.873	0.981
		N	98	98	97	96	98
	Boys	Coefficient	-0.178	-0.211	-0.110	-0.065	-0.154
		P	0.116	0.063	0.340	0.570	0.176
		N	79	79	77	78	79
tHcy µmol/L	All	Coefficient	-0.022	0.006	0.025	0.003	-0.051
		P	0.767	0.933	0.741	0.966	0.502
		N	177	177	174	174	177
	Girls	Coefficient	0.021	0.069	-0.038	-0.074	-0.075
		P	0.840	0.502	0.711	0.472	0.465
		N	98	98	97	96	98
	Boys	Coefficient	-0.062	-0.067	0.084	0.085	0.026
		P	0.588	0.560	0.466	0.457	0.822
		N	79	79	77	78	79
Total cysteine µmol/L	All	Coefficient	0.150	0.151	0.183	0.131	0.134
		P	**0.046**	**0.044**	**0.015**	0.086	0.075
		N	177	177	174	174	177
	Girls	Coefficient	0.162	0.170	0.155	0.095	0.103
		P	0.110	0.094	0.129	0.358	0.314
		N	98	98	97	96	98
	Boys	Coefficient	0.152	0.161	0.205	0.133	0.187
		P	0.182	0.156	0.074	0.247	0.099
		N	79	79	77	78	79

Abbreviations: BMI body mass index. Circ. Circumference. DMG: dimethylglycine MMA: methylmalonic acid tHcy: fasting plasma total homocysteine

Bold values highlight p <0.05. All metabolites are determined in plasma except for red blood cell folate.

[a]From tetrapolar bioelectrical impedance.

[b]From skinfold measurements. (Nagy et al., 2016)

To see whether similar correlations to those of between 1C components and fat mass are also found with muscle mass, fat-free mass, height and other body circumferences different from waist circumference, further correlations were investigated in **Table 32**.

Plasma betaine was correlated with fat free mass, muscular mass, height and other body circumferences different from waist circumference except for the head circumference. Stratifying by sex the association was significant in girls for all measurements and in boys only in one measurement. Plasma DMG was not associated, and the DMG/betaine ratio was inversely associated only in girls in 3 measurements.

Choline was correlated with 4 measurements looking at all of the children together (fat free mass, muscular mass, head and trunk circumference) and only in girls (fat free mass, muscular mass, height and trunk circumference) .

The other nutrients/metabolites (methionine, folate, red blood cell folate, cobalamin, MMA, tHcy and total cysteine) were not correlated or they were correlated with only one anthropometric measurement.

Table 32. Spearman's rank-order correlations between child 1C metabolism and other anthropometric measurements.

			Fat free mass (kg)[a]	Muscular mass (kg)[b]	Height (cm)	Head circ. (cm)	Arm circ. (cm)	Trunk circ. (cm)
Betaine µmol/L	All	Coefficient	0.277	0.289	0.209	0.120	0.319	0.230
		P	**<0.001**	**<0.001**	**0.005**	0.111	**<0.001**	**0.002**
		N	174	171	177	177	177	173
	Girls	Coefficient	0.354	0.372	0.330	0.218	0.301	0.327
		P	**<0.001**	**<0.001**	**<0.001**	**0.031**	**0.003**	**0.001**
		N	97	94	98	98	98	96
	Boys	Coefficient	0.194	0.193	0.038	0.087	0.326	0.157
		P	0.091	0.092	0.742	0.447	**0.003**	0.174
		N	77	77	79	79	79	77
DMG µmol/L	All	Coefficient	0.060	0.078	0.037	-0.020	0.096	0.082
		P	0.429	0.311	0.625	0.792	0.204	0.286
		N	174	171	177	177	177	173
	Girls	Coefficient	0.049	0.073	0.091	-0.044	0.053	0.076
		P	0.635	0.484	0.371	0.664	0.602	0.460
		N	97	94	98	98	98	96
	Boys	Coefficient	0.092	0.091	-0.047	0.032	0.148	0.135
		P	0.425	0.430	0.681	0.782	0.194	0.242
		N	77	77	79	79	79	77
DMG/ Betaine	All	Coefficient	-0.122	-0.109	-0.071	-0.119	-0.140	-0.104
		P	0.109	0.156	0.350	0.114	0.063	0.174
		N	174	171	177	177	177	173
	Girls	Coefficient	-0.224	-0.205	-0.123	-0.203	-0.183	-0.176
		P	**0.028**	**0.048**	0.226	**0.045**	0.072	0.086
		N	97	94	98	98	98	96
	Boys	Coefficient	0.004	0.002	-0.012	-0.080	-0.087	-0.020
		P	0.975	0.984	0.919	0.485	0.446	0.866
		N	77	77	79	79	79	77
Methionine µmol/L	All	Coefficient	0.081	0.107	0.069	-0.146	0.129	0.054
		P	0.290	0.163	0.360	0.053	0.088	0.483
		N	174	171	177	177	177	173
	Girls	Coefficient	0.139	0.183	0.125	-0.033	0.150	0.186
		P	0.175	0.078	0.221	0.744	0.140	0.069
		N	97	94	98	98	98	96
	Boys	Coefficient	0.048	0.048	0.006	-0.235	0.088	-0.063
		P	0.677	0.677	0.958	**0.037**	0.440	0.585
		N	77	77	79	79	79	77

			Fat free mass (kg)[a]	Muscular mass (kg)[b]	Height (cm)	Head circ. (cm)	Arm circ. (cm)	Trunk circ. (cm)
Choline µmol/L	All	Coefficient	0.163	0.182	0.131	0.150	0.131	0.177
		P	**0.032**	**0.017**	0.082	**0.047**	0.083	**0.020**
		N	174	171	177	177	177	173
	Girls	Coefficient	0.266	0.306	0.272	0.147	0.167	0.239
		P	**0.008**	**0.003**	**0.007**	0.150	0.100	**0.019**
		N	97	94	98	98	98	96
	Boys	Coefficient	0.013	0.014	-0.048	0.066	0.097	0.041
		P	0.907	0.906	0.673	0.564	0.397	0.726
		N	77	77	79	79	79	77
Folate nmol/L	All	Coefficient	-0.059	-0.075	-0.106	-0.010	-0.003	0.013
		P	0.440	0.332	0.162	0.891	0.966	0.868
		N	174	171	177	177	177	173
	Girls	Coefficient	0.069	0.050	-0.060	0.043	0.184	0.072
		P	0.499	0.629	0.559	0.675	0.069	0.488
		N	97	94	98	98	98	96
	Boys	Coefficient	-0.223	-0.223	-0.176	-0.164	-0.224	-0.070
		P	0.051	0.051	0.120	0.148	**0.047**	0.547
		N	77	77	79	79	79	77
Red blood cell folate nmol/L	All	Coefficient	-0.025	-0.032	-0.025	-0.004	-0.001	-0.069
		P	0.743	0.683	0.747	0.955	0.988	0.371
		N	172	169	175	175	175	171
	Girls	Coefficient	0.105	0.108	0.103	0.001	0.079	-0.006
		P	0.309	0.303	0.316	0.994	0.439	0.952
		N	96	93	97	97	97	95
	Boys	Coefficient	-0.181	-0.181	-0.165	-0.051	-0.087	-0.146
		P	0.117	0.117	0.150	0.660	0.450	0.209
		N	76	76	78	78	78	76
Cobalamin pmol/L	All	Coefficient	-0.039	-0.035	-0.037	-0.028	-0.101	-0.065
		P	0.611	0.655	0.629	0.711	0.184	0.396
		N	173	170	176	176	176	172
	Girls	Coefficient	0.035	0.052	0.101	-0.006	-0.034	-0.029
		P	0.733	0.620	0.324	0.954	0.742	0.781
		N	97	94	98	98	98	96
	Boys	Coefficient	-0.144	-0.145	-0.202	-0.159	-0.185	-0.129
		P	0.213	0.212	0.076	0.165	0.106	0.266
		N	76	76	78	78	78	76

			Fat free mass (kg)[a]	Muscular mass (kg)[b]	Height (cm)	Head circ. (cm)	Arm circ. (cm)	Trunk circ. (cm)
MMA µmol/L	All	Coefficient	-0.001	0.018	0.090	-0.110	-0.010	0.009
		P	0.992	0.816	0.234	0.146	0.893	0.908
		N	174	171	177	177	177	173
	Girls	Coefficient	0.067	0.104	0.094	-0.096	0.041	0.104
		P	0.511	0.317	0.357	0.345	0.688	0.311
		N	97	94	98	98	98	96
	Boys	Coefficient	-0.067	-0.067	0.096	-0.129	-0.073	-0.125
		P	0.564	0.565	0.399	0.258	0.523	0.277
		N	77	77	79	79	79	77
tHcy µmol/L	All	Coefficient	-0.055	-0.034	-0.043	-0.054	-0.001	-0.068
		P	0.473	0.663	0.571	0.479	0.987	0.372
		N	174	171	177	177	177	173
	Girls	Coefficient	-0.095	-0.063	-0.126	-0.084	-0.068	-0.087
		P	0.353	0.546	0.217	0.413	0.505	0.397
		N	97	94	98	98	98	96
	Boys	Coefficient	0.017	0.017	0.045	0.054	0.077	-0.061
		P	0.886	0.887	0.696	0.639	0.499	0.599
		N	77	77	79	79	79	77
Total cysteine µmol/L	All	Coefficient	0.073	0.069	0.004	-0.008	0.169	0.101
		P	0.338	0.373	0.954	0.918	**0.024**	0.185
		N	174	171	177	177	177	173
	Girls	Coefficient	0.058	0.044	-0.050	0.026	0.140	0.083
		P	0.572	0.676	0.626	0.796	0.170	0.421
		N	97	94	98	98	98	96
	Boys	Coefficient	0.106	0.106	0.019	0.003	0.185	0.127
		P	0.357	0.359	0.866	0.981	0.102	0.270
		N	77	77	79	79	79	77

Abbreviations: Circ. Circumference. DMG: dimethylglycine MMA: methylmalonic acid tHcy: fasting plasma total homocysteine

Bold values highlight p <0.05.

[a]From tetrapolar bioelectrical impedance.

5.3. Identification of metabolites associated with adiposity in 7.5 year-old children

From an initial list of 20 nutrients/metabolites and their correlation with five measures of adiposity, **Table 33** shows the correlation of 7 selected nutrients/metabolites. Correlations with at least 3 measurements of fat mass that were significant or approaching significance ($p<0.1$) (in boys and girls together), are reported. The objective was to identify potential metabolites involved in the fat mass metabolism that might be relevant for the study and prevention of metabolic syndrome. Some of them do not have previous direct results associating them with adiposity. Those metabolites associated with more fat measurements are shown on the top of the table.

The only metabolite that was associated with all fat mass measurements was HAA. Stratifying by sex, the 5 correlations are significant in girls and in boys is associated only with fat mass percentage. The kynurenic acid, part of the same pathway of HAA, is also associated with 2 fat measurements (and 2 other measurements have a $p<0.1$). Stratifying by sex, the correlations are only seen in boys opposite to the HAA acid that was correlated in all measurements in girls. The kynurenic acid is the fourth metabolite more frequently associated with the fat measurements. From the same pathway, the sixth metabolite of the table, picolinic acid was inversely associated with one fat measurement and follow a similar trend for other 3 measurements ($p<0.1$) but the association was not observed splitting by sex.

The second metabolite more frequently correlated with the fat measurements is histidine, we found a positive correlation with four fat measurements, and stratifying by sex the associations were only seen in girls. The next metabolite more frequently associated with the fat measurements was trigonelline. The associations were inverse. Stratification by sex found the correlations only significant in boys. ADMA was positively associated with 2 fat measurements looking at all (and 2 other measurements have a $p<0.1$). Stratifying by sex, ADMA was significantly correlated with fat measurements only for girls. Finally, neopterin looking at girls and boys together was inversely correlated with one fat measurement and

other 2 measurements followed the same trend (p<0.1). The association was not observed splitting by sex.

Table 33. Spearman's rank-order correlation of some child metabolites with adiposity measures.

			Waist circ.(cm)	Waist/ height	Fat mass (%)[a]	Fat mass index[b]	BMI (kg/m²)
HAA nmol/L	All	coefficient	0.336	0.264	0.367	0.350	0.328
		P	**<0.001**	**0.008**	**<0.001**	**<0.001**	**<0.001**
		N	99	99	97	98	99
	Girls	coefficient	0.470	0.367	0.412	0.464	0.363
		P	**<0.001**	**0.006**	**0.002**	**<0.001**	**0.006**
		N	55	55	54	54	55
	Boys	coefficient	0.179	0.131	0.317	0.205	0.277
		P	0.244	0.397	**0.038**	0.183	0.069
		N	44	44	43	44	44
Histidine µmol/L	All	coefficient	0.162	0.110	0.230	0.206	0.222
		P	**0.031**	0.145	**0.002**	**0.006**	**0.003**
		N	177	177	174	174	177
	Girls	coefficient	0.275	0.228	0.324	0.262	0.300
		P	**0.006**	**0.024**	**0.001**	**0.010**	**0.003**
		N	98	98	97	96	98
	Boys	coefficient	0.048	-0.043	0.078	0.133	0.122
		P	0.675	0.709	0.499	0.245	0.284
		N	79	79	77	78	79
Trigonelline µmol/L	All	coefficient	-0.224	-0.190	-0.119	-0.234	-0.201
		P	**0.026**	0.059	0.247	**0.021**	**0.046**
		N	99	99	97	98	99
	Girls	coefficient	-0.039	-0.041	0.036	-0.065	-0.039
		P	0.778	0.765	0.797	0.642	0.776
		N	55	55	54	54	55
	Boys	coefficient	-0.517	-0.481	-0.310	-0.403	-0.484
		P	**<0.001**	**<0.001**	**0.043**	**0.007**	**<0.001**
		N	44	44	43	44	44
Kynurenic acid nmol/L	All	coefficient	0.197	0.076	0.255	0.229	0.189
		P	0.051	0.454	**0.012**	**0.024**	0.061
		N	99	99	97	98	99
	Girls	coefficient	0.146	0.082	0.205	0.152	0.101
		P	0.287	0.551	0.137	0.272	0.464
		N	55	55	54	54	55
	Boys	coefficient	0.293	0.039	0.383	0.384	0.322
		P	0.054	0.802	**0.011**	**0.010**	**0.033**
		N	44	44	43	44	44

			Waist circ.(cm)	Waist/ height	Fat mass (%)[a]	Fat mass index[b]	BMI (kg/m^2)
ADMA µmol/L	All	coefficient	0.223	0.126	0.139	0.124	0.167
		P	**0.003**	0.095	0.067	0.104	**0.026**
		N	177	177	174	174	177
	Girls	coefficient	0.273	0.166	0.219	0.195	0.223
		P	**0.007**	0.103	**0.031**	0.058	**0.027**
		N	98	98	97	96	98
	Boys	coefficient	0.158	0.020	0.102	0.101	0.086
		P	0.163	0.864	0.378	0.378	0.449
		N	79	79	77	78	79
Picolinic acid nmol/L	All	coefficient	-0.173	-0.249	-0.194	-0.148	-0.167
		P	0.086	**0.013**	0.057	0.145	0.099
		N	99	99	97	98	99
	Girls	coefficient	-0.205	-0.252	-0.214	-0.200	-0.212
		P	0.134	0.063	0.121	0.147	0.120
		N	55	55	54	54	55
	Boys	coefficient	-0.105	-0.251	-0.157	-0.044	-0.080
		P	0.499	0.100	0.316	0.775	0.605
		N	44	44	43	44	44
Neopterin nmol/L	All	coefficient	-0.144	-0.117	-0.119	-0.161	-0.190
		P	0.085	0.163	0.159	0.055	**0.023**
		N	144	144	141	142	144
	Girls	coefficient	-0.149	-0.134	-0.175	-0.191	-0.205
		P	0.183	0.231	0.120	0.090	0.066
		N	81	81	80	80	81
	Boys	coefficient	-0.102	-0.078	-0.072	-0.169	-0.137
		P	0.428	0.545	0.584	0.190	0.283
		N	63	63	61	62	63

Abbreviations: BMI body mass index. Circ. Circumference. HAA: 3-hydroxyanthranilic acid. ADMA: Asymmetric dimethylarginine

[a] From tetrapolar bioelectrical impedance.

[b]From skinfold measurements (Nagy et al., 2016).

6. Discussion

6.1. Pregnancy cobalamin status, homocysteine, folate and mid childhood outcomes

The discussion of this section is part of the publication:

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The following is an extended discussion.

Principal findings

Moderately elevated pregnancy tHcy was positively associated with MetSco in boys, and specifically zFMI and zHOMA-IR. Low plasma holoTC and high plasma MMA in the 1st trimester were positively associated with MetSco, 1st trimester low plasma holoTC with zFMI and zHOMA-IR, and 1st trimester high plasma MMA with dyslipidemia in boys. The pregnancy tHcy-child zFMI association was not mediated by birthweight. Low versus mid-high 3rd trimester plasma cobalamin was associated with lower FMI and low versus mid-high plasma folate with lower dyslipidemia in boys. In girls only, low versus mid-high 1st trimester plasma folate was positively associated with zHOMA-IR.

Comparison with previous studies

Child Adiposity.

Overall, these findings in participants with a low prevalence of cobalamin deficiency support previous observations from studies in countries where cobalamin deficiency is highly prevalent. However, none of those were stratified by sex. Indian studies reported no association between pregnancy tHcy and percentage body fat or other anthropometric measurements (e.g. truncal, leg fat mass and percentage body fat) in the offspring in

mid-childhood (Krishnaveni et al., 2014; Yajnik et al., 2008). In our study, birthweight was not a mediator of the association between maternal tHcy and child zFMI. A previous study refuted birthweight as a mediator in the association between pre-pregnancy obesity and anthropometric outcomes in children (Adane et al., 2019).

We observed no association between pregnancy folate status and offspring FMI. However, a USA study observed that postpartum maternal plasma folate in the highest quartile compared to the lowest quartile, protected against high BMI z-score and probability of overweight or obesity in the offspring. This was especially evident among obese mothers. These associations were weaker when child adiposity was included in the models, leading the authors to suggest that offspring adiposity mediates the observed pregnancy-offspring associations (G. Wang et al., 2016).

Child insulin resistance.

The association between pregnancy tHcy and insulin resistance in boys agrees with the findings for child postload glucose concentrations, plasma insulin concentrations, and HOMA-IR reported in an Indian study, although not stratified by sex (Krishnaveni et al., 2014). Maternal plasma cobalamin status was not associated with insulin resistance in the offspring in our study, agreeing with one Indian study (Krishnaveni et al., 2014) but not with another (Yajnik et al., 2008) or a Nepalese study (Stewart et al., 2011). The difference with these studies may be due to the important differences in cobalamin status between these cohorts. Cobalamin deficiency (plasma cobalamin <150 pmol/L), affected 60% and 70% of the Pune cohort mothers at 18 and 28 GW respectively (Yajnik et al., 2008). This contrasted with 2.6% of our mothers in the 1st trimester and 12.1% in the 3rd trimester. However, we observed that low pregnancy holoTC (fraction of cobalamin bound to trans-cobalamin II for tissue uptake) (Nexo & Hoffmann-Lücke, 2011) status was associated with insulin resistance in boys.

The observed association between low pregnancy plasma folate status and higher HOMA-IR in the children (specifically girls) agrees with a USA study that reported higher insulin

resistance in children born to obese mothers with low folate status (G. Wang et al., 2016). Our results disagree with those from the Indian studies reporting an association between high pregnancy folate status and insulin resistance in the offspring (Krishnaveni et al., 2014; Yajnik et al., 2008). In an animal study, folic acid-deficient diets led to increased steatosis in mice (associated with insulin resistance) (Christensen et al., 2010). However, unlike our study where the low pregnancy folate–child insulin resistance association was limited to girls, in the mice the effects were more frequent and severe in males.

Child plasma lipids.

The lack of association between maternal tHcy and offspring dyslipidemia agrees with a previous Indian study (Krishnaveni et al., 2014).

On the other hand, pregnancy plasma MMA was positively associated with MetSco and dyslipidemia in boys. The low versus mid-high pregnancy plasma folate status was associated with lower dyslipidaemia in the children (specifically in boys) and this finding disagrees with a Dutch study that reported no association between pregnancy folate and triglyceride status in the child (Krikke et al., 2016). However, high folic acid diets provoked alterations in hepatocyte lipid metabolism consistent with increased lipogenesis in male mice (Christensen et al., 2015).

Child blood pressure.

Blood pressure is an outcome that is not included in our metabolic score because it is not available for the PreC cohort. However, in the RTBC cohort, child point blood pressure did not vary with pregnancy tHcy status. Other studies from Europe and from India, reported no association between pregnancy tHcy (van den Hil et al., 2013) or between pregnancy folate, vitamin B12 or homocysteine and offspring blood pressure (Krishnaveni et al., 2014). In the Boston Birth Cohort, maternal plasma Hcy after delivery was associated with systolic blood pressure in children showing a U pattern (higher risk of elevated systolic blood pressure

amongst those in the lowest Hcy quartile vs. quartile 2-3, and also higher risk of elevated systolic blood pressure in those in the highest Hcy quartile vs. quartile 2-3). The association was stronger in children born to obese mothers with Hcy in quartile 4 versus non-obese mother in quartile 2 and 3 of tHcy (H. Wang et al., 2017). The prevalence of maternal obesity (BMI≥30kg/m^2) in our sample is lower than the Boston Birth Cohort (4.4% versus 25.2%) and therefore we have too few mothers to investigate whether the Boston birth Cohort association is replicated in our study.

Interpretation

Elevated tHcy has been associated with endothelial dysfunction, affecting placental vasculature, and offspring cardiometabolic health (Gaillard et al., 2014; H. Wang et al., 2017). Previously, we reported a greater strain of pregnancy on cobalamin reserves (reflected by higher plasma MMA) in women starting pregnancy with low plasma holoTC status (Murphy et al., 2007). Here low pregnancy plasma holoTC and high MMA are associated with higher MetSco in boys. High MMA is also associated with dyslipidemia in boys and low holoTC with increased FMI and HOMA-IR. The holoTC and MMA findings suggest that the pregnancy tHcy–child MetSco association may reflect impaired cobalamin status as reported in previous studies (Stewart et al., 2011; Yajnik et al., 2008). When metabolic syndrome develops in adults, anomalies in glucose metabolism have been reported to occur before obesity and dyslipidemia (Barceló et al., 2017). Low foetal cobalamin supply leading to reduced protein synthesis and increased lipogenesis has been hypothesised to link maternal cobalamin deficiency to increased insulin resistance in the offspring (Krishnaveni et al., 2014; Sobczyńska-Malefora et al., 2022; Yajnik et al., 2008). Regarding fat metabolism, animal studies showed that severe hepatic steatosis occurred, secondary to cobalamin deficiency in which elevated MMA inhibits the oxidation of free fatty acids within the liver (Clare et al., 2019; Kennedy et al., 1994). This is unlikely in our study because cobalamin deficiency was infrequent. However, 1-CM and impaired glucose and adiposity have been linked (Sobczyńska-Malefora et al., 2022; Yajnik et al., 2008). An alternate hypothesis to a role for 1-CM should be considered. However, maternal–child

associations (MetSco and its components) were independent of birthweight and maternal BMI, which has been associated with offspring central fat and cardiometabolic risk (Gaillard et al., 2014; Perng et al., 2014; H. Wang et al., 2017).

Low 1st trimester cobalamin status, according to its indicators, holoTC and MMA, was associated with adverse metabolic outcomes in the child. However, lowest tertile 3rd trimester cobalamin status was associated with lower FMI in boys and lowest tertile folate status with lower dyslipidemia. Cord plasma cobalamin and folate are higher than circulating cobalamin and folate, respectively, in the mother at labour (Solé-Navais et al., 2018). Low status in plasma concentrations of these nutrients in late pregnancy may reflect placental uptake of the vitamins rather than impaired status (Graber et al., 1971). We hypothesise that early pregnancy status in cobalamin is a more accurate reflection of the mother's underlying status in this nutrient than late pregnancy status. This may also be true for folate but would be affected by current trends in early pregnancy folic acid supplement use.

Sex specific findings

Mostly, the observed pregnancy–offspring outcomes were specific to boys. Male animals (Tiffin et al., 1991) and human embryos (P. F. Ray et al., 1995) proliferate to the blastocyst stage at a faster rate than females. This difference in proliferation has been linked to the Y chromosome and was termed 'Growth factor Y' by Erickson. Also, it is important to consider that male and female preimplantation embryos have different antigens, indicating sex differences in gene expression (Erickson, 1997). Male preimplantation embryos are more responsive to intrauterine undernutrition than females (Kwong et al., 2000). A key factor potentially influencing intrauterine sex differences is the placenta and placenta genes are differentially expressed in male and female mice on different folic acid supplementation regimes (Luan et al., 2021). Adult hepatocyte phosphatidylethanolamine N-methyltransferase differs between sexes (Resseguie et al., 2007) and sex differences in other 1-CM enzymes have been described in mice (Sadre-Marandi et al., 2018). In a study of ewes, periconceptional dietary restrictions in B12, folate and methionine led to genome-wide epigenetic modifications in DNA methylation in the offspring, with more than

half of the affected loci specific to males and associated with adiposity, insulin resistance, altered immune function, and high blood pressure. The observed effects were stronger in the adult male than female offspring (Sinclair et al., 2007). Glucose tolerance in the female rat offspring was unaffected by restricted diets but insulin was higher in males born to pregnant rat dams fed similar diets (Maloney et al., 2011).

Sex specific findings were reported in a Canadian study that observed lower rates of weight gain during the first 6 months after birth in boys but not in girls born to women with cobalamin deficiency (<148 pmol/L) at 36 weeks of gestation (Wu et al., 2013). Also in Bangladesh, supplementation in pregnancy with 15 vitamins and minerals including cobalamin and folate, reduced stunting during 0-54 months in boys, but not in girls (Khan et al., 2011). In Gambian women, periconceptional maternal supplementation with 14 vitamins and minerals including cobalamin and folate, causes changes in the epigenome with clear differences between the girls and boys (Khulan et al., 2012).

In a Spanish study (n= 976, 10–15 years of age) the incidence of metabolic syndrome was higher in boys than in girls (5.38% vs. 3.85%). It is remarkable that none of the 20 female subjects diagnosed with metabolic syndrome had serum triglyceride concentrations 150 mg/dl or more (González-Jiménez et al., 2015).

Further investigation is required to determine whether similar maternal–offspring associations occur in girls but may be masked by the physiological factors that drive differences in FMI between girls and boys from 3 years onwards (Nagy et al., 2014).

Birthweight as a mediator of maternal tHcy and child outcomes

Birthweight was not a mediator of the maternal tHcy - child outcomes in our analysis. However, the coefficients of the linear regressions were in line with our *a priori* hypothesis. We expected children born to mothers with high pregnancy tHcy to have lower birthweight z-score compared to those with tHcy in the mid-low tertiles (negative coefficient *a*). We also expected an inverse association between birthweight z-score and child metabolic score or its components (negative coefficient *b*). Also, we expected that children born to mothers in the

highest tHcy tertile would have a higher metabolic score (or any of its components) compared to children born to mothers in the mid-low tertiles (positive coefficient *c'*). Our findings are similar to those of another study that looks at the mediation effect of birthweight in the association of pre-pregnancy obesity and anthropometric outcomes in children aged 8.6 years (SD:3.0. N=1618 pairs). It found that the effect of maternal obesity on child anthropometry was due to a direct effect and not mediated through birthweight (Adane et al., 2019). We also must consider that elevated prenatal tHcy might be a biomarker of adverse foetal development but not the cause.

Strengths and limitations

This study collected data prospectively from early pregnancy until mid-childhood in mother–child dyads unexposed to mandatory fortification with folic acid and with a low prevalence of cobalamin deficiency. The cohorts were recruited before (PreC) and after (RTBC) periconceptional supplementation with folic acid recommendations were implemented. Nevertheless, they were from the same hospitals, samples were collected and processed using identical protocols, and all folate and cobalamin status determinations were by the same methods. The cohorts were combined to improve statistical power. Sensitivity analysis confirmed that the reported associations occurred when the RTBC mother–child dyads were analysed alone (not shown). Waist circumference or waist-to-height ratio are recommended for total body fat assessment (Nagy et al., 2014). However, FMI can also be used (Nagy et al., 2014) and skinfold measurements are better alternatives to waist circumference and BMI (Kriemler et al., 2010) and predict obesity well (Sardinha et al., 1999) in children and adolescents. Waist circumference is unavailable for the PreC cohort, but we confirmed the association between pregnancy tHcy and offspring body fat using waist circumference z-score in RTBC (data not shown). We assessed overweight–obesity using Spanish tables (Carrascosa et al., 2008) because the participants were almost exclusively Spanish. When applying the criteria of international obesity task force tables (Cole & Lobstein, 2012), the prevalence of overweight–obesity in our population was higher (23.7 versus 20.2%). We considered the use of the Spanish tables appropriate because we use

population-specific curves to determine birthweight and BMI z-scores and the aim of the study was to investigate maternal–offspring outcomes and not to compare prevalence between different countries. Residual confounding from factors not considered in our models may occur. However, our models were controlled for numerous maternal and child factors that influence offspring growth (González-Jiménez et al., 2015). The dichotomous nature of the definition of metabolic syndrome results in loss of information (Steinberger et al., 2009). For this reason, a metabolic score was studied. We compared the original metabolic score proposed by Ahrens in 135 RTBC children and there was no difference in the overall result compared to the modified score (MetSco) used in the thesis that does not include blood pressure and replaces waist circumference with fat mass index derived from skinfold measurement.

Conclusions

Moderately elevated pregnancy tHcy and low holoTC status were positively associated with MetSco, zFMI, and zHOMA-IR in boys. High pregnancy MMA was also positively associated with MetSco and dyslipidemia in boys. The association between pregnancy tHcy and child zFMI was not mediated by birthweight.

Differences between mid-childhood (7.5 y) plasma metabolite status according to maternal pregnancy plasma cobalamin and tHcy status.

Main findings

We studied whether child nutrients/metabolites in plasma at 7.5 years differed according to pregnancy plasma cobalamin and tHcy status. When maternal cobalamin was in the lowest tertile at 34 GW, child plasma betaine and sarcosine were higher compared to the mid-high cobalamin tertile. When the same analysis was repeated using MMA (highest vs. low-mid tertile) above results were confirmed. Plasma tryptophan was lower in children born to mothers with 1st trimester tHcy in the highest tertile compared to those born to mothers with 1st trimester tHcy in the low-mid tertiles.

Comparison with other studies

After searching the literature, we did not find studies looking at the association between pregnancy plasma cobalamin and tHcy with child plasma betaine, sarcosine or tryptophan in mid-childhood. However, the relationship between choline and cobalamin during pregnancy was investigated in a controlled feeding study. High choline intake led to increased serum holoTC. The authors proposed that this was due to increased betaine status and that this would in turn, reduce the cobalamin requirements for methionine synthase (King et al., 2019).

Furthermore, women with the *BHMT* c.716G>A polymorphism (AA or GA), had lower serum holoTC concentrations compared to the GG genotype (King et al., 2019). Our results agree with these. We found that women with the AA genotype had lower plasma holoTC at labour and in the cords compared to GA genotype. Given the relationship between betaine and cobalamin during pregnancy, our results linking pregnancy plasma cobalamin with the difference in infant plasma betaine concentrations in middle childhood should be studied further.

6.2. Betaine and the DMG pathway

6.2.1. Pregnancy and cord betaine and DMG/betaine and birthweight

Principal findings

Plasma betaine at ≤ 12 GW was not associated with birthweight. However, plasma betaine at 34 GW were inversely associated with birthweight in the crude model. The significance of the associations was lost with further adjustments.

Cord plasma betaine was inversely associated with birthweight in all models for girls and boys together except for the model that included hypertension. The same happened in the case of boys. In girls, betaine was inversely associated in the crude model and when preterm babies were excluded.

Plasma betaine in the cord interacted with folate in their association with birthweight in all of the children and in girls alone. A stratified analysis above and below folate median showed that cord betaine and birthweight were inversely associated when folate is above the median but not below (girls and boys together, and girls).

Plasma DMG/betaine ratio at 12 GW was positively associated with birthweight looking at girls and boys together (“All”) but the association was non significant when adjusted for hypertension and the statistical power was reduced. There was no association at 34 GW. Cord plasma DMG/betaine ratio was positively associated with birthweight looking at “All'' and in boys in all models.

Comparison with previous studies

Two studies in the Netherlands did not observe an association between maternal betaine and birthweight. One of them studied pregnancy plasma betaine at 30-34 gestational weeks (n=366) (Hogeveen et al., 2013) and the other at 34-36 gestational weeks (n=1331)

(Moltó-Puigmartí et al., 2021). On the contrary, we observed an inverse association between plasma betaine at 34 GW and birthweight in the crude model. Our results are similar to an Asian cohort that found that maternal betaine status at 26-28 gestational weeks was positively associated with smaller infant birth size (n=955, β= −57.6 g per 5-μmol/L increment; 95% CI= −109.9, −5.3 g) and with less abdominal fat mass (n = 307) (L. van Lee et al., 2016). Similar results were reported from a Chinese cohort (n=115) looking at maternal betaine at 22.7–33.0 gestational weeks and birthweight. Further analysis stratifying by sex or maternal tHcy, found that the reduction in birthweight was seen only in males or those who had maternal tHcy >= 5.1 μmol/L (Du et al., 2019). There are some differences between studies that need to be considered. In one of the Asian studies, nobody smoked (self-reported) (Du et al., 2019) and in the other the models were not adjusted for smoking, even when the information was collected (L. van Lee et al., 2016). The Dutch studies and our study included smoking as a confounder factor.

Similar to our results, one of the above Dutch studies found an inverse association between standardised cord betaine and birthweight (n=1126, β = −65 (95% CI=−94, −36). The coefficient was calculated using z-scores of betaine and represents the decrease in birthweight (grams) following 1 SD increase in cord betaine) (Hogeveen et al., 2013). They did not stratify by sex. Repeating our analysis using similar covariables used by this study (sex, gestational age, gravidity, age, smoking, plasma folate) and analysing standardised cord betaine, we observed a similar association (n=572, β=-55 (95% CI=-85, -25)).

Ours is the only study that looked at maternal plasma betaine in early pregnancy (12 GW) as well as in late pregnancy (34 GW) and in the cord. None of the previous studies reported the association of the DMG/betaine ratio but one of the Dutch studies found that DMG in cord was positively associated with birthweight (Hogeveen et al., 2013).

Interpretation

Pregnant South Asian women living in Canada had higher betaine and DMG status than women of European ethnicity, suggesting ethnic differences in methyl donor metabolisms during pregnancy that might be explained by dietary patterns, supplement use, as well as genetic variants (Mujica-Coopman et al., 2019). It is possible that the inverse association between maternal betaine during pregnancy and birthweight is more pronounced in situations of higher betaine status, such as that seen in Asian women. European women may have lower betaine status than Asian women and this might be the reason for the lack of association between pregnancy betaine and birthweight in the European cohorts (Hogeveen et al., 2013; Moltó-Puigmartí et al., 2021) compared to the observed inverse association between pregnancy betaine and birthweight in the Asian cohorts (Du et al., 2019; L. van Lee et al., 2016). Regarding possible genetic variants involved, the *BHMT* polymorphism c.716G>A (rs3733890) has a similar frequency between European, South Asian and East Asian populations (A allele frequency 32%, 29% and 32%) (Howe et al., 2021) and therefore, might not be the cause of the differences in betaine and DMG status reported in the Canadian study.

It has been suggested that the inverse association between cord betaine and birthweight, might reflect a disturbed placental function (Hogeveen et al., 2013).

We observed that the inverse association between betaine and birthweight was significant in the cord when folate was above the median (analysing boys and girls together, and only girls). Previously, in our cohort we found that in the presence of low folate status the inverse association between betaine and tHcy is stronger (Fernàndez-Roig et al., 2013). Our current findings stratifying by median folate suggest that mothers that have maintained the betaine pool (because they use folate for methionine synthesis), seem to have foetuses that weigh less but we do not know why. A possible mechanism may emerge from the Singapore study which reported that the inverse association between pregnancy plasma betaine (26–28 GW) and birthweight was associated with less abdominal fat mass determined in 307 children (L. van Lee et al., 2016).

In further agreement with the Singapore study, a study of 1C metabolites and nutrients in breast milk (SAM, SAH, methionine, cystathionine, choline) reported that only betaine was negatively correlated with infant growth at 12 months in two different cohorts (Ribo et al., 2021). However, these findings do not necessarily imply negative consequences, because studies in mice found that higher milk betaine was associated with lower adiposity and improved glucose metabolism in adulthood, at the same time with an increase in *Akkermansia* spp in caecal samples. Similarly, in humans, babies that consumed milk of higher betaine content had a higher abundance of *Akkermansia muciniphila* in faecal samples (Ribo et al., 2021).

Another possible mechanism is that betaine modifies insulin and glucose metabolism because adults in the highest quartile of serum betaine had lower risk of type 2 diabetes compared with the lowest quartile (Lu et al., 2022). Growth hormone might also be involved because betaine has been found to increase its concentration by 45.6% in pigs fed high betaine diets, leading to decreased fat deposition and activity of lipogenic enzymes as well as increased activity of hormone-sensitive lipase in adipose tissue (Q.-C. Huang et al., 2006).

Singapore's study mentioned before suggested that another mechanism explaining that higher maternal betaine status was generally associated with smaller infant birth size and less abdominal fat mass, was via reduced availability of acetyl-CoA (necessary for fatty acid synthesis) due to increased methylation of homocysteine to methionine with betaine as 1C donor (L. van Lee et al., 2016). However, in our study, when we stratified by median folate, the reduction in birthweight was mainly found when folate was above the median, suggesting that betaine may not be necessary for homocysteine remethylation under those circumstances. Reinforcing the previous result, when we compared the geometric mean of cord DMG/betaine ratio above and below cord folate median (26.3 nmol/L), we found that the ratio was higher below than above median folate (data not shown).

On the other hand, in our sample, the cord plasma DMG/betaine ratio was positively associated with birthweight (looking at girls and boys together, and when separated by sex only in boys) suggesting that when betaine is indeed used for methionine synthesis foetal growth is stimulated. That considering that the ratio allows an inference of

betaine-dependent remethylation of Hcy (Gillies et al., 2022) and higher ratios suggest higher remethylation of Hcy. Regarding the sex differences in this result, other researchers have hypothesised that males are more dependent on exogenous sources of betaine (*in utero*, through breast milk, or from the child's diet) than females (Moltó-Puigmartí et al., 2021). Overall, our findings are in agreement with a study that observed that women with estimated foetal weight <5th percentile and with abnormal uterine artery doppler studies, had lower serum DMG (Porter et al., 2020). Furthermore, in piglets with intrauterine growth restriction, recent studies found that postnatal dietary supplementation with DMG sodium salt, improved growth performance, redox status, immune dysfunction and hepatic mitochondrial dysfunction (Bai, Jiang, & Wang, 2022; Bai, Jiang, Li, et al., 2022a, 2022b; Bai, Jiang, Wang, et al., 2022; C. Feng et al., 2018). In humans, there are no studies investigating DMG supplementation during pregnancy and foetal growth.

Strengths and limitations

This study collected data prospectively from early pregnancy in mother–baby dyads unexposed to mandatory fortification with folic acid.

The reduction in birthweight, apparently due to betaine in cord, is small and represents an average reduction of 55 grams in birthweight for a 1 SD increase in cord plasma betaine. However, in boys the correlation of cord plasma betaine with birthweight and with birthweight z-score is the strongest out of all of the correlations between 1C metabolites.

6.2.2. *BHMT* c.716G>A polymorphism and birthweight

Birthweight did not differ between maternal or baby *BHMT* c.716 G>A genotypes. The polymorphism has been associated with adverse developmental outcomes such as cleft lip and spina bifida. The results of those studies are conflicting and are discussed later, in the analysis of the polymorphism with SGA babies.

6.2.3. Pregnancy and cord betaine and SGA babies

Main findings

Maternal plasma betaine at 34 GW was associated with increased risk of SGA in the crude model but not in the adjusted models. Cord plasma betaine was associated with increased risk of SGA babies and the sex-stratified analysis showed that the association was only observed in boys.

Comparison with previous studies

Similar to our crude model at 34 GW, a Singapore study (n=955) also reported that maternal betaine status at 26-28 GW was associated with a higher risk of SGA babies (per 5-µmol/L betaine increment, OR= 1.57; 95% CI= 1.05, 2.35) (L. van Lee et al., 2016). In contrast, a Chinese study reported no association between maternal betaine during pregnancy (22.7–33.0 GA) and risk of SGA (Du et al., 2019).

We found that higher cord plasma betaine was associated with a small but significant risk of SGA (in girls and boys together and in boys alone). A previous study reported that cord betaine in the highest quartile compared with the lowest quartile was associated with increased risk of low birthweight (≤2500 grams, standardized for gestational age and adjusted for sex) (OR: 4.93 CI=1.04; 23.24) (Hogeveen et al., 2013). The effect size in this study of 1126 mothers was higher than in ours (we studied betaine as continuous variable different from them) and the models were not adjusted for the same confounders as ours (they did not include maternal BMI, anaemia, plasma folate and plasma cobalamin).

Interpretation

For the positive association between cord betaine and risk of SGA we propose similar mechanisms to those described in the inverse association between cord betaine and birthweight like impaired placental function (Hogeveen et al., 2013); a reduction in abdominal fat mass (L. van Lee et al., 2016) that might be explained because betaine has been found to increase growth hormone by 45.6% in pigs fed high betaine diets, leading to decreased fat deposition and activity of lipogenic enzymes as well as increased activity of hormone-sensitive lipase in adipose tissue (Q.-C. Huang et al., 2006). Another possible mechanism is that betaine modifies insulin and glucose metabolism because adults in the highest quartile of serum betaine had lower risk of type 2 diabetes compared with the lowest quartile (Lu et al., 2022).

6.2.4. *BHMT* c.716G>A polymorphism and SGA babies

Babies born to mothers with the maternal AA genotype compared to GG (codominant model) and in AA compared to GG+GA (recessive model) were at increased risk of SGA. Also each copy of A increased the risk of SGA by an additive amount (additive model). The dominant (GG vs. GA+AA) was never significant.

There was no association between the polymorphism in the baby and SGA.

Comparison with other studies.

To the best of our knowledge, no other studies have investigated the association between the *BHMT* c.716G>A polymorphism and birthweight or SGA babies. However, the polymorphism has been associated with developmental outcomes such as cleft lip and spina bifida, although with conflicting results. A Polish study reported a decreased risk of cleft lip in individuals with the AA genotype versus GG or GG+GA genotypes (Mostowska, Hozyasz, Wojcicki, et al., 2010) but in the same population the prevalence of the homozygote variant genotype did not differ between the mothers. However, there was another maternal polymorphism in the intronic sequence of *BHMT2* that reduces the risk of cleft lip so while the protective effect in the foetus was associated with the *BHMT* c.716G>A variant allele, the protective effect brought by the mothers was in a variant allele of a polymorphism in *BHMT2* (Mostowska, Hozyasz, Biedziak, et al., 2010). Another study from China observed no allelic or genotypic association between *BHMT* c.716G>A genotype and cleft lip. However, they reported that another polymorphism (rs3797546) in the *BHMT* gene increases the genetic risk of cleft lip in a recessive manner (Hu et al., 2011). Finally, a meta-analysis of 47 studies reported no association between c.716G>A and orofacial cleft (includes cleft lip) in populations of European ancestry (Slavec et al., 2022). Similarly in the case of spina bifida, a Canadian study found a non-significant decrease in risk with the AA versus GG genotype in mothers and also in children (Morin et al., 2003). On the other hand, a USA study reported an increased risk in children with the AA versus GG genotype (Shaw et al., 2009). Also, a family-based study USA study (2006) of neural tube defects (NTD) reported that the polymorphism increased their risk in general, and most significantly when mothers took folate-containing nutritional supplements before conception or parents preferentially

transmitted the *MTHFR* rs1801133 T allele. They propose that the *BHMT* c.716G>A polymorphism increases the risk even more in women supplemented with folic acid due to another unknown variable related with supplementation or alternatively, the polymorphism could have created a highly efficient variant that overfunction when combined with high folate status (Boyles et al., 2006). In an NTD study in China from a population recruited between 2005 and 2011, the polymorphism was associated with increased risk but only in the case of no folic acid supplementation (J. Liu et al., 2014). The polymorphism was also associated with omphalocele in both African-Americans (GA vs. GG; and also for AA vs. GG) and Asians (GA vs. GG) living in the United states but no association was seen in white non-Hispanic or Hispanic populations (Mills et al., 2012).

Other conflicting results have been reported for the association of the *BHMT* c.716G > A polymorphism with Down syndrome, with some studies in Brazil reporting a reduced risk (Amorim et al., 2013; Zampieri et al., 2012) but an Indian study reported an increased risk (Jaiswal et al., 2017). Also, in Brasil, GA or AA genotypes were associated with lower Alu DNA methylation and mothers of individuals with Down syndrome have lower Alu methylation (Mendes et al., 2021).

During pregnancy, the maternal AA genotype has been associated with increased risk of placental abruption (OR 2.82, 95% CI 1.84, 4.97) (Ananth et al., 2007).

Outside of pregnancy the polymorphism has been associated with a protective effect against coronary artery disease in people over 60 years of age in the United States (Weisberg et al., 2003) and with uterine cancer incidence in Poland (Mostowska et al., 2011). An Indian study reported an increased risk of coronary artery disease in people with the *BHMT* c.716G > A polymorphism and younger than 60 (Singh et al., 2011).

Interpretation

The association between maternal AA genotype and higher risk of SGA babies might be explained due to the effect of this genotype on DMG (a marker of the methylation of Hcy to methionine by BHMT enzyme). In our sample, plasma DMG was generally lower in women with the AA compared to the other genotypes and a similar trend was observed for the

DMG/betaine ratio. DMG is only produced during the methylation of Hcy to methionine by BHMT enzyme (Lever et al., 2011) and our results suggest that the conversion of betaine to DMG is lower due to the polymorphism. Previously, in our cohort we described the same pattern (Colomina et al., 2016) and similar results were found in a population study in Norway that reported that the polymorphism had a significant effect on DMG, that decreased according to the number of A alleles (Fredriksen et al., 2007).

A controlled feeding study in women (26 third-trimester pregnant, 28 lactating, and 21 non-pregnant) consuming choline in accordance with (480 mg) and exceeding (930 mg) daily intake recommendations, reported that the *BHMT* c.716G>A polymorphism stimulates the use of dietary choline for phosphatidylcholine synthesis through the cytidine diphosphate-choline (CDP-PC) pathway at the expense of betaine synthesis. The authors propose that the *BHMT* c.716G>A variant causes the Km (an inverse measure of affinity of the enzyme for its substrate) to be lower, thus increasing the affinity for both its substrates betaine and homocysteine. They also propose that participants with the variant allele will require less betaine for maximal BHMT enzyme activity while possibly sparing more choline (Ganz et al., 2017). The same mechanism was proposed to explain the association between *BHMT* c.716G>A and NTD's that found an increased risk (Boyles et al., 2006). Alternatively, they propose an interaction between *BHMT* and *MTHFR* polymorphisms in both genes for increasing the NTD risk (Boyles et al., 2006). We did not find an interaction between the two polymorphisms (data not shown). Also, we did not see differences in the maternal or baby *MTHFR* rs1801133 polymorphism between mothers of babies SGA compared to those no SGA.

Studies in animal models support the involvement of the BHMT enzyme in foetal growth. In a model of intrauterine growth restriction (IUGR) in Yucatan miniature piglets, the liver of affected animals had significantly lower activities of the enzymes BHMT and cystathionine γ-lyase (CGL) than larger littermates. mRNA expression of *BHMT* did not differ but *CGL* expression was lower in the IUGR compared to larger piglets. This suggests that the changes in BHMT activity between IUGR and large piglets might involve post-transcriptional modifications. Considering that in this study IUGR piglets showed a tendency to have higher transglutaminase activity than the large piglets, the authors propose that liver

transglutaminases may crosslink with BHMT subunits and reduce its activity as seen in vitro previously (MacKay et al., 2012).

Contrary to the above animal model, we did not see an association between the polymorphism in the baby and SGA. In vitro *BHMT* expression in human foetal livers and brain (approximately second trimester) is lower than in mature liver and brain. Also, BHMT activity in neonatal liver was in the range of that of mature liver (Gaull et al., 1973), supporting other studies reporting that BHMT activity and protein increases with gestational age (Q. Feng et al., 2011). However, the involvement of foetal BHMT in 1C metabolism is not well understood. We observed differences in cord plasma betaine according to foetal genotype (GA higher than GG) and in DMG/betaine (GG higher than GA). A previous study also reported higher betaine in the GA compared to GG genotype but not significantly so, probably due to smaller sample size than ours (Visentin et al., 2015).

Strengths and limitations

This study collected data prospectively from early pregnancy in mother–babies dyads unexposed to mandatory fortification with folic acid.

The associations studied may be susceptible to residual confounding due to the observational study design. Nevertheless, analyses were adjusted for known confounders and few studies have investigated this polymorphism in mother child dyads.

6.2.5. Betaine and DMG/betaine during pregnancy and in cord and mid childhood outcomes

Principal findings

Pregnancy and cord plasma betaine and DMG/betaine were not associated with metabolic score in the children at 7.5 years.

Comparison with previous studies

Few studies have examined the association between pregnancy or cord betaine status and outcomes in the offspring beyond birthweight. A study in the Netherlands reported that 3rd trimester plasma betaine was positively associated with weight and overweight in the offspring at 1 and 2 years of age. Subsequently, it was associated with being overweight at 6–8 y in boys only (Moltó-Puigmartí et al., 2021). Unlike our study, child betaine status was not taken into account. Similar to the Dutch study we found a positive trend ($p<0.1$), between pregnancy betaine and child zFMI. The differences with our study might be due to our smaller sample size. Also, a limitation of that study, and difference with respect to ours, is that the blood samples were collected in the non fasting state. After a meal betaine status can increase by 10-15 % (Meyer, 2021b).

The findings of the Dutch study are related to those of another study of the association between cord plasma metabolites and early childhood obesity risk, in Project Viva (United States). They compared children with the highest change in weight-for-age until 6 months of age and with a BMI higher than the 85th percentile in mid-childhood (around 7.7 years) versus children with normal postnatal weight until 6 months and normal mid-childhood weight. They observed that 16 of 415 metabolites tested were different between cases and controls. These included DMG and N-acetylmethionine, 2 methyl donors, that were lower in cases. Also they compared the biological pathways of the metabolites between cases and controls and found that main differences were in five routes including the betaine pathway (Isganaitis et al., 2015).

Interpretation

Plasma betaine concentrations during pregnancy and in the cord were associated with birthweight, as described previously. However, these were not associated with zFMI and other metabolic outcomes in mid-childhood. The same lack of association occurred in the case of DMG/betaine. For future research, it would be interesting to study the association between plasma betaine during pregnancy and in the cord on health before 7.5 years of age. Possible effects of pregnancy/cord betaine on postnatal health, according to our results, may not persist into mid-childhood and postnatal factors such as breastfeeding, physical activity, sleep and diet might be more dominant for child health.

Strengths and limitations

Strengths of this study include the prospective collection of data from early pregnancy until mid-childhood in mother–child dyads unexposed to mandatory fortification with folic acid. Additionally, a metabolic score was studied because the dichotomous nature of the definition of metabolic syndrome results in loss of information (Steinberger et al., 2009). Residual confounding from factors not considered in our models may occur. However, our models were controlled for numerous maternal and child factors that influence offspring growth (González-Jiménez et al., 2015).

6.2.6. Testing the associations between child plasma betaine and other 1C metabolites with adiposity and other anthropometric measures in the child

Correlation of betaine in children and other 1C metabolites with 5 fat mass measurements.

Main findings

Child plasma betaine at 7.5 years is positively associated with 5 fat mass measurements and the association is reported also when stratifying by sex. The related metabolite plasma DMG and the DMG/betaine ratio were associated with only one fat measurement. Out of the other metabolites studied, only total cysteine was correlated with more than one fat measurement (3 in total).

Choline was associated with one fat measurement and the other nutrients and metabolites (methionine, folate, red blood cell folate, cobalamin, MMA, tHcy) were not correlated with any.

Comparison with other studies.

Our findings are in agreement with a study in six-year-old Iranian girls that found that total betaine intake was associated with risk of being overweight (Jafari et al., 2021). In our study, child plasma choline was associated with waist circumference while the same Iranian study mentioned above, did not find an association between child choline intake and overweight or obesity (Jafari et al., 2021).

The positive correlation between cysteine and adiposity that we found, is in agreement with other studies in which cysteine was positively associated with waist circumference in children (da Silva et al., 2013), obesity in adolescents (Elshorbagy et al., 2012) and in adults (Elshorbagy et al., 2009). We did not find correlations between tHcy and adiposity measures. However, a study in Brazil reported that prepubertal children with a waist circumference above the 90th percentile (7.3 mmol/L) were 2.4 times more likely to have increased tHcy (da Silva et al., 2013).

Our lack of correlation between methionine and adiposity measures is in agreement with a study from the Viva la Familia study (USA) that found no correlation between methionine and fat mass in children and adolescents (4-19 y) (Elshorbagy et al., 2012).

Correlation of betaine in children and other 1C metabolites with other anthropometric measurements.

Betaine was not only positively correlated with fat measurements mentioned in the section above but also with fat free mass, muscular mass, height, arm circumference and trunk circumference. Stratifying by sex the correlation of plasma betaine with above measurements was significant mainly only in girls. Choline was correlated looking at girls and boys together with 4 measurements (fat free mass, muscular mass, head circumference and trunk circumference) and the correlation was similar stratifying by sex in girls (fat free mass, muscular mass, height and trunk circumference). The other metabolites did not correlate or some of them correlated with a single anthropometric measurement (methionine, folate, red blood cell folate, cobalamin, MMA, tHcy, total cysteine).

Comparison with previous studies

Our findings are in agreement with a study in Iranian girls that found that total betaine intake but not choline was associated with mid-arm circumference (Jafari et al., 2021).

Regarding the choline positive correlations we found, they are similar to the results of a study in which serum choline was positively correlated with height-for-age z score in Malawian children (1-5 year-old) (Semba et al., 2016).

Interpretation

The positive association between child plasma betaine and the 10 reported anthropometric measurements (adiposity and others) might be explained by considering that in pigs fed high betaine in its diets, there was an increase of around 42-58% in growth hormone, insulin-like growth factor I, free triiodothyronine, free thyroxine and insulin (Q.-C. Huang et al., 2006). The positive correlation of child plasma betaine with fat measurements and the lack of correlation with DMG or the correlation of the DMG/betaine ratio with a single measurement of fat; suggest that the main mechanism involved in the correlation child

plasma betaine-fat might be mainly through the role of betaine as an osmolyte and not as a 1C donor.

Strengths and limitations

Blood samples were collected from fasting children, which is particularly important for betaine determinations, since after a meal, betaine status can increase by 10-15% (Meyer 2021b). We reported various measurements of adiposity and not all of them have the same association with the metabolic syndrome. For example, waist circumference is more strongly correlated than BMI (H. J. Schneider et al., 2007). However, the use of BMI is the main tool for the assessment of fatness in clinical practice due to its ease of calculation (Cornier et al., 2011). Fat mass determined through bioelectric impedance, does not give information regarding body fat distribution, while waist circumference does. Therefore, the use of different measurements provides complementary information. This study has some risk of random findings due to its observational design. Finally, this was an initial exploratory analysis and future studies should consider potential confounders.

6.3. Identification of metabolites associated with adiposity in 7.5 year-old children

Child plasma nutrient/metabolites correlations with adiposity

From 20 metabolites, 7 were correlated with different fat measurements ($p<0.1$ in at least 3 measures), 3 of them (HAA, kynurenic acid and picolinic acid) are from the kynurenine pathway that is the main route in which the amino acid tryptophan is catabolized (Stone and Darlington 2002). Remarkably, HAA was directly correlated with the 5 fat measurements. When stratified by sex, HAA was mainly correlated in girls, while kynurenic acid was mainly positively correlated in boys. The inverse correlation with picolinic acid was only significant looking at girls and boys together.

We found a positive correlation between child histidine and fat measurements, and stratifying by sex the association was only observed in girls. ADMA was positively associated with 4 adiposity measures. Stratifying by sex the association was only found in girls. Two other metabolites were inversely correlated with adiposity measures: trigonelline with 4 adiposity measures and when stratify by sex only significant in boys, and neopterin with 3 adiposity measures. The inverse correlation with neopterin was only observed in girls and boys together.

Comparison with other studies - Kynurenine pathway (HAA, kynurenic acid, picolinic acid)

Kynurenic acid and HAA are higher in adult men compared to women probably due to sex hormone influence (Tan, Tint, Kothandaraman, Yap, et al., 2022). We did not see differences in girls and boys in the mean values of these metabolites (data not shown). However, another study in 8-year-old prepubertal Asian children found higher plasma HAA in boys than in girls. Similar to our results, they found that kynurenine pathway metabolites (kynurenic acid (KA), xanthurenic acid (XA), HAA and quinolinic acid) were positively associated with body fat percentage and metabolic syndrome scores (Tan, Tint, Kothandaraman, Yap, et al., 2022). Furthermore, they found that kynurenine pathway metabolites in cord were associated with early childhood adiposity (Tan, Tint, Kothandaraman, Michael, et al., 2022). The same pathway was positively associated with

BMI and fat mass in a paediatric cohort in Vienna (Lischka et al., 2022). Regarding another metabolite of this pathway, picolinic acid, to the best of our knowledge, there are no reports studying it in association with fat mass in children.

Comparison with other studies - Histidine

The positive correlation between child histidine and fat measurements is counterintuitive because histidine is an essential amino acid with anti-inflammatory and antioxidant properties (Holeček, 2020). Stratifying by sex the association was only observed in girls. With the analysis presented, we cannot say whether the correlation is reflecting the normal growth process or not, and further analysis looking at normal weight versus obese children should be performed. However, similar to our results, in a study with Japanese American adults, histidine was associated with a greater area of visceral fat only in women (Tran et al., 2021). Opposite to our results, a negative correlation was found between serum histidine and BMI z-score in Australian children (mean age 11.9 years). When they stratified by sex it was negatively associated only in girls (Saner et al., 2019).

Comparison with other studies - Trigonelline

With the search carried out in PubMed, there are no studies in children that investigate the association of this phytohormone with adiposity (**Table 34**). Possible mechanisms that would explain the correlation we found are described in an *in vitro* study with mouse preadipocytes that found that trigonelline inhibits adipogenesis (Ilavenil et al., 2014). Another study found that trigonelline induced browning of mouse white adipocytes, a process that is a new therapeutic target in obesity treatment (Choi et al., 2021). Additionally, in mice fed a high-fat diet, a trigonelline-enriched yoghurt led to a reduction in fat accumulation and improved insulin resistance (Costa et al., 2020).

Comparison with other studies - ADMA

The positive correlation we found between plasma ADMA and fat measurements is similar to another study in children (mean age 11 years) with excess body weight that have higher ADMA than normal weight children. Also they found an inverse correlation between serum ADMA and serum nitric oxide. Additionally, they reported that females with excess body

weight had lower levels of nitric oxide than males (Czumaj et al., 2019). This finding is coherent with the fact that when we stratified by sex the ADMA-adiposity correlations were only significant in girls. In adults a similar positive correlation ADMA-obesity has been reported (**Table 34**).

Comparison with other studies - Neopterin

Plasma neopterin inverse correlation with fat measurements is similar to a study shown in **Table 34**. They found that serum neopterin was decreased in overweight/obese males (10 ≤ 18 years) and that is the opposite of what is seen in adults with atherosclerosis and cardiovascular disease where the levels are increased (Mangge et al., 2014). The authors proposed that the inverse association between neopterin and overweight/obesity in young males, suggests a low or subnormal activity of T helper cell type 1 (Th1-type) immunity which may be due to a T helper cell type 2 (Th-2-type) prevalence.

Remarkably, previous studies in adults have connected neopterin with tryptophan and kynurenine pathway. They found an inverse relationship between tryptophan and neopterin concentrations, which is probably also connected to a positive correlation observed between neopterin and the activity of the enzyme IDO (which converts tryptophan to kynurenine) (Brandacher et al., 2007).

Final considerations

In **Table 34** we summarise the correlations we found for the 7 nutrient/metabolites, compare them with at least one other study and show the number of articles retrieved on the subject in PubMed to give an idea of how much knowledge there is about the nutrient/metabolite in association with obesity. We also show the information separating between children versus *in vitro* studies, animal models or adults. The information is presented in this way, since in some cases the correlation that has been reported in the literature is opposite in children to adults (e.g., neopterin). The metabolites associated with more fat measurements in our analysis are shown at the top of the table, with HAA being the most frequently associated and neopterin being the least frequently associated.

The approach to this analysis of 20 metabolites was exploratory to identify whether they were associated with adiposity in children. Previous studies regarding these metabolites in children are scarce. Random findings is a risk of such an approach and it can be reduced by post-hoc correction for multiple comparisons. For this initial exploratory analysis we prioritised first identifying the nutrients/metabolites that correlated with at least 3 adiposity measures and accepting p value of <0.1 without Bonferroni correction. For example, it is well established that plasma total cysteine is associated with waist circumference in children (da Silva et al., 2013) and with obesity in adolescents (Elshorbagy et al., 2012) and in adults (Elshorbagy et al., 2009). In our sample (of mainly healthy children), it was weakly correlated with waist circumference (0.150, p=0.046), waist/height ratio (0.151, p=0.044), fat mass from bioimpedance (0.183, p=0.015) and with p-values<0.1 for 2 other measures (fat mass index from skinfold measures=0.131 p=0.086 and BMI=0.134 p=0.075). When analysed the 7 nutrient/metabolites selected using our criteria, HAA, trigonelline and ADMA showed stronger correlations with waist circumference, compared to total cysteine. Furthermore, four metabolites from the same pathway (kynurenine) were correlated with adiposity measures in agreement with the few studies done in children associating this pathway with obesity (Barat et al., 2016; Butte et al., 2015; Lischka et al., 2022; Mangge et al., 2014; Tan, Tint, Kothandaraman, Yap, et al., 2022). The next step in determining the relevance of the associations would be to replicate them in other studies and explore whether they occur in studies of overweight/obese children.

Table 34. Child metabolites correlations with adiposity and comparison with other studies.

No	Metabolite	Function/Indication(s)	Correlation we found with adiposity	Metabolite association with obesity in literature			
				Humans/In vitro/animal models		Children	
				Association found in an example article(s)	Articles found [a]	Association found in an example article(s)	Articles found [b]
1	3-hydroxyanthranilic acid (HAA)	Part of the tryptophan catabolism (Stone & Darlington, 2002).	+	+ (Theofylaktopoulou et al., 2013)	7	+ (Tan, Tint, Kothandaraman, Yap, et al., 2022)	1
2	histidine	Anti-inflammatory and antioxidant essential amino acid (Holeček, 2020).	+	- (Menon et al., 2020) + (Tran et al., 2021)	234	- (Saner et al., 2019)	18
3	trigonelline	Phytohormone often used as a marker of coffee consumption (Midttun et al., 2018).	-	- (Choi et al., 2021)	32		0
4	kynurenic acid	Part of the tryptophan catabolism (Stone & Darlington, 2002).	+	+ (Favennec et al., 2015)	54	+ (Tan, Tint, Kothandaraman, Yap, et al., 2022)	2
5	asymmetric dimethylarginine (ADMA)	Endogenous inhibitor of nitric oxide synthase. Useful for the assessment of endothelial function (Böger et al., 1998).	+	+ (Avci et al., 2020)	167	+ (Czumaj et al., 2019)	21
6	picolinic acid	Part of the tryptophan catabolism (Stone & Darlington, 2002).	-	- (not significant) (Favennec et al., 2015)	33		0
7	neopterin	Assessment of cellular immune response. Increased concentrations are found in infections and other diseases (Capuron et al., 2014).	-	+ (Avci et al., 2020)	53	- (boys) (Mangge et al., 2014)	13

[a] Pubmed search 2022: (metabolite) AND (obesity)

[b] Pubmed search 2022: ((metabolite) AND (obesity)) AND (children)

6.4. General comment

This thesis consists of three parts. The first focused on the association between some maternal 1C metabolites (plasma cobalamin, holoTC, MMA, folate, betaine, DMG/betaine, tHcy) during pregnancy and metabolic health in mid-childhood. The second studied the association between plasma betaine and the DMG pathway during pregnancy and in the cord with foetal growth. The third part explores the association of child plasma metabolites in mid-childhood with anthropometry with a focus on adiposity due to its importance in the metabolic syndrome.

High tHcy during pregnancy and low plasma cobalamin status during early pregnancy are positively associated with mid-childhood metabolic score in the offspring. Regarding maternal plasma pregnancy betaine and mid-childhood metabolic score, we observed no association between the two. However, similar to a previous study, we found a positive (non-significant) trend between pregnancy betaine and child zFMI. Possibly, we do not have enough statistical power. Fortunately, sample size in the RTBC is expected to increase because the child recruitment phase is still ongoing. The follow-up of the offspring at different ages will be interesting to understand how our findings evolve during the growth process.

Regarding the second part of the thesis, we observed an inverse association between cord plasma betaine and foetal growth. Stratifying by median cord plasma folate, the inverse association was only significant above the median. It is possible that the inverse association is not due to betaine's role in 1C metabolism. It seems that mothers that maintained the betaine pool throughout pregnancy (because they use folate for methionine synthesis) have foetuses that weigh less. On the other hand, the cord plasma DMG/betaine ratio was positively associated with birthweight suggesting that when betaine is indeed used for methionine synthesis, it is positively associated with foetal growth. This consideration is based on the hypothesis that the ratio allows inference of betaine-dependent remethylation of Hcy (Gillies et al., 2022) and higher ratios suggest higher remethylation of Hcy. The positive DMG/betaine-birthweight association agrees with another finding of the thesis, that babies born to mothers with the *BHMT* c.716 G>A polymorphism have increased risk of SGA.

More precisely, the recessive (AA vs. GG+GA) and codominant (AA vs. GG) models increased the risk of SGA. Our group previously reported that the A allele was associated with lower plasma DMG both during pregnancy and in the cord, suggesting that this allele impedes the conversion of betaine to DMG during pregnancy (Colomina et al., 2016). Overall, our findings are in agreement with a study that observed that women with estimated foetal weight <5th percentile and with abnormal uterine artery doppler studies, had lower serum DMG (Porter et al., 2020). Furthermore, in piglets with intrauterine growth restriction, recent studies found that postnatal dietary supplementation with DMG sodium salt, improved growth performance, redox status, immune dysfunction and hepatic mitochondrial dysfunction (Bai, Jiang, & Wang, 2022; Bai, Jiang, Li, et al., 2022a, 2022b; Bai, Jiang, Wang, et al., 2022; C. Feng et al., 2018). In humans, there are no studies investigating DMG supplementation during pregnancy and foetal growth.

Regarding the third part of the thesis, we observed that child plasma betaine at 7.5 years was correlated with growth (10 anthropometric measurements including 5 of adiposity). Additionally, studying child metabolites, we observed that 7 are correlated with different adiposity measures at 7.5 years of age. Of these, 3 are from the kynurenine pathway (involved in tryptophan catabolism). In children, tryptophan catabolism via the kynurenine pathway was positively associated with BMI z-score and serotonin negatively correlated with BMI (Lischka et al., 2022). This is a recent finding in a paediatric population and future research needs to address how tryptophan catabolism and obesity are related.

Interestingly, some findings throughout the thesis when stratified by sex, were observed only in males but not in females. Similarly, some animal studies observed that periconceptional 1C nutrient restriction, altered DNA methylation in the offspring and more than half of the altered loci were male-specific (Sinclair et al., 2007). These differences between the sexes need to be investigated further to understand their causes and possible consequences.

In future research, the association between pregnancy plasma betaine status and child health beyond birth and the kynurenine pathway with metabolic health in children needs to be further studied.

7. Conclusions

The conclusions of this thesis are summarised in the list below:

- *To investigate the association between maternal pregnancy plasma tHcy, cobalamin status, folate and betaine with a metabolic score in children aged 6–7.5 years.*

  Fasting plasma total homocysteine during pregnancy and early pregnancy low plasma cobalamin status were associated with mid-childhood metabolic score and its components (fat mass, insulin resistance and dyslipidemia) in the offspring. Stratification by sex showed that these findings were limited to male offspring.

  The maternal-offspring associations were observed for the functional markers of cobalamin status (holotranscobalamin and methylmalonic acid) and total homocysteine, not for plasma cobalamin concentration.

  Neither pregnancy nor cord plasma betaine or dimethylglycine/betaine ratio were associated with an infancy metabolic score that includes measures of fat mass, insulin resistance, and dyslipidemia.

- *To determine the association between maternal pregnancy plasma betaine, DMG/betaine ratio and the BHMT c.716G>A polymorphism with foetal growth (birthweight and small for gestational age).*

  3rd trimester plasma betaine was inversely associated with birthweight but the association lost significance after adjusting for confounding factors.

  Cord plasma betaine was inversely associated with birthweight and was associated with increased risk of small for gestational age. Stratifying by sex showed that the increased risk was only observed in boys.

  The inverse association between cord plasma betaine and birthweight remained significant when cord plasma folate was above the median but not below. The cord plasma dimethylglycine/betaine ratio was positively associated with birthweight and only significant in boys, in the stratified analysis by sex.

  Birthweight did not differ with maternal or cord betaine-homocysteine S-methyltransferase c.726 G>A genotype. However, small for gestational age risk was

higher in the offspring of mothers with betaine-homocysteine S-methyltransferase c.726 AA versus GG and also AA versus GG+GA genotypes.

The betaine-homocysteine S-methyltransferase c.726 G>A polymorphism in the foetus was not associated with risk of small for gestational age.

- *To describe the association between child plasma betaine and related 1C metabolites with adiposity and other anthropometric measures in the child during mid-childhood.*

  Plasma betaine was positively correlated with 5 adiposity measures.

  Plasma dimethylglycine, the dimethylglycine/betaine ratio and choline were associated with only one adiposity measure.

  Plasma total cysteine was correlated with 3 fat measurements.

  Plasma methionine, folate, red blood cell folate, plasma cobalamin and methylmalonic acid were not associated with any adiposity measures.

  Regarding other anthropometric measurements, plasma betaine was positively correlated with fat free mass, muscular mass, height, arm circumference and trunk circumference.

  Plasma total free choline was correlated with 4 measurements (fat free mass, muscular mass, head circumference and trunk circumference) and the other metabolites (methionine, folate, red blood cell folate, cobalamin, methylmalonic acid, total homocysteine, total cysteine) did not correlate or only correlated with a single anthropometric measurement.

- *To investigate differences between mid-childhood (7.5 y) plasma metabolite status according to maternal pregnancy plasma cobalamin and tHcy status and to identify child plasma markers of adiposity.*

  Child plasma betaine was highest in children born to mothers in the lowest vs mid-high plasma cobalamin tertile at 34 GW while plasma tryptophan at 7.5 years was lowest in the highest vs low-mid 1st trimester plasma total homocysteine tertile.

  Child plasma 3-hydroxyanthranilic acid, kynurenic acid, histidine and asymmetric dimethylarginine were positively correlated with adiposity (affecting at least one of the indicators: waist circumference, waist/height, fat mass %, fat mass index, BMI).

Plasma picolinic acid, trigonelline and neopterin were inversely correlated with adiposity. Three of the above metabolites are from the kynurenine pathway.

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Scientific and academic contributions and other merits

Articles

Rojas-Gómez, Alejandra, Pol Solé-Navais, Pere Cavallé-Busquets, Gemma Ornosa-Martin, Carme Grifoll, Carla Ramos-Rodriguez, Joan Fernandez-Ballart, et al. 2022. "Pregnancy Homocysteine and Cobalamin Status Predict Childhood Metabolic Health in the Offspring." Pediatric Research, May. https://doi.org/10.1038/s41390-022-02117-5.

Cavallé-Busquets, Pere, Montserrat Inglès-Puig, Joan D. Fernandez-Ballart, Júlia Haro-Barceló, **Alejandra Rojas-Gómez**, Carla Ramos-Rodriguez, Monica Ballesteros, Klaus Meyer, Per M. Ueland, and Michelle M. Murphy. 2020. "Moderately Elevated First Trimester Fasting Plasma Total Homocysteine Is Associated with Increased Probability of Miscarriage. The Reus-Tarragona Birth Cohort Study." Biochimie 173: 62–67.

Rojas-Gómez, Alejandra, Amy Tan, Yvonne Lamers, and Michelle Murphy. 2020. "Comparison of Folate Status between Countries with Mandatory versus Voluntary or No Fortification Policy." https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=193308.

International conference contributions

Conference: Folic Acid, Vitamin B12, And One-Carbon Metabolism Conference. July 29-August 3, 2018. Western Shore, Nova Scotia, Canada.
Authors: Alejandra Rojas-Gomez, Julia Haro-Barcelo, Gemma Ornosa-Martin, Pol Sole-Navais, Monica Ballesteros, Montserrat Ingles-Puig, Per M. Ueland, Klaus Meyer, Joan D. Fernandez-Ballart, Pere Cavallé-Busquets, Michelle M. Murphy.
Titol: Associations between prenatal 1C metabolism and mid-childhood metabolic profile in the Reus-Tarragona Birth Cohort Study.
Format: Short presentation and poster.

Conference: Folic Acid, Vitamin B12, And One-Carbon Metabolism Conference. July 29-August 3, 2018. Western Shore, Nova Scotia, Canada.
Authors: Julia Haro-Barcelo, **Alejandra Rojas-Gomez,** Gemma Ornosa-Martin, Pol Sole-Navais, Monica Ballesteros, Montserrat Ingles-Puig, Per M. Ueland, Klaus Meyer, Joan D. Fernandez-Ballart, Pere Cavallé-Busquets, Michelle M. Murphy.
Titol: Associations between maternal and paternal 1C metabolism and mid-childhood growth and health in the Reus-Tarragona Birth Cohort Study.
Format: Poster.

Conference: 12th International Conference on One Carbon Metabolism, B Vitamins and Homocysteine. 9-16 June, 2019. Montbrió del Camp, Catalonia, Spain
Authors: Alejandra Rojas-Gomez, Julia Haro-Barcelo, Carla Ramos-Rodríguez, Gemma Ornosa-Martin, Pol Sole-Navais, Monica Ballesteros, Montserrat Ingles-Puig, Per M. Ueland, Luis Masana, Mercedes Heras, Joan D. Fernandez-Ballart, Pere Cavallé-Busquets, Michelle M. Murphy.

Titol: Associations between prenatal plasma homocysteine and metabolic score at mid-childhood.
Format: Poster**.**

Conference: 12th International Conference on One Carbon Metabolism, B Vitamins and Homocysteine. 9-16 June, 2019. Montbrió del Camp, Catalonia, Spain
Authors: Julia Haro-Barcelo, **Alejandra Rojas-Gomez**, Carla Ramos-Rodríguez, Gemma Ornosa-Martin, Pol Sole-Navais, Monica Ballesteros, Montserrat Ingles-Puig, Per M. Ueland, Luis Masana, Mercedes Heras, Joan D. Fernandez-Ballart, Pere Cavallé-Busquets, Michelle M. Murphy.
Titol: Genetic and metabolic alterations in paternal one carbon metabolism and development of pregnancy complications of placental origin.
Format: Poster.

Conference: 12th International Conference on One Carbon Metabolism, B Vitamins and Homocysteine. 9-16 June, 2019. Montbrió del Camp, Catalonia, Spain
Authors: Montserrat Inglès-Puig, Joan Fernandez-Ballart, Pere Cavallé-Busquets, Júlia Haro-Barceló, Mónica Ballesteros, **Alejandra Rojas-Gómez**, Carla Ramos-Rodriguez, Per M Ueland, Klaus Meyer, Michelle M Murphy.
Titol: Moderately elevated 1st trimester fasting plasma total homocysteine is associated with increased probability of miscarriage. A prospective cohort study.
Format: Poster

Conference: Virtual FASEB. The Folic Acid, Vitamin B12 and One-Carbon Metabolism Conference. 2020
Authors: Michelle M Murphy, Júlia Haro-Barceló, Luis Adolfo Santos, **Alejandra Rojas-Gómez,** Carla Ramos-Rodriguez, Joan D. Fernandez-Ballart, Marta Herrero, Pol Solé-Navais, Mónica Ballesteros, Pere Cavallé-Busquets.
Titol: Preconception and first trimester folic acid supplement use and folate status. The Reus Tarragona Birth Cohort.
Format: Poster.

Conference: 13th International conference one-carbon metabolism, B vitamins and homocysteine. September 12-16 2021. Poznan, Poland
Authors: Alejandra Rojas-Gomez, Carla Ramos-Rodríguez, Pere Cavallé-Busquets, Monica Ballesteros, Per M Ueland, Luis Masana, Mercedes Heras, Joan D Fernandez-Ballart, and Michelle M Murphy
Titol: Associations between pregnancy homocysteine and cobalamin status and metabolic score in the offspring.
Format: Oral presentation.

Conference: The Folate, Vitamin B12, and One-Carbon Metabolism Conference August 14–19, 2022. Asheville, NC.
Authors: Carla Ramos-Rodríguez, Luis Adolfo Santos-Calderón, Pere Cavallé-Busquets, Julia Haro Barceló, Alejandra Rojas-Gómez, Per M Ueland, Joan D Fernandez-Ballart and Michelle M Murphy.

Titol: Maternal and paternal One-Carbon metabolism, L-Arginine analogs and hypertensive pregnancies.
Format: Poster.

Teaching and academic activities

Medicine Grade in the Faculty of Medicine and Health Sciences of Universitat Rovira i Virgili

(approximately 60 hours/year; 2018-2021)

- Research and Documentation Bases (70%)

- General Epidemiology (30%)

Other contributions

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

Involvement in scientific projects

TItle: To investigate the association between prenatal maternal status in methyl donor nutrients and methylation profile in leukocyte extracted DNA from the children at 7.5-8 years. EpiBrain: Epigenetic effects of B-vitamins on brain health throughout life

Principal investigator: Yvonne Lamers, Michelle Murphy 2019 - 2022.

Outreach activities

- Explanation of the research project and the COFUND program. Mirada Directa Europa Tarragona Ràdio. February 2019
- Explanation of the research project and the COFUND program. Canal Reus TV. July 2020
  https://www.youtube.com/watch?v=sTQNFI2vfXI&t=284s
- Explanation of the research project – Fellow of the week. September 2020
  https://www.facebook.com/login/?next=https%3A%2F%2Fwww.facebook.com%2F135877696485772%2Fposts%2Fwhat-maternal-exposures-during-pregnancy-may-be-relevant-for-children-health-thi%2F4633161693423994%2F
- "Viatge a l'invisible: com extreure i veure ADN a casa / Journey to the Invisible: How to Extract and View DNA at Home".
  - European Researchers' Night. Tarragona. November 24, 2020.
  - Aula Gent Gran. URV. Reus. March 15, 2022.
  - Asociación Aspercamp. July 12, 2022.

Appendices

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CLINICAL RESEARCH ARTICLE

Pregnancy homocysteine and cobalamin status predict childhood metabolic health in the offspring

Alejandra Rojas-Gómez[1], Pol Solé-Navais[1,7], Pere Cavallé-Busquets[2,3], Gemma Ornosa-Martin[1], Carme Grifoll[2], Carla Ramos-Rodriguez[1], Joan Fernandez-Ballart[1,3], Luis Masana[4], Mónica Ballesteros[5], Per Magne Ueland[6] and Michelle M. Murphy[1,3]✉

BACKGROUND: Inadequate pregnancy cobalamin status has been associated with adverse offspring metabolic health in Indian and Nepalese studies. Studies of pregnancy cobalamin status and mid-childhood health outside of Asia are scarce.
METHODS: Associations between pregnancy fasting plasma total homocysteine (tHcy), cobalamin status (plasma cobalamin, holotranscobalamin (holoTC), methylmalonic acid (MMA)) and mid-childhood metabolic score (MetSco) ((including fat mass index (zFMI), homeostatic model assessment of insulin resistance (zHOMA-IR) and dyslipidemia (zTG − zHDLc)/2) *z*-scores)) were investigated in a prospective study of 293 mother–child dyads.
RESULTS: Highest versus low–mid pregnancy tHcy tertile was associated with higher mid-childhood MetSco, specifically with higher child zFMI. Stratifying by sex, the maternal tHcy–child MetSco association was limited to boys and confirmed for zFMI and zHOMA-IR. The maternal tHcy-child zFMI association was not mediated by birth weight *z*-score. First trimester plasma cobalamin was not associated with child outcomes, but other indicators of cobalamin status were. Lowest versus mid–high plasma holoTC tertile was associated with MetSco (specifically zFMI and zHOMA-IR) and highest versus low–mid plasma MMA tertile with higher MetSco and dyslipidemia in boys.
CONCLUSIONS: Moderately elevated pregnancy tHcy and low cobalamin status were associated with mid-childhood metabolic score in boys. The pregnancy tHcy–child zFMI association was not mediated by birth weight.

Pediatric Research; https://doi.org/10.1038/s41390-022-02117-5

IMPACT:

- Fasting plasma total homocysteine (tHcy) during pregnancy and low cobalamin status during early pregnancy are associated with mid-childhood metabolic score and its components in the offspring. These findings were only significant in male offspring.
- The study provides new evidence that impaired one carbon metabolism during pregnancy is associated with negative health outcomes in the offspring, in a population with low prevalence of cobalamin deficiency.
- The maternal–offspring associations were observed in the functional markers of cobalamin status (holotranscobalamin and methylmalonic acid) and tHcy, not with plasma cobalamin concentration.
- Screening for low pregnancy cobalamin status should be considered.

INTRODUCTION

Low birth weight has been linked to cardiovascular disease,[1,2] type 2 diabetes,[3,4] hypertension,[3–5] and elevated triglycerides.[3] Elevated pregnancy fasting plasma total homocysteine (tHcy) has been associated with low birth weight and intrauterine growth retardation risk.[6,7] In regions where cobalamin deficiency is prevalent, low pregnancy cobalamin status has been associated with impaired glucose metabolism in the mother and the offspring during childhood.[8] Similar results were reported in Bangladeshi pregnant women, living in the UK.[9] Combined with cobalamin deficiency, high folate status during pregnancy has been associated with gestational diabetes[10] and exacerbation of high adiposity and insulin resistance in the offspring.[8] Pregnancy tHcy has also been associated with impaired glucose metabolism and insulin resistance in the offspring.[11] Cobalamin deficiency is less prevalent in European women,[12–14] but we reported interactions between folic acid supplement regime and low first trimester plasma cobalamin status (≤221 pmol/L) leading to worse cobalamin status as pregnancy progressed in women exceeding 400 µg/day of folic acid compared to those who adhered to the recommended dose.[12] Few studies outside Asia have investigated how pregnancy one-carbon metabolism

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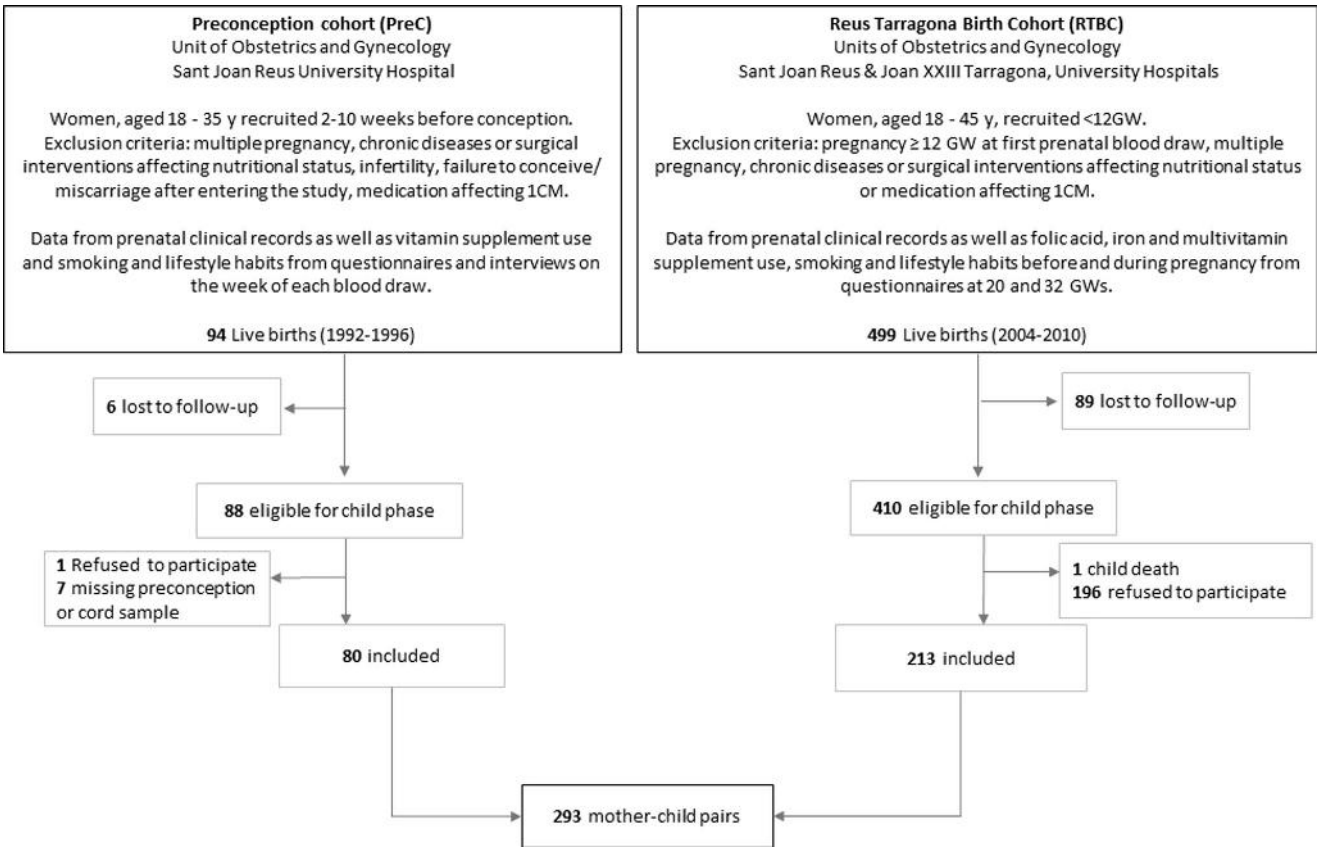

Fig. 1 Participant recruitment and follow-up. 1CM: 1 carbon metabolism.

(1-CM) status affects childhood metabolic and growth outcomes. In a multi-ethnic Dutch cohort where 11.9 and 13.8% of the mothers were folate and cobalamin deficient, respectively, maternal folate was inversely associated with body mass index (BMI) and cobalamin with heart rate in the children.[15] Low postpartum maternal folate status was associated with increased risk of childhood overweight/obesity in the offspring in a USA study.[16] In animal studies, folate- and cobalamin-deficient diets during pregnancy or lactation lead to impaired glucose and lipid metabolism in the offspring.[17,18]

We hypothesized that moderately elevated pregnancy tHcy and cobalamin is associated with alterations in metabolic parameters in the offspring. We aimed to investigate the association between pregnancy tHcy, cobalamin status, and metabolic score in children aged 6–8 years.

METHODS

Participants

Mother–child dyads ($n = 293$) from the PreC (Preconception) and RTBC (Reus-Tarragona Birth Cohort [registered at www.clinicaltrials.gov, NCT01778205]) studies participated from preconception/early pregnancy over 7–9 years (Fig. 1). The studies were approved by the Sant Joan Reus (SJR) and Joan XXIII Tarragona (JXXIII) University Hospitals' joint Ethics Committees (internal reference 22/2016, approved on 20/10/2016 and revised on 30/10/2019), and conducted according to the Declaration of Helsinki guidelines with informed consent from participants. Parents provided consent, and the children, verbal assent, for the child phase.

Recruitment, described previously (PreC[7,19,20] and RTBC[12,21]), was by the Unit of Preventive Medicine and Public Health, Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili and the Units of Obstetrics and Gynecology, SJR and JXXIII Hospitals. Non-pregnant women volunteered for the PreC study in response to local city hall and media advertisements. None of them took folic acid supplements periconceptionally because the study was before the introduction of current recommendations.[22] Some took folic acid-containing supplements coinciding with iron supplementation in mid–late pregnancy and 35 women never took folic acid supplements throughout pregnancy.

For the RTBC, participants were recruited from the high-risk obstetrics units and University/Hospital staff and contacts. They were advised at their first prenatal check-up to take supplements containing 400 µg folic acid/day and 2 µg cyanocobalamin/day for the first trimester and 40 mg iron/day after 12 gestational weeks (GW). Women with anemia were treated with iron supplements by their clinicians, and the iron doses were recorded.

Health check-up at 6–8 years

Child participation was at 6 (PreC) or 7.5 years of age (RTBC). Clinical data including anthropometric measurements were collected at the study check-up as well as from health records and lifestyle habits by interview with the parents.

Height was measured by stadiometer (with a precision of 0.1 cm). Children stood still, with their heels together and feet facing outwards at a 60° angle, head in the Frankfort plane, and palms of their hands placed on their legs.

Weight was measured on a mechanical beam scale with height rod (Pesperson model) (PreC) and electronic scale with a precision of 0.100 g (Tanita BC-420MA, Tanita Corporation, Tokyo, Japan) (RTBC).

Means of triplicate triceps (halfway between the acromion and the olecranon process at the back of the arm) and subscapular (20 mm below the tip of the scapula, at an angle of 45° to the lateral side of the body) skinfold thicknesses were measured by a Harpenden skinfold calliper (Holtain Ltd, Crymych, Wales), with an accuracy of 0.2 mm. Fat mass percentage (x) was determined from the sum of triceps (mm) and subscapular (mm) skinfold thicknesses (y)[23]. Fat mass percentage was used to calculate fat mass index[24]:

For $y \le 35$ mm:

$$x(Boys) = 1.21y - 0.008y^2 - 1.7$$

$$x(Girls) = 1.33y - 0.013y^2 - 2.5$$

For $y > 35$ mm:

$$x(Boys) = 0.783y + 1.6$$

$$x(Girls) = 0.546y + 9.7$$

$$Fat\ mass\ index\ (FMI) = \frac{(weight\ in\ kg) \times \left(\frac{sex\ specific\ x}{100}\right)}{(height\ in\ m)^2}$$

Blood sample collection, processing and storage

Fasting blood samples were collected from the mothers at <12 GW (both cohorts), 32 GW (PreC), 34 GW (RTBC), and children in EDTA-K2 evacuated tubes, kept at 4 °C, and plasma separated within 1–2 h. Plasma samples were stored at −20 °C (PreC) and −80 °C (RTBC) until all samples from the same pregnancy were analysed in the same batch.

Biochemical determinations

tHcy was determined by immunoassay (PreC) (IMx autoanalyzer, Abbott, Chicago, USA)[19] and liquid-tandem mass spectrometry (RTBC).[25] Plasma methylmalonic acid (MMA) was determined by gas chromatography mass spectrometry with methylchloroformate derivatization,[25] folate and cobalamin by microbiological assays with *Lactobacillus casei*[26] and *Lactobacillus leichmannii*,[27] respectively, and holoTC by immunoassay (AxSym autoanalyzer, Abbott Chicago, USA) in SJR Hospital.[28] Plasma MMA measurements were not available for the children from the PreC cohort and plasma holoTC was not available for the children from the RTBC cohort. Plasma insulin concentration was determined by Iso-Insulin ELISA Kit (a solid-phase two-site enzyme immunoassay, Mercodia, Sweden) and glucose by the glucose oxidase (GOD) peroxidase (POD) method (Spinreact, Sant Esteve de Bas, Spain). Insulin resistance was calculated as HOMA-IR [homeostasis model assessment of insulin resistance] = (FPI [fasting plasma insulin concentration, mU/L] × FPG [fasting plasma glucose, mmol/L])/22.5.[29] Plasma total cholesterol and high-density lipoprotein cholesterol (HDLc) were determined by enzymatic colorimetric techniques (Spinreact, Sant Esteve de Bas, Spain), and triglycerides (TG) by glycerol phosphate oxidase (GPO) peroxidase (POD) technique (Spinreact, Sant Esteve de Bas, Spain). Low-density lipoprotein cholesterol was calculated using the Friedewald formula (Total cholesterol − HDLc − triglycerides mg/dL/5).[30] Plasma lipoprotein(a) (Lp(a)) was determined by quantitative turbidimetric test Lp(a)-turbilatex (Spinreact, Sant Esteve de Bas, Spain) and Apolipoprotein A1 (ApoA1) and B (ApoB) by turbidimetry technique (ABX Pentra, France).

Metabolic score

A modification of the risk score used in the IDEFICS cohort[31] was used:

$$\text{Metabolic score (MetSco)} = \text{zFMI} + \frac{\text{zTG} - \text{zHDLc}}{2} + \text{zHOMA} - \text{IR}$$

The IDEFICS score includes waist circumference (WC) and blood pressure. These were unavailable for PreC, so FMI was used and blood pressure was omitted. Dyslipidemia was measured as (zTG − zHDLc)/2, where HDLc is inversely associated with the metabolic risk profile. We derived *z*-scores (standardized residuals) from a generalized linear model (GLM) of each component (FMI, Lipids, HOMA-IR) as dependent variables, including age and sex as the predictors.

Sample size calculation

A priori, by way of orientation, sample size calculation was based on the hypothetical association between elevated pregnancy tHcy and childhood obesity. A type 1 error of 5% and power of 80% in unilateral contrast tests were assumed, for an expected odds ratio of ≥4 for childhood obesity for pregnancy tHcy in the highest tertile, compared to the other tertiles combined. We expected 38% of the children to be overweight and the others to have normal weight. Based on a pilot study, 10% of the mothers of normal weight children were expected to have had highest tertile pregnancy tHcy (unpublished data).

Statistical analysis

Variable distribution normality was tested by the Kolmogorov–Smirnov test and ln-transformation to approach normality applied as required for parametric tests. Quantitative variables were compared between categories by the Student's unpaired *t* test, medians by the Median test for *K* independent samples (SPSS), and proportions by the Chi-square test. Correlations between variables are reported as Spearman's rank-order correlation coefficients. Associations between pregnancy tHcy, cobalamin, and folate status and mid-childhood outcomes (MetSco and its components) were investigated by multiple linear regression analysis. Associations were determined for the highest maternal tertiles of plasma tHcy and MMA compared to the low–mid tertiles (combined) and lowest maternal tertiles of plasma cobalamin, holoTC, and folate compared to the mid–high tertiles (combined).

Models were adjusted for maternal characteristics (preconception (PreC) and first trimester (RTBC) BMI, socioeconomic status, pregnancy smoking pattern (never (reference group), first trimester only, throughout pregnancy), and child characteristics (breastfeeding (yes/no), BMI *z*-score[32] as a substitute for energy intake that is unavailable for the PreC cohort, and tHcy). Mediation analysis was used to test whether the pregnancy tHcy–offspring outcome associations were mediated by birth weight *z*-score (Spanish birth weight tables).[33]

Assumptions in linear regression (linearity, homogeneity of variance (homoscedasticity), normality of errors, independence of errors between the two cohorts, model specification, and multicollinearity) were checked.

Unusual and influential data were detected by inspecting scatterplots of the independent and dependent variables for potential outliers and residuals, to exclude those with a Cook's distance >4/*n* (*N* = 4). SPSS version 27.0 for Windows, with the PROCESS macro[34] for the mediation analysis, was used.

RESULTS

Participant characteristics according to pregnancy tHcy status are reported in Table 1. Maternal (including age, BMI, parity, smoking habits, and socioeconomic status) and child (including male sex prevalence, birth weight *z*-score, low birth weight (<P10), and breastfeeding regime) characteristics were similar between the pregnancy tHcy categories. Prevalence of overweight–obesity according to Spanish tables[32] was higher in children born to mothers in the highest tHcy tertile in the first trimester of pregnancy compared to the low–mid tertiles but there was no difference among the third trimester tHcy tertiles. Detailed maternal and child characteristics of both cohorts are reported in Supplemental Table S1. RTBC mothers were slightly older, less of them smoked but more of the smokers continued smoking throughout pregnancy, and they had higher socioeconomic status. Generally, the biochemical indicators of first trimester 1-CM status were better in the RTBC, except for plasma MMA that did not differ between the two cohorts. The same was true for third trimester indicators, except for plasma folate that was lower in the RTBC. The prevalence of low birth weight was lower in the RTBC and less of the babies had been breastfed for at least 1 month. Child plasma tHcy and triglycerides were lower in the RTBC, and HDLc and glucose were higher. None of the other metabolic or biochemical parameters differed between the two cohorts. Pregnancy tHcy, plasma cobalamin, and holoTC were each weakly correlated with the same corresponding variables in the children (Supplemental Table S2). Maternal plasma holoTC was relatively strongly correlated with plasma cobalamin and tHcy, compared to plasma MMA. Plasma holoTC, cobalamin, and tHcy were all only weakly correlated with plasma MMA. The child holoTC–cobalamin correlation was relatively strong, and stronger than any of the other correlations among child nutrients or tHcy. Child folate, holoTC, and cobalamin were

Table 1. Participant characteristics according to pregnancy tHcy status.

	All	First trimester		Third trimester	
		tHcy low–mid tertiles[a]	tHcy highest tertile	tHcy low–mid tertiles[b]	tHcy highest tertile
Mothers during pregnancy					
Age (years)[c,d]	32.0 (27.0, 37.0) [289]	32.0 (27.9, 37.0) [178]	30.5 (26.0, 36.1) [98]	32.0 (27.0, 37.0) [173]	31.0 (26.7, 36.0) [86]
BMI (kg/m^2)[c,d]	23.0 (19.9, 27.3) [285]	22.8 (20.1, 27.1) [178]	23.3 (19.7, 28.1) [95]	22.7 (20.1, 27.0) [171]	22.9 (19.2, 28.7) [85]
Parity (nulliparous)[e]	52.6 (46.8, 58.2) [152/289]	48.9 (41.6, 56.2) [87/178]	56.1 (46.3, 65.5) [55/98]	51.4 (44.0, 58.8) [89/173]	52.3 (41.9, 62.6) [45/86]
Smoking during pregnancy[e]					
Never	78.8 (73.7, 83.1) [227/288]	79.8 (73.3, 85.0) [142/178]	77.6 (68.3, 84.7) [76/98]	79.8 (73.2, 85.1) [138/173]	79.1 (69.3, 86.3) [68/86]
Periconception/first trimester only	6.9 (4.5, 10.5) [20/288]	6.2 (3.5, 10.7) [11/178]	7.1 (3.5, 14.0) [7/98]	7.5 (4.4, 14.4) [13/173]	5.8 (2.5, 12.9) [5/86]
Throughout	14.2 (10.7, 18.7) [41/288]	14.0 (9.7, 19.9) [25/178]	15.3 (9.5, 23.7) [15/98]	12.7 (8.5, 18.5) [22/173]	15.1 (9.1, 24.2) [13/86]
Socioeconomic status[e]					
Low	10.4 (7.4, 14.5) [30/288]	7.3 (4.3, 12.1) [13/178]	14.3 (8.7, 22.6) [14/98]	9.8 (6.2, 15.2) [17/173]	10.5 (5.6, 18.7) [9/86]
Middle	42.0 (36.5, 47.8) [121/288]	42.1 (35.1, 49.5) [75/178]	41.8 (32.6, 51.7) [41/98]	39.9 (32.9, 47.3) [69/173]	41.9 (32.0, 52.4) [36/86]
High	47.6 (41.9, 53.3) [137/288]	50.6 (43.3, 57.8) [90/178]	43.9 (34.5, 53.7) [43/98]	50.3 (42.9, 57.7) [87/173]	47.7 (37.4, 58.1) [41/86]
Children at birth and infancy					
Boys[e]	48.4 (42.7, 54.2) [140/289]	46.6 (39.4, 54.0) [83/178]	52.0 (42.3, 61.7) (51/98)	49.1 (41.8, 56.5) (85/173)	46.5 (36.3, 57.0) (40/86)
Birth weight *z*-score[d,f]	−0.073 (−1.055, 1.224) [286]	−0.042 (−0.992, 1.279) [178]	−0.097 (−1.178, 1.185) [97]	−0.097 (−1.015, 1.190) [173]	−0.002 (−0.996, 1.385) [86]
Birth weight <P10[e,f]	6.6 (4.3, 10.1) [19/286]	5.6 (3.1, 10.0) [10/178]	7.2 (3.5, 14.2) [7/97]	5.8 (3.2, 10.3) [10/173]	5.8 (2.5, 12.9) [5/86]
Breastfed: Yes (min. 1 month)[e]	72.4 (66.9, 77.3) [202/279]	69.7 (62.5, 76.0) [122/175]	74.5 (64.8, 82.2) [70/94]	75.1 (68.1, 81.1) [127/169]	67.5 (56.8, 76.6) [56/83]
Mid-childhood check-up					
Age (months)[d]	89.0 (72.0, 91.0) [289]	89.0 (72.0, 91.0) [178]	88.0 (72, 92) [98]	89.0 (72.0, 91.6) [173]	88.0 (72.0, 92.0) [86]
BMI (kg/m^2)[d]	16.3 (14.1, 19.7) [287]	16.2 (14.1, 19.8) [177]	16.4 (14.2, 20.2) [97]	16.3 (14.2, 19.3) [171]	16.4 (14.1, 21.2) [86]
z-score BMI[d,g]	−0.409 (−2,449, 2.042) [287]	−0.559 (−2.430, 1.866) [177]	−0.277 (−2.404, 2,733) [97]	−0.435 (−2.442, 1.742) [171]	−0.523 (−2.375, 3.458) [86]
Overweight–obesity[e,g]	20.2 (16.0, 25.2) [58/287]	16.4 (11.7, 22.5) [29/177]	27.8 (19.9, 37.5)* [27/97]	19.3 (14.1, 25.9) [33/171]	23.3 (15.6, 33.2) [20/86]

Comparison between low–mid tertile versus highest tertile in each trimester: Proportions were compared by the Chi-square test, continuous variables were compared using the Median test for *K* independent samples (SPSS).
BMI body mass index, *tHcy* fasting plasma total homocysteine.
*$P < 0.05$
[a]RTBC <5.7 µmol/L, PreC <7.1 µmol/L.
[b]<5.7 µmol/L both cohorts.
[c]At the beginning of pregnancy.
[d]P50 (P10, P90) [*N*].
[e]% (95% CI) [*N*].
[f]Based on Spanish tables.[33]
[g]Based on Spanish tables.[32] *N*s vary between the data reported for all participants and the stratified analysis and also between each trimester due to unattended blood draws or unreturned questionnaires.

Table 2. Nutritional and metabolic markers in maternal and child fasting plasma samples, according to pregnancy tHcy status.

	First trimester			Third trimester		
	All	tHcy low–mid tertiles[a]	tHcy highest tertile	All	tHcy low–mid tertiles[b]	tHcy highest tertile
Mothers during pregnancy						
Cobalamin (pmol/L)[c]	348.1 (201.2, 496.4) [274]	361.8 (213.4, 521.2) [177]	324.2 (189.2, 482.7) [97]	233.3 (142.7, 375.9) [257]	238.2 (147.1, 384.6) [172]	230.9 (140.1, 348.8) [85]
Cobalamin deficiency[d,e]	2.6 (1.2, 5.2) [7/274]	2.3 (0.9, 5.7) [4/177]	3.1 (1.1, 8.7) [3/97]	12.1 (8.6, 16.6) [31/257]	11.0 (7.2, 16.6) [19/172]	14.1 (8.3, 23.1) [12/85]
HoloTC (pmol/L)[c]	65.5 (36.9, 106.9 [220]	67.8 (42.4, 110.2) [145]	58.7 (28.1, 96.0) [73]	61.4, (32.6, 103.2) [210]	63.8 (37.3, 104.4) [139]	54.9 (27.5, 90.7) [71]
MMA (μmol/L)[c]	0.110 (0.080, 0.159) [274]	0.108 (0.080, 0.148) [177]	0.113 (0.084, 0.175) [97]	0.140 (0.100, 0.211) [259]	0.140 (0.100, 0.200) [173]	0.141 (0.103, 0.228) [86]
Folate (nmol/L)[c,f]	22.5 (8.2, 50.7) [270]	25.9 (10.6, 51.7) [175]	15.3 (5.7, 47.8) [89]***	10.2 (4.6, 42.3) [246]	15.8 (5.5, 50.3) [164]	6.5 (4.0, 15.9) [78]***
Folate deficiency[d,f,g]	7.4 (4.8, 11.2) [20/270]	3.4 (1.6, 7.3) [6/175]	13.5 (7.9, 22.1) [12/89]**	32.1 (26.6, 38.2) [79/246]	20.1 (14.7, 26.9) [33/164]	55.1 (44.1, 65.7) [43/78]***
Children at check-up						
tHcy (μmol/L)[c]	5.6 (4.2, 7.3) [222]	5.4 (4.2, 7.1) [126]	5.8 (4.3, 7.4) [85]*	5.6 (4.2, 7.3) [222]	5.4 (4.1, 7.2) [126]	5.9 (4.5, 7.4) [70]*
Cobalamin (nmol/L)[c]	579.2 (393.0, 870.8) [221]	583.3 (393.1, 891.9) [125]	570.6 (392.5, 875.0) [85]	579.2 (393.0, 870.8) [221]	576.1 (386.0, 816.2) [125]	594.4 (398.9, 943.3) [70]
Folate (pmol/L)[c]	17.1 (9.3, 32.5) [217]	17.1 (9.6, 32.7) [126]	17.1 (9.2, 32.3) [81]	17.1 (9.3, 32.5) [217]	17.9 (9.3, 33.1) [125]	15.6 (8.6, 27.9) [67]
Folate deficiency[d,g]	4.6 (2.5, 8.3) [10/217]	4.8 (2.2, 10.0) [6/126]	4.9 (1.9, 12.0) [4/81]	4.6 (2.5, 8.3) [10/217]	4.8 (2.2, 10.1) [6/125]	4.5 (1.5, 12.4) [3/67]
HDLc (mmol/L)[c]	1.6 (1.2, 2.0) [235]	1.7 (1.2, 2.0) [135]	1.5 (1.2, 1.9) [87]	1.6 (1.2, 2.0) [235]	1.6 (1.2, 2.0) [133]	1.6 (1.3, 2.0) [74]
Triglycerides (mmol/L)[c]	0.6 (0.4, 1.0) [235]	0.6 (0.4, 1.0) [135]	0.6 (0.4, 1.1) [88]	0.6 (0.4, 1.0) [235]	0.6 (0.4, 1.0) [133]	0.6 (0.4, 1.0) [74]
Insulin (mU/L)[c]	5.5 (4.9, 6.9) [234]	5.5 (4.9, 6.9) [135]	5.6 (4.8, 7.4) [87]	5.5 (4.9, 6.9) [234]	5.5 (4.9, 6.9) [133]	5.6 (4.9, 7.6) [74]
Glucose (mmol/L)[c]	5.1 (4.6, 5.5) [234]	5.1 (4.6, 5.5) [135]	5.2 (4.7, 5.6) [88]**	5.1 (4.6, 5.5) [234]	5.0 (4.4, 5,4) [133]	5.2 (4.6, 5.6) [74]*
HOMA-IR[c]	1.2 (1.0, 1.6) [234]	1.2 (1.0, 1.6) [135]	1.3 (1.0, 1.7) [87]	1.2 (1.0, 1.6) [234]	1.2 (1.0, 1.5) [133]	1.3 (1.1, 1.7) [74]*
Fat mass index[c]	2. 3 (1.5, 4.7) [274]	2.3 (1.5, 4.3) [171]	2.4 (1.5, 5.0) [95]	2. 3 (1.5, 4.7) [274]	2.3 (1.6, 4.7) [167]	2.4 (1.4, 5.6) [82]
Metabolic score[c,h]	−0.530 (−2.018, 2.168) [223]	−0.683 (−2.181, 1.532) [130]	−0.224 (−1.776, 2.934) [86]	−0.530 (−2.018, 2.168) [223]	−0.693 (−2.144, 1.524) [131]	−0.233 (−1.676, 3.203) [70]
Total cholesterol (mmol/L)[c]	4.3 (3.5, 5.3) [235]	4.3 (3.5, 5.3) [135]	4.4 (3.5, 5.3) [87]	4.3 (3.5, 5.3) [235]	4.3 (3.5, 5.3) [133]	4.4 (3.5, 5.4) [74]
Lipoprotein (a) (mg/dL)[c]	5.5 (1.0, 33.2) [235]	6.4 (0.9, 30.7) [135]	5.0 (1.0, 33.3) [87]	5.5 (1.0, 33.2) [235]	5.1 (0.8, 30.3) [133]	5.5 (1.3, 33.3) [74]
ApoA1 (mg/dL)[c]	139.0 (116.0, 164.8) [223]	141.0 (115.0, 163.0) [129]	135.5 (117.5, 169.5) [84]	139.0 (116.0, 164.8) [223]	138.0 (114.7, 161.3) [126]	140.0 (116.2, 168.9) [70]
ApoB (mg/dL)[c]	74.0 (57.2, 93.8) [231]	72.5 (56.5, 94.0) [134]	75.5 (57.7, 89.3) [86]	74.0 (57.2, 93.8) [231]	73.0 (56.2, 93.0) [131]	76.0 (58.4, 98.6) [73]
LDLc—Friedewald (mmol/L)[c]	2.4 (1.8, 3.2) [235]	2.3 (1.8, 3.3) [135]	2.5 (1.8, 3.1) [87]	2.4 (1.8, 3.2) [235]	2.3 (1.7, 3.3) [133]	2.5 (1.9, 3.3) [74]

Numbers vary between the data reported in the "All" column and the stratified analysis by tHcy tertiles because tHcy measurements in each trimester were not available for all women. Comparison between low-mid tertile versus highest tertile in each trimester: proportions using Chi-square test and continuous variables using Median test for *K* independent samples (SPSS).
ApoA1 apolipoprotein A1, *ApoB* apolipoprotein B, *HDLc* high-density lipoprotein cholesterol, *HoloTC* holotranscobalamin, *LDLc* low-density lipoprotein cholesterol, *MMA* methylmalonic acid, *tHcy* fasting plasma total homocysteine.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
[a]RTBC: low–mid <5.7 μmol/L, high ≥5.7 μmol/L; PreC low–mid <7.1 μmol/L, high ≥7.1 μmol/L. First trimester tHcy tertile values differed between the cohorts due to different folic acid supplementation patterns.
[b]Both cohorts: low–mid <5.7 μmol/L, high ≥5.7 μmol/L.
[c]P50 (P10, P90) [*N*].
[d]% (95% CI) [*N*].
[e]<148 pmol/L.
[f]Not including mothers with plasma folate below the limit of detection (2 nmol/L, first trimester $N = 5$, third trimester $N = 6$).
[g]<7.0 nmol/L.
[h]Metabolic score: zFMI + ((zTG − zHDLc)/2) + zHOMA-IR.

inversely correlated with tHcy in that decreasing order of strength of correlation. Pregnancy 1-CM status and child biochemical data by pregnancy tHcy status is reported in Table 2. Only folate status differed significantly between the corresponding tHcy categories in both trimesters. Folate status was lower and deficiency more prevalent in mothers in the highest versus low–mid tHcy tertiles. More mothers had cobalamin and folate deficiency in the third trimester compared to the first. None of the children had cobalamin deficiency (data not shown) but 4.6% had folate deficiency. Offspring tHcy and plasma glucose concentration were higher when pregnancy tHcy was in the highest versus low–mid tertiles and this was also true for HOMA-IR when mothers had high tertile tHcy in the third trimester. No differences were observed in any of the child lipid parameters ((total cholesterol, plasma lipoprotein (a), ApoA1 and ApoB, LDL cholesterol) by pregnancy tHcy status.

Determinants of first trimester maternal tHcy are reported in Supplemental Table S3. Plasma folate concentration was the strongest.

Associations between pregnancy tHcy status, child MetSco, and its components are reported in Table 3. Offspring of mothers with highest versus low–mid tHcy tertiles had higher MetSco and zFMI. Stratifying by sex, the associations were only significant in boys. Furthermore, in boys only, zHOMA-IR was higher when mothers had third trimester tHcy in the highest versus the low–mid tertiles.

Associations between pregnancy indicators of cobalamin status and childhood outcomes are reported in Table 4. First trimester plasma cobalamin was not associated with any child outcomes but boys born to mothers with low third trimester plasma cobalamin status had lower mid-childhood FMI. On the other hand, first trimester holoTC in the lowest versus mid–high tertiles was associated with higher MetSco, FMI, and insulin resistance in boys. Highest first trimester MMA tertile versus low–mid tertiles was associated with increased child metabolic score and dyslipidemia ((zTG − zHDLc)/2) in boys. Associations between pregnancy folate status and mid-childhood outcomes are reported in Supplemental Table S4. Children of mothers with first trimester plasma folate concentration in the lowest (<14.6 nmol/L) versus mid–high tertiles had higher insulin resistance, and stratifying by sex, this was limited to girls. Boys born to mothers with third trimester plasma folate in the lowest compared to mid–high tertiles had lower dyslipidemia.

Mediation analysis was used to explore whether the associations between first trimester tHcy and zFMI are partially mediated via fetal growth (birth weight *z*-score) (Fig. 2). The direct effect (tHcy outcome, coefficient *c'*), indirect effect ((tHcy–birth weight *z*-score outcome, coefficient *a* (tHcy–birth weight), and coefficient *b* (birth weight *z*-score outcome)) and total effect (tHcy outcome, coefficient *c*, unadjusted for birth weight *z*-score) are illustrated. The indirect effect ($a \times b$) represents the association between tHcy and child zFMI via the sequence tHcy–birth weight outcome. The Monte Carlo confidence interval for the indirect effect includes 0, indicating that birth weight does not play a role in the association between early pregnancy tHcy and fat mass index in the offspring.

DISCUSSION

Principal findings

Moderately elevated pregnancy tHcy was positively associated with MetSco in boys, and specifically zFMI and zHOMA-IR. First trimester low holoTC and high MMA were positively associated with MetSco, first trimester holoTC with zFMI and zHOMA-IR, and first trimester MMA with dyslipidemia in boys. The pregnancy tHcy-child zFMI association was not mediated by birth weight. Low third trimester plasma cobalamin was associated with lower FMI and low plasma folate with lower dyslipidemia in boys. In girls only, low first trimester plasma folate was positively associated with zHOMA-IR.

Comparison with previous studies

Overall, these findings in participants with a low prevalence of cobalamin deficiency support previous observations from studies in countries where cobalamin deficiency is highly prevalent. However, none of those were stratified by sex. Indian studies reported no association between pregnancy tHcy and percentage body fat or other anthropometric measurements in the offspring in mid-childhood.[8,11] We observed no association between pregnancy folate and offspring FMI. However, a USA study

Table 3. Association between maternal tHcy highest tertile versus low-mid (reference)[a] and child metabolic outcomes at 6–8 years by multiple linear regression analysis.

		All		Girls		Boys	
		Adjusted R^2	*B* coefficients[b] (SE)[c]	Adjusted R^2	*B* coefficients[b] (SE)[c]	Adjusted R^2	*B* coefficients[b] (SE)[c]
First trimester[d]	Metabolic score	0.437***	0.418 (0.189)*	0.399***	0.325 (0.315)	0.514***	0.462 (0.224)*
	zFat Mass Index	0.680***	0.211 (0.073)**	0.749***	0.150 (0.099)	0.637***	0.276 (0.108)*
	zHOMA-IR	0.152***	0.081 (0.091)	0.127**	0.044 (0.156)	0.155**	0.109 (0.104)
	(zTG–zHDLc)/2	0.014	0.252 (0.226)	−0.036	0.263 (0.370)	0.065	0.154 (0.286)
Third trimester[e]	Metabolic score	0.521***	0.435 (0.183)*	0.545***	0.446 (0.280)	0.519***	0.511 (0.236)*
	zFat Mass Index	0.664***	0.190 (0.081)*	0.727***	0.111 (0.113)	0.632***	0.312 (0.113)**
	zHOMA-IR	0.202***	0.157 (0.087)†	0.205***	0.129 (0.138)	0.140*	0.238 (0.114)*
	(zTG–zHDLc)/2	0.053*	0.176 (0.227)	0.076	0.413 (0.339)	0.051	−0.077 (0.308)

Models adjusted for: maternal age, maternal body mass index, socioeconomic status, pregnancy smoking periconceptionally versus never, pregnancy smoking during pregnancy versus never, breastfeeding (yes/no), zBMI at childhood check-up, child tHcy at check-up.
HDLc high-density lipoprotein cholesterol, *HOMA-IR* homeostasis model assessment of insulin resistance, *TG* triglycerides.
*$P < 0.05$, **$P < 0.01$,***$P < 0.001$, †$P = 0.07$.
[a]First trimester RTBC: low–mid <5.7 μmol/L, highest ≥5.7 μmol/L; PreC low–mid <7.1 μmol/L, highest ≥7.1 μmol/L; first trimester tHcy tertile values differed between the cohorts due to different folic acid supplementation patterns. Third trimester (both cohorts): low–mid <5.7 μmol/L, highest ≥5.7 μmol/L.
[b]Unstandardized *B* coefficients of maternal tHcy highest versus low–mid tertiles (reference).
[c]Standard errors.
[d]All ($n = 197$), girls ($n = 103$), boys ($n = 94$).
[e]All ($n = 182$), girls ($n = 95$), boys ($n = 87$).

Table 4. Association between indicators of cobalamin status (plasma cobalamin, HoloTC, MMA) and child outcomes by multiple linear regression analysis.

			All		Girls		Boys	
			Adjusted R^2	*B* coefficients[b] (SE)[c]	Adjusted R^2	*B* coefficients[b] (SE)[c]	Adjusted R^2	*B* coefficients[b] (SE)[c]
First trimester	Cobalamin[a]	Metabolic score	0.420***	0.097 (0.210)	0.397***	−0.236 (0.321)	0.486***	0.464 (0.274)
		zFat Mass Index	0.664***	−0.008 (0.083)	0.736***	−0.122 (0.105)	0.592***	0.113 (0.137)
		zHOMA-IR	0.128***	0.009 (0.101)	0.101*	−0.030 (0.162)	0.154**	0.063 (0.124)
		(zTG − zHDLc)/2	0.020	0.191 (0.244)	−0.035	−0.166 (0.371)	0.093*	0.574 (0.337)
	HoloTC[d]	Metabolic score	0.432***	0.332 (0.241)	0.376***	−0.172 (0.373)	0.542***	0.897 (0.328)**
		zFat Mass Index	0.695***	0.115 (0.089)	0.767***	−0.098 (0.108)	0.645***	0.323 (0.157)*
		zHOMA-IR	0.144***	0.087 (0.117)	0.117*	−0.059 (0.188)	0.171**	0.306 (0.152)*
		(zTG − zHDLc)/2	0.016	0.260 (0.279)	−0.071	−0.028 (0.421)	0.075	0.536 (0.421)
	MMA[e]	Metabolic score	0.422***	0.122 (0.206)	0.398***	−0.229 (0.329)	0.497***	0.529 (0.241)*
		zFat Mass Index	0.679***	0.049 (0.079)	0.753***	−0.061 (0.104)	0.597***	0.175 (0.120)
		zHOMA-IR	0.130***	−0.107 (0.099)	0.114*	−0.224 (0.165)	0.148**	0.004 (0.110)
		(zTG − zHDLc)/2	0.028	0.359 (0.239)	−0.036	0.111 (0.380)	0.118*	0.699 (0.293)*
Third trimester	Cobalamin[f]	Metabolic score	0.509***	−0.082 (0.184)	0.513***	−0.036 (0.280)	0.513***	−0.213 (0.265)
		zFat Mass Index	0.667***	−0.133 (0.080)	0.720***	−0.021 (0.112)	0.615***	−0.269 (0.127)*
		zHOMA-IR	0.176***	−0.064 (0.088)	0.162**	−0.092 (0.137)	0.218**	−0.100 (0.122)
		(zTG − zHDLc)/2	0.066*	0.230 (0.220)	0.028	0.154 (0.330)	0.083	0.312 (0.335)
	HoloTC[g]	Metabolic score	0.514***	0.060 (0.208)	0.508***	−0.030 (0.293)	0.527***	0.173 (0.318)
		zFat Mass Index	0.686***	0.002 (0.087)	0.720***	0.014 (0.114)	0.647***	−0.038 (0.144)
		zHOMA-IR	0.173***	0.036 (0.102)	0.180**	−0.034 (0.147)	0.159*	0.139 (0.151)
		(zTG − zHDLc)/2	0.063*	0.043 (0.250)	0.028	−0.020 (0.340)	0.068	0.145 (0.419)
	MMA[h]	Metabolic score	0.514***	−0.263 (0.189)	0.522***	−0.367 (0.284)	0.509***	0.007 (0.257)
		zFat Mass Index	0.662***	−0.049 (0.084)	0.720***	0.018 (0.114)	0.592***	−0.029 (0.126)
		zHOMA-IR	0.180***	−0.101 (0.091)	0.177**	−0.199 (0.139)	0.212**	0.040 (0.118)
		(zTG − zHDLc)/2	0.065*	−0.225 (0.227)	0.039	−0.372 (0.335)	0.072	−0.008 (0.325)

Models adjusted for: maternal age, maternal body mass index, maternal plasma folate in the corresponding trimester, pregnancy smoking periconceptionally versus never, pregnancy smoking during pregnancy versus never, socioeconomic status, zBMI at childhood check-up, child tHcy. Models were not adjusted for breastfeeding due to the limited sample size.
HDLc high-density lipoprotein cholesterol, *HOMA-IR* homeostatic model assessment of insulin resistance, *TG* triglycerides.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
[a]Lowest (<286.8 pmol/L) versus mid–high tertiles (reference). All ($n = 196$), girls ($n = 105$), boys ($n = 91$).
[b]Unstandardized *B* coefficient.
[c]Standard error.
[d]Lowest (<53.3 pmol/L) versus mid–high tertiles (reference). All ($n = 159$), girls ($n = 82$), boys ($n = 77$).
[e]Highest (≥0.12 µmol/L) versus low–mid tertiles (reference). All ($n = 195$), girls ($n = 105$), boys ($n = 90$).
[f]Lowest (<194.6 pmol/L) versus mid–high tertile (reference). All ($n = 180$), girls ($n = 97$), boys (n = 83).
[g]Lowest (<47.6 pmol/L) versus mid–high tertile (reference). All ($n = 150$), girls ($n = 81$), boys ($n = 69$).
[h]Highest (≥0.16 µmol/L) versus low–mid tertile (reference). All ($n = 180$), girls ($n = 97$), boys ($n = 83$).

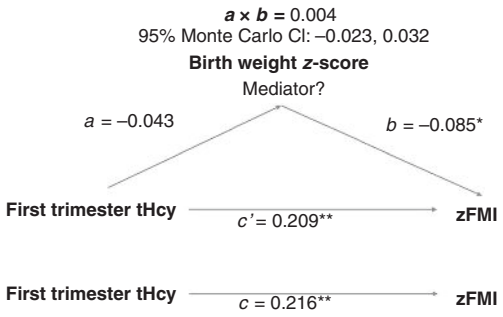

Fig. 2 Mediation analysis[34]: association between first trimester tHcy–child *z*-fat mass index via birth weight *z*-score. *a*, *b*, *c*′, and *c* (linear regression analysis *B* coefficients adjusting for maternal age, BMI, socioeconomic status, smoking (*a*), and birth weight *z*-score, breastfeeding, and child zBMI, tHcy (*b* and *c*′) and excluding birth weight (*c*)). $N = 196$ mother–child dyads. $a \times b$ = indirect effect of tHcy on zFMI via birth weight *z*-score. *$P < 0.05$, **$P < 0.01$.

observed that postpartum maternal folate protected against high BMI *z*-score and probability of overweight or obesity in the offspring. This was especially evident among obese mothers.[16] The association between pregnancy tHcy and insulin resistance in boys agrees with the findings for child postload glucose concentrations, plasma insulin concentrations, and HOMA-IR reported in an Indian study.[11] Maternal cobalamin status was not associated with insulin resistance in the offspring in our study, agreeing with one Indian study[11] but not with another[8] or a Nepalese study.[35] However, we observed that low pregnancy holoTC (fraction of cobalamin bound to trans-cobalamin II for tissue uptake)[36] status was associated with insulin resistance in boys. The observed association between low pregnancy folate status and higher HOMA-IR in the children (specifically girls) agrees with a USA study that reported higher insulin resistance in children born to obese mothers with low folate status.[16] Our results disagree with those from the Indian studies reporting an association between high pregnancy folate status and insulin resistance in the offspring.[8,11]

Folic acid-deficient diets led to increased steatosis in mice (associated with insulin resistance).[37] However, unlike our study where the low pregnancy folate–child insulin resistance association was limited to girls, in the mice the effects were more frequent and severe in males.

The lack of association between maternal tHcy and offspring dyslipidemia agrees with a previous Indian study.[11] On the other hand, pregnancy MMA was positively associated with MetSco and dyslipidemia in boys. The low pregnancy folate status–lower dyslipidemia in childhood (specifically boys) association disagrees with a Dutch study reporting no association between pregnancy folate and child triglycerides.[15] High folic acid diets provoked alterations in hepatocyte lipid metabolism consistent with increased lipogenesis in male mice.[38]

Birth weight was not a mediator of the association between maternal tHcy and child zFMI. A previous study refuted birth weight as a mediator in the association between pre-pregnancy obesity and anthropometric outcomes in children.[39]

Interpretation

Elevated tHcy has been associated with endothelial dysfunction, affecting placental vasculature, and offspring cardiometabolic health.[40] Previously, we reported a greater strain by pregnancy on cobalamin reserves (reflected by higher MMA) in women starting pregnancy with low holoTC status.[13] Here low pregnancy holoTC and high MMA are associated with higher MetSco in boys. High MMA is also associated with dyslipidemia in boys and low holoTC with increased FMI and HOMA-IR. The holoTC and MMA findings suggest that the pregnancy tHcy–child MetSco association may reflect impaired cobalamin status as reported in previous studies.[8,35] When metabolic syndrome develops in adults, anomalies in glucose metabolism have been reported to occur before obesity and dyslipidemia.[41] Low fetal cobalamin supply leading to reduced protein synthesis and increased lipogenesis has been hypothesized to link maternal cobalamin deficiency to increased insulin resistance in the offspring.[8,9] Regarding fat metabolism, animal studies showed that severe hepatic steatosis occurred, secondary to cobalamin deficiency in which elevated MMA inhibits the oxidation of free fatty acids within the liver.[42,43] This is unlikely in our study because cobalamin deficiency was infrequent. However, 1-CM and impaired glucose and adiposity have been linked.[8,9,11] An alternate hypothesis to a role for 1-CM should be considered. However, maternal–child associations (MetSco and its components) were independent of birth weight and maternal BMI, which has been associated with offspring central fat and cardiometabolic risk.[40,44,45]

Low first trimester cobalamin status, according to its indicators, holoTC and MMA, was associated with adverse metabolic outcomes in the child. However, lowest tertile third trimester cobalamin status was associated with lower FMI in boys and lowest tertile folate status with lower dyslipidemia. Cord plasma cobalamin and folate are higher than circulating cobalamin and folate, respectively, in the mother at birth.[12] Low status in plasma concentrations of these nutrients in late pregnancy may reflect placental uptake of the vitamins rather than impaired status.[46] We hypothesize that early pregnancy status in cobalamin is a more accurate reflection of the mother's underlying status in this nutrient than late pregnancy status. This may also be true for folate but would be affected by current trends in early pregnancy folic acid supplement use.

Mostly, the observed pregnancy–offspring outcomes were specific to boys. Male animal[47] and human[48] embryos proliferate to the blastocyst stage at a faster rate than females and sex differences in gene expression in preimplantation embryos occur.[49] Male preimplantation embryos are more responsive to intrauterine undernutrition than females.[50] Also, placenta genes are differentially expressed in male and female mice on different folic acid supplementation regimes.[51] Adult hepatocyte phosphatidylethanolamine *N*-methyltransferase differs between sexes[52] and sex differences in other 1-CM enzymes have been described in mice.[53] In animal studies, dietary restrictions in 1-CM nutrients during pregnancy led to genome-wide epigenetic modifications in offspring DNA methylation. More than half of the affected loci were specific to males and stronger effects were observed for insulin resistance, adiposity, altered immune function, and high blood pressure in males than in females.[54] Glucose tolerance in the female rat offspring was unaffected by restricted diets but insulin was higher in males born to pregnant rat dams fed similar diets.[55]

Further investigation is required to determine whether similar maternal–offspring associations occur in girls but may be masked by the physiological factors that drive differences in FMI between girls and boys from 3 years onwards.[56]

Strengths and limitations

This study collected data prospectively from early pregnancy until mid-childhood in mother–child dyads unexposed to mandatory fortification with folic acid and with a low prevalence of cobalamin deficiency.

The cohorts were recruited before (PreC) and after (RTBC) periconceptional supplementation with folic acid recommendations were implemented. Nevertheless, they were from the same hospitals, samples were collected and processed using identical protocols, and all folate and cobalamin status determinations were by the same methods. The cohorts were combined to improve statistical power. Sensitivity analysis confirmed that the reported

associations occurred when the RTBC mother–child dyads were analyzed alone (not shown).

WC or waist-to-height ratio are recommended for total body fat assessment.[56] However, FMI can also be used[56] and skinfold measurements are better alternatives to WC and BMI[57] and predict obesity well[58] in children and adolescents. WC is unavailable for the PreC cohort, but we confirmed the association between pregnancy tHcy and offspring body fat using WC *z*-score in RTBC (data not shown).

We assessed overweight–obesity using Spanish tables[32] because the participants were almost exclusively Spanish. By using the international obesity task force tables,[59] the prevalence of overweight–obesity in our population was higher (23.7 versus 20.2%). We considered the use of the Spanish tables appropriate because we use population-specific curves to determine birth weight and BMI *z*-scores and the aim of the study was to investigate maternal–offspring outcomes and not to compare prevalence between different countries.

Residual confounding from factors not considered in our models may occur. However, our models were controlled for numerous maternal and child factors that influence offspring growth.[60]

CONCLUSIONS

Moderately elevated pregnancy tHcy and low holoTC status were positively associated with MetSco, zFMI, and zHOMA-IR in boys. High pregnancy MMA was also positively associated with MetSco and dyslipidemia in boys. The association between pregnancy tHcy and child zFMI was not mediated by birth weight.

DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are not publicly available because participant consent covers data exploration in response to hypothesis testing within a defined field and with the compromise that this will be vetted by the Principal Investigator (M.M.M.). The corresponding author (M.M.M.) is willing to provide the data to interested parties on reasonable request and agreement that it will be exploited under the terms of participant consent and following further approval by the Ethics Committees if required.

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AUTHOR CONTRIBUTIONS

A.R.-G. and M.M.M. conceptualized and designed the study, the data collection instruments, collected data, analyzed data, drafted the initial manuscript, and reviewed and revised the manuscript. J.F.-B., P.C.-B., P.S.-N., G.O.-M., M.B., C.G., and C.R.-R. participated in the conceptualization and design of the study, designed the data collection instruments, collected data, and reviewed and revised the manuscript. P.M.U. and L.M. participated in the conceptualization and design of the study and critically reviewed the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Signed informed consent to participate in the study was obtained from all participants, from either parent on behalf of the children and verbal assent from the children.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41390-022-02117-5.

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12 semanas

Estudio NUTCIR 1

Nombre..... ***Fecha .... …***

CUESTIONARIO DE FRECUENCIA DE CONSUMO ALIMENTARIO 1

INSTRUCCIONES PARA CONTESTAR

Procure contestar tranquilamente este cuestionario. Tómese el tiempo que considere necesario.

Este cuestionario le pregunta la frecuencia con que usted consumía de forma **habitual** determinados alimentos antes del embarazo.

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)		

	CUANTAS VECES COME…?	
	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…)		

	¿CUÁNTAS VECES...?	
	A LA SEMANA	AL MES
Come en fiambrera de plástico o “Tupper”		
Calienta la comida en fiambrera de plástico o “Tupper”		
Consume alimentos en Tetrabrik o envase de plástico (Zumos, leche, sopas, batidos…)		
Consume pescado /marisco en lata (atún, sardinas, berberechos, mejillones....)		
Consume alimentos en lata tipo conserva (lentejas, fabada,....)		
Consume alimentos envasados en plástico (fruta envasada, carne o pescado en bandejas…)		
Come comida rápida (Fast-food)		

Nom: Data:

ENCUESTA 1 SOBRE HÁBITOS Y ESTILO DE VIDA
(referida a la primera mitad del embarazo)

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** ☐
- ¿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 fuma	☐	☐	☐	☐
Fumaba durante los 12 meses antes del embarazo	☐	☐	☐	☐

	No	Antes de los 3 meses	Entre los 3 y los 6 meses	Después de los 6 meses
Ha dejado de fumar durante el embarazo	☐	☐	☐	☐

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	☐	☐	☐	☐

	No	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	☐	☐	☐	☐

PESO, ALTURA, EDAD, ORIGEN Y PARTICIPACIÓN EN ESTUDIOS

	Antes del embarazo	Última vez que se pesó antes de realizar la entrevista (fecha; SG)
Peso:	. Kg	. Kg (/ / ; SG)

Altura: . m
Fecha de nacimiento:
Participación en otros estudios:

Origen padres:
Origen abuelos:

Nom: Data:

SUSTANCIAS TÓXICAS

- ¿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: _____

	No	Ocasionalmente	Regularmente
Actualmente toma sustancias tóxicas	☐	☐	☐
En los 12 meses antes del embarazo tomaba sustancias tóxicas	☐	☐	☐

	No	Antes de los 3 meses	Entre los 3 y los 6 meses	Después de los 6 meses
Lo ha dejado durante el embarazo	☐	☐	☐	☐

ACTIVIDAD FÍSICA (durante el embarazo)

- ¿Qué actividad física hace en el trabajo, estudio o trabajo de casa?

Horas/semana

- 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? (anotar la prioritaria si dos actividades coinciden en horas)

Horas/semana

- 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, gimnasia, juegos de pelota o deportes vigorosos regularmente..... ☐ _____

- Entrenamiento deportivo regular para competición ..... ☐ _____

- Durante los últimos 12 meses

	Nunca	Esporádicamente	Habitualmente
¿Ha tenido la costumbre de tomar el Sol?	☐	☐	☐

Nom: Data:

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 orales antes del embarazo? _____
(Número de reglas desde que dejó de tomar anticonceptivos hasta que se quedó embarazada)

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 €
☐	☐	☐	☐	☐

Anote cualquier duda relacionada con esta encuesta:

UNIVERSITAT ROVIRA I VIRGILI

Reus & Tarragona Birth Cohort

Unitat de Medicina Preventiva i Salut Pública

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Data de entrevista (d d m m a a)

Hora antropometria

Data de naixement (d d m m a a)

Pes (kg)		
Alçada (cm)		
Circumferència cranial(cm)		
Circumferència coll (cm)		
Circumferència mitjana del braç(cm)		
Circumferència del pit (cm)		
Circumferència de la cintura (WHO) (cm)		
Circumferència de la cintura (CDC) (cm)		
Circumferència malucs (cm)		
Circumferència de la cuixa (cm)		

Plec tricipital (mm)			
Plec subescapular (mm)			
Plec bicipital (mm)			
Plec suprailíac (mm)			
Plec de la cuixa (mm)			

Pressió arterial Sistòlica (mmHg)			
Pressió arterial Diastòlica (mmHg)			

Informació impedància bioelèctrica

Ha ingerit líquids avui? ○ Si ○ No

Quantes hores fa de l'últim cop?

Ha orinat aquest matí? ○ Si ○ No

Ha realitzat activitat física avui? ○ Si ○ No

Quantes hores fa?

A quina resistència? _____kHz

Només en casos excepcionals es realitzarà l'antropometria si el nen NO està en dejú.

Está en dejú? ○ Si ○ No

ID ☐☐☐

Iniciales ☐☐☐

Genero ☐

Centro ☐

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Fecha entrevista ☐☐ ☐☐ ☐☐
d d m m a a

Fecha nacimiento ☐☐ ☐☐ ☐☐
d d m m a a

Historia clínica

1. ¿Quién responde la Historia Clínica?

☐ Madre
☐ Padre
☐ Otro familiar. Especificar
☐ Tutor/a Responsable no familiar
☐ Otros. Especificar

2.

	Si	No	Motivo
Extracción sanguínea			
Carnet de Salud			
BIA			
Cuaderno de actividad física			
MAPA			
Diario de actividad física			
Desarrollo Neurológico 1			
Neurodesarrollo 2			
Espirometria			
Diente			

3. ¿Ha estado diagnosticado de alguna enfermedad o malformación congénita?

_____

4. ¿Ha estado diagnosticado de alguna enfermedad crónica?

_____

5. ¿Ha estado hospitalizado por algún motivo?

○ No ○ Sí Especificar: _____

Edad durante la hospitalización (años)_____

6. ¿Se le ha realizado algún tipo de cirugía?

○ No ○ Sí

Tipo y fecha _____

7. ¿Se alimentó el/la niño/a con leche materna?

○ No ○ Sí

¿Durante cuantos meses de manera exclusiva? (nº de meses) _____
¿Durante cuantos meses en total? _____

8. ¿Qué edad tenía cuando se introdujeron alimentos sólidos a su dieta? (nº de meses) _____

9. ¿Su hijo/a tiene problemas visuales?

○ No ○ Sí ¿Necesita llevar gafas? _____

10. ¿Su hijo/a tiene problemas auditivos?

○ No ○ Sí

11. Ha estado diagnosticado/a o ha tenido en algún momento:

	No	Sí	Antes de los 2 años	Entre 2 y 5 años	Pasados los 5 años	Nº de veces en los últimos 12 meses
Resfriado con fiebre	○	○	○	○	○	
Infección de oído	○	○	○	○	○	
Infección de garganta	○	○	○	○	○	
Neumonía	○	○	○	○	○	
Bronquitis	○	○	○	○	○	
Bronquiolitis	○	○	○	○	○	
Gastroenteritis	○	○	○	○	○	
Anemia	○	○	○	○	○	
Caries	○	○	○	○	○	
Sarampión	○	○	○	○	○	
Varicela	○	○	○	○	○	
Rubéola	○	○	○	○	○	
Otras. Especificar:	○	○	○	○	○	

12. ¿Tiene alguna alergia alimentaria?

	Sí	No
- Leche de vaca	○	○
- Huevos de gallina	○	○
- Soja	○	○
- Cacahuetes	○	○
- Frutos Secos	○	○
- Trigo	○	○
- Pescado	○	○
- Marisco	○	○
- Otros:	○	○

13. ¿Ha disminuido o suprimido el consumo de algún alimento de la dieta del niño/a debido a la alergia?

○ No ○ Sí Cual/es? _____

¿Ha sustituido este/os alimento/s por algún otro? _____

14. ¿Durante el último año ha estado tomando algún medicamento?

○ No ○ Sí ¿Cual/es? _____

15. En la actualidad, ¿está tomando algún medicamento?

○ No ○ Sí ¿Cual/es? _____

16. Algún familiar directo ha estado diagnosticado de algún trastorno psicopatológico (ejemplos trastorno psicótico, depresión, trastorno de ansiedad, retraso mental, trastorno por déficit de atención con hiperactividad, consumo de tóxicos, trastorno de la personalidad)?

○ No ○ Sí ¿Quién? _____ ¿Qué tipo de trastorno? _____

Cuestionario básico de sibilancias

a. ¿Ha tenido alguna vez sibilancias o silbidos en el pecho durante cualquier momento del pasado? ○ Sí ○ No

SI SU RESPUESTA ES "NO" PASE A LA PREGUNTA F

b. ¿Ha tenido alguna vez sibilancias o silbidos en el pecho en los últimos 12 meses?

○ Sí ○ No

SI SU RESPUESTA ES "NO" PASE A LA PREGUNTA F

c. Cuantos ataques de sibilancias ha tenido vuestro/a hijo/a en los últimos 12 meses?

○ Ninguno ○ 1 a 3 ○ 4 a 12 ○ Más de 12

d. En los últimos 12 meses, cuantas veces, de promedio, el sueño de vuestro/a hijo/a ha sido perturbado a causa de las sibilancias?

○ Nunca se ha despertado con sibilancias

○ Menos de una noche por semana

○ Una noche o más noches por semana

e. En los últimos 12 meses, ¿las sibilancias han sido lo suficientemente severas como para limitar el habla del niño/a únicamente una o dos palabras entre respiraciones?

○ Sí ○ No

f. ¿Vuestro/a hijo/a ha tenido alguna vez asma? ○ Sí ○ No

g. En los últimos 12 meses, ¿el tórax de vuestro/a hijo/a ha sonado sibilino durante o después de la realización de ejercicio físico? ○ Sí ○ No

h. En los últimos 12 meses, vuestro/a hijo/a ha tenido tos seca por las noches, sin haber estado resfriado o con una infección de tórax? ○ Sí ○ No

Cuestionario básico de rinitis

a. ¿Ha tenido alguna vez estornudos, la nariz "moqueaba" o estaba tapado/a sin tener la gripe ni estar resfriado? Sí ○ ○ No

SI SU RESPUESTA ES "NO" PASE A LA PREGUNTA F

b. En los últimos 12 meses, ha tenido alguna vez estornudos, la nariz "moqueaba" o estaba tapado/a sin tener la gripe ni estar resfriado? ○ Si ○ No

ID:
Iniciales:

SI SU RESPUESTA ES "NO" PASE A LA PREGUNTA F

c. ¿En los últimos 12 meses, estos problemas en la nariz han ido acompañados de picor en los ojos u ojos llorosos? ○ Sí ○ No

d. ¿En cuál de los últimos 12 meses se dieron estos problemas en la nariz?

○ Enero ○ Febrero ○ Marzo ○ Abril ○ Mayo ○ Junio ○ Julio
○ Agosto ○ Septiembre ○ Octubre ○ Noviembre ○ Diciembre

e. ¿En los últimos 12 meses, cuantas veces estos problemas de nariz han interferido como las actividades diarias de vuestro/a hijo/a?

○ Una vez ○ Un poco ○ Unas cuantas veces ○ Muchas veces

f. ¿Vuestro/a hijo/a ha tenido alguna vez alergia al polen? ○ Sí ○ No

<u>Cuestionario básico de eczema</u>

a. ¿Ha tenido alguna erupción cutánea con picores que aparecen y desaparecen al menos durante 6 meses? ○ Sí ○ No

SI SU RESPUESTA ES "NO" PASE A LA PREGUNTA G

b. ¿Ha tenido alguna erupción cutánea con picores en algún momento durante los últimos 12 meses? ○ Sí ○ No

SI SU RESPUESTA ES "NO" PASE A LA PREGUNTA G

c. Esta erupción cutánea con picores ha afectado alguna de las siguientes zonas del cuerpo: ¿los pliegues de los codos, detrás de las rodillas, delante de los tobillos, debajo las nalgas o alrededor del cuello, orejas u ojos? ○ Sí ○ No

d. ¿A qué edad surgió la primera erupción cutánea con picores?

○ Antes de los 2 años ○ Entre 2-4 años ○ 5 años o más

e. ¿Esta erupción se clareó completamente en cualquier momento durante los últimos 12 meses? ○ Sí ○ No

f. En los últimos 12 meses, ¿cuantas veces de promedio, su hijo/a se ha quedado despierto durante la noche a causa de esta erupción?

○ Nunca en los últimos 12 meses ○ Menos de una noche por semana
○ Una o más noches por semana

g. ¿Ha tenido alguna vez un eczema? ○ Sí ○ No

ID:
Iniciales:

Carnet de Salud

Crecimiento

Fecha	Peso (g)	Talla (cm)

Calendario de vacunaciones

	DTPa	Poliomelitis	Haemophilus influenza tipo B	Meningitis C conjugada	XRP	Hepatitis B
2m	✓	✓	✓	✓		✓
4m	✓	✓	✓			✓
6m	✓	✓	✓	✓		✓
12m					✓	
15m				✓		
18m	✓	✓	✓			
4a					✓	
4-6a	✓					

ID:
Iniciales:

Verificar información embarazada

17. Hábito tabáquico durante el embarazo

¿Era fumadora activa 3 meses antes de quedarse embarazada?

○ No ○ Sí ¿Mantuvo el hábito durante todo el embarazo? _____

○ No ○ Sí Si no, ¿cuándo lo dejó? _____

¿Estuvo en contacto con alguna persona que fumara delante suyo durante el embarazo?

○ No ○ Sí ¿Durante cuantas horas diarias aproximadamente? _____

18. Hábito tabáquico en el entorno del niño/a

¿La madre fuma actualmente o ha fumado a lo largo de la vida del niño/a?

○ No ○ Sí

Dentro de casa, ¿en qué sitio se suele fumar?

Si ha dejado de fumar, ¿edad del niño/a? _____

○ Fuera de casa
○ En casa pero sin estar delante del niño/a
○ Delante del niño/a
○ Otros

	0 cigs/día	1-5 cigs/día	6-10 cigs/día	> 10 cigs/día
¿Cuántos cigarrillos al día fuma?	○	○	○	○

19. ¿El padre fuma actualmente o ha fumado a lo largo de la vida del niño/a?

○ No ○ Sí

Dentro de casa, ¿en qué sitio se suele fumar?

Si ha dejado de fumar, ¿edad del niño/a? _____

○ Fuera de casa
○ En casa pero sin estar delante del niño/a
○ Delante del niño/a
○ Otros

	0 cigs/día	1-5 cigs/día	6-10 cigs/día	> 10 cigs/día
¿Cuántos cigarrillos al día fuma?	○	○	○	○

20. ¿Otras personas del entorno del niño/a actualmente fuman o han fumado a lo largo de la vida del niño/a?

○ No ○ Sí

Dentro de casa, ¿en qué sitio se suele fumar?

Si ha dejado de fumar, ¿edad del niño/a? _____

○ Fuera de casa
○ En casa pero sin estar delante del niño/a
○ Delante del niño/a
○ Otros

	0 cigs/día	1-5 cigs/día	6-10 cigs/día	> 10 cigs/día
¿Cuántos cigarrillos al día fuma?	○	○	○	○

ID:
Iniciales:

Datos sociodemográficos

21. ¿Cuál es su trabajo actual y que nivel de estudios máximos ha completado?

	Madre	Padre
Trabajo actual/ últimos 12 meses		
Nivel de estudios	○ Sin estudios	○ Sin estudios
	○ Primaria incompleta	○ Primaria incompleta
	○ Primaria (ESO, EGB)	○ Primaria (ESO, EGB)
	○ EGB, bachillerato elemental	○ EGB, bachillerato elemental
	○ Formación profesional I o II	○ Formación profesional I o II
	○ BUP, bachillerato superior	○ BUP, bachillerato superior
	○ COU, PREU	○ COU, PREU
	○ Estudios universitarios de grado medio (Diplomatura)	○ Estudios universitarios de grado medio (Diplomatura)
	○ Estudios universitarios de grado superior (licenciatura, máster, doctorado)	○ Estudios universitarios de grado superior (licenciatura, máster, doctorado)

22. ¿Cuántas personas forman la unidad familiar? _____

23. Ingresos netos anuales totales en casa

Ejemplo: si la mujer tenía un sueldo de 20000€, el marido uno de 18000€ y había un abuelo que vivía con la familia y recibía una pensión de 6000€

< 9000 €	>9000 € - 19000 €	>19000 € - 25000 €	>25000 € - 35000 €	> 35000 €
○	○	○	○	◉

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

ID:
Iniciales:

ID ☐☐☐ **TP-90**

Inicials ☐☐☐☐

Centre ☐

Data de compleció ☐☐ ☐☐ ☐☐
d d m m a a

(A omplir per l'investigador)

Reus & Tarragona Birth Cohort

Cuestionario de actividad física y desarrollo

Este cuaderno lo tiene que completar las madres i/o padres, o bien el tutor legal con la ayuda del niño. Las preguntas hacen referencia a la actividad física, i al desarrollo de vuestro hijo.

Las respuestas que figuren dentro de este cuaderno serán confidenciales y nunca estarán ligadas al nombre del niño.

Muchas gracias por su colaboración.

UNIVERSITAT ROVIRA I VIRGILI

Cuestionario de actividad física

Queremos conocer el nivel de actividad física de su hijo durante los últimos 7 días (la semana anterior). Esto incluye deportes o bailes que le hagan sudar o que provoquen que sienta las piernas cansadas, o bien juegos que le hacen respirar rápidamente como pilla-pilla, saltar a la comba, correr, escalar u otros.

Recuerden:

1. No hay respuestas correctas o incorrectas – No se trata de ningún examen.
2. Por favor, respondan todas las preguntas tan honesta i precisamente como puedan.

1. Actividad física durante el tiempo libre: ¿Su hijo ha realizado alguna de las siguientes actividades en los **últimos 7 días** (semana pasada)? Si la respuesta es SI, ¿cuántas veces? (Marquen un solo circulo por fila).

	No	1-2	3-4	5-6	7 veces o más
Saltar a la cuerda	○	○	○	○	○
Patinar	○	○	○	○	○
Juegos: Tocar i parar, ...	○	○	○	○	○
Caminar (ejercicio)	○	○	○	○	○
Bicicleta	○	○	○	○	○
Correr/ Footing	○	○	○	○	○
Natación	○	○	○	○	○
Bailar	○	○	○	○	○
Montar en monopatín	○	○	○	○	○
Futbol	○	○	○	○	○
Hockey sobre césped.....	○	○	○	○	○
Hockey sobre patines	○	○	○	○	○
Básquet	○	○	○	○	○
Patinaje artístico	○	○	○	○	○
Esquí	○	○	○	○	○
Hockey sobre hielo.....	○	○	○	○	○
Otros:					

_____ ○ ○ ○ ○ ○
_____ ○ ○ ○ ○ ○

2. En los **últimos 7 días**, en las clases de Educación Física, ¿cuántas veces estuvo muy activo (jugando intensamente, corriendo, saltando, haciendo lanzamientos)? (Marquen una sola respuesta.)

No hace educación física ○
Prácticamente nunca ○
Alguna vez ○
A menudo ○
Siempre ○

3. En los **últimos 7 días**, ¿que hacia la mayoría de veces a la hora del patio? (Marquen una sola respuesta.)

Estar sentado (hablar, leer, hacer los deberes) ○
Pasear ○
Correr o jugar un poco ○
Correr o jugar bastante ○
Correr o jugar casi siempre ○

4. En los **últimos 7 días**, ¿que hacia la mayoría de las veces en la hora de comer? (a parte de comer) (Marquen una sola respuesta.)

Estar sentado (hablar, leer, hacer los deberes) ○
Pasear ○
Correr o jugar un poco ○
Correr o jugar bastante ○
Correr o jugar casi siempre ○

5. En los últimos 7 días, inmediatamente después de la escuela hasta las 6, ¿cuántos días jugó a algún juego, hizo deporte o bailes en los que estuviera muy activo? (Señale sólo una)

- Ninguna
- Una vez en la última semana
- 2 o 3 veces en la última semana
- 4 veces en la última semana
- 5 veces en la última semana

6. En los últimos 7 días, cuántos días a partir de media tarde (entre las 6 y las 10) hizo deportes, baile o jugó a juegos en los que estuviera muy activo? (Señale sólo una)

- Ninguna
- Una vez en la última semana
- 2 o 3 veces en la última semana
- 4 veces en la última semana
- 5 veces en la última semana

7. En e**l último fin de semana**, ¿cuántas veces practicó deporte, baile (danza) o jugó a juegos en los cuales estaba muy activo? (Marquen una sola respuesta.)

- Ninguna
- Una vez en la última semana
- 2 o 3 veces en la última semana
- 4 veces en la última semana
- 5 veces en la última semana

8. ¿Cuál de las siguientes afirmaciones le describen mejor en los **últimos 7 días**? Lean las 5 afirmaciones antes de decidir qué respuesta la describe.

a. Todo, o prácticamente su tiempo libre lo dedicó a actividades que requieren poco esfuerzo físico ○

b. Alguna vez (1-2 veces) ha practicado actividad física en su tiempo libre (Ej.: hacer deporte, correr, nadar, bicicleta o aeróbic) ○

c. A menudo (3-4 veces) ha practicado actividad física en su tiempo libre ○

d. Bastante a menudo (5-6 veces) ha practicado actividad física en su tiempo libre ○

e. Muy a menudo (7 o mas veces) ha practicado actividad física en su tiempo libre ○

9. Señala cuántas veces ha realizado alguna actividad física (tales como deporte, juegos, bailar o cualquier otra actividad física) para cada día durante la **última semana**.

	Ninguna (0)	Poca (1)	Normal (2)	Bastante (3)	Mucha (4)
Lunes	○	○	○	○	○
Martes	○	○	○	○	○
Miércoles	○	○	○	○	○
Jueves	○	○	○	○	○
Viernes	○	○	○	○	○
Sábado	○	○	○	○	○
Domingo	○	○	○	○	○

10. ¿Ha estado enfermo durante ésta última semana, o alguna cosa le ha impedido practicar alguna actividad física? (Marquen una casilla.)

Si ○
No ○

Si la respuesta es si, ¿qué lo ha impedido?

11. ¿Cómo acostumbra a ir el niño a la escuela?

- Bicicleta ○
- Coche/ moto ○
- Andando ○
- Autobús ○

12. ¿Cuánto tarda en llegar a la escuela?

- 1 a 5 minutos ○
- 6 a 10 minutos ○
- 11 a 15 minutos ○
- Mas de 15 minutos ○

13. Piensen en una **semana habitual.** Indiquen cuántas horas al día el niño realiza las acciones siguientes:

	Entre semana (media de los cinco días)	Fines de semana (media de los dos días)
Mirar la televisión (DVD, vídeos i películas al ordenador)		
Jugar al ordenador (consolas,..)		
Hacer deberes (sin ordenador)		
Dormir		
Leer		

14. ¿Tiene televisión en su habitación?

- Si ○
- No ○

Desarrollo físico

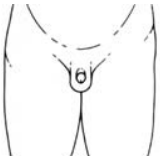
Nos gustaría evaluar el desarrollo físico de vuestro hijo usando las figuras que se representan en esta página.
Éstas indican estadios de pubertad comúnmente usados por médicos para evaluar el desarrollo y crecimiento de los niños.

Los niños pasan por distintos estadios de desarrollo físico en diferentes edades. Algunos de ellos empiezan tan pronto como a los 6 años, y otros no lo hacen hasta los 16.

Las figuras siguientes muestran distintas cantidades de vello púbico masculino.
Los niños pueden pasar por cada uno de los diferentes estadios representados.

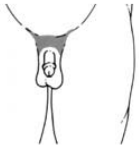
Por favor, miren atentamente cada una de las diferentes figuras. Es importante que lean también las descripciones.

Señalad la casilla que más concuerde con el estadio de vuestro hijo.



○ Ligera vellosidad infantil.

○ Pelo escaso, liso y ligeramente de color oscuro, usualmente arraigado al penis.



○ Pelo rizado, escasamente desarrollado, pero oscuro, y arraigado al penis.

○ No estoy seguro.

Monitoreo Ambulatorio de la Presión Arterial - MAPA

Se trata de una técnica no invasiva que obtiene medidas de la presión arterial durante 24 horas.

El uso del MAPA no tiene que provocar ninguna alteración del estilo de vida de la niña, y le tiene que permitir realizar todas las actividades cotidianas de manera habitual.

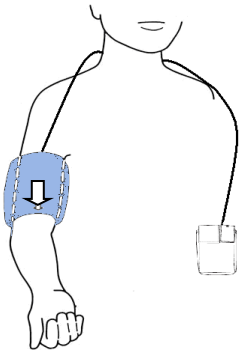
Se debe mantener especial cuidado en el mantenimiento del MAPA, y procurar que no quede fuera de la bandolera.

Para apagarlo, colocad la pestaña de la parte superior en el "0", y para encender, moverla hacia el otro lado. Siempre que el MAPA esté en el brazo, tiene que permanecer encendido.

Qué hago si...

... el brazalete se mueve de sitio?

La flecha de color blanco debe quedar a unos 2 cm por encima del pliegue del brazo, y señalando al punto medio tal y como se muestra en el dibujo.



...el MAPA presiona muy fuerte a la hora de tomar la medida?

Será necesario aflojar el brazalete, sacando el "velcro" y colocándolo de manera que no presione demasiado, pero que no quede móvil.

Para cualquier duda, o problema que surja, 626886314.

¡¡¡Muchas gracias por participar!!!

Unidad de Medicina Preventiva i Salud Pública

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

Alejandra Rojas-Gomez[1,2], Julia Haro-Barcelo[1,2], Gemma Ornosa-Martin[1,2], Pol Sole-Navais[1,2], Monica Ballesteros[3], Montserrat Ingles-Puig[4], Per M. Ueland[5], Klaus Meyer [6], Joan D Fernandez-Ballart[1,2], Pere Cavallé-Busquets[2,4] and Michelle M Murphy [1,2]

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Background: There is increasing evidence that research into healthy ageing should start from very early life because plasticity is most prominent at the time of cell differentiation. It is not well understood how prenatal micronutrient balance within the 1C metabolic network is associated with metabolic outcomes in children.

Aims: To investigate the transgenerational association between maternal 1C metabolism (1CM) in the 1st trimester of pregnancy and fasting plasma glucose, HOMA-IR and atherogenic index in children aged 7.5 years.

Methods: 212 children from the Reus Tarragona Birth cohort participated at follow up aged 7.5 years. Mothers were recruited before 12 gestational weeks (GW) and extensive lifestyle and clinical data as well as fasting blood samples at <12 GW were collected. Plasma folate and cobalamin concentrations were determined by microbiological assays and *MTHFR* 677C>T and *TCII* 776C>G genotypes were determined. Fasting blood samples were collected from 158 children. They also had a health check-up. Metabolic profile including plasma insulin (solid phase two-site enzyme immunoassay), glucose (glucose oxidase) and atherogenic index from total cholesterol/ HDL cholesterol (enzymatic colorimetric techniques), were determined as well as HOMA-IR. Child physical activity was assessed by questionnaires completed by the parents.

The probabilities of high glucose, HOMA-IR or atherogenic index in the children associated with low 1st trimester B12 status and genetic maternal factors were assessed by multiple logistic regression analysis (MLRA) adjusting for maternal characteristics (BMI, 1st trimester plasma folate and B12 status, smoking during pregnancy and *MTHFR* C677T or *TCII* C776G genotype) and subsequently for child variables (birthweight percentile, breastfeeding and level of physical activity). Only results from significant models are reported.

Results

42/158 children had high plasma glucose (5.29 mmol/L for girls; 5.44 mmol/L for boys). None of the variables studies were associated with fasting plasma glucose. 15/158 children had HOMA-IR above P90 (1.87 for girls; 1.61 for boys). 1st trimester plasma B12 in the 1st tertile (<319.5 pmol/L), compared to the 2nd and 3rd tertiles, was associated with increased probability of high HOMA-IR in the child (OR: 3.9, CI: 1.0,15.2). 15/158 children had an atherogenic index above P90 (3.4 for girls; 3.3 for boys). No association between maternal 1CM parameters and child atherogenic index was observed.

Conclusions: Low B12 maternal status in the 1st trimester was associated with high HOMA-IR in the children.

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

Alejandra Rojas-Gomez[1,2], Julia Haro-Barcelo[1,2], Carla Ramos-Rodríguez[1,2], Gemma Ornosa-Martin[1,2], Pol Sole-Navais[1,2], Monica Ballesteros[2,3], Montserrat Ingles-Puig[2,4], Per M Ueland [5], Luis Masana[2,6], Mercedes Heras[2,6], Joan D Fernandez-Ballart[1,2,7], Pere Cavallé-Busquets[2,4,7] and Michelle M Murphy [1,2,7].

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Background
Moderately elevated fasting plasma total homocysteine (tHcy) during pregnancy has been associated with low birth weight in the offspring but studies investigating its association with child health are scarce.
Aims
To study the association between maternal tHcy in the first trimester of pregnancy and metabolic score in children aged 6-8 years.
Methods
A total of 295 mother-child dyads from the Reus Tarragona Birth Cohort (RTBC) and the preconception (PRE-C) cohort were followed from the first trimester of pregnancy until the children were aged 6-8 years. Pregnancy was confirmed by ultrasound scan and fasting blood samples collected from the mothers before 12 gestational weeks. 241 children agreed to provide fasting blood samples at follow up. A modified metabolic score (MetSco), from the Identification and prevention of Dietary- and lifestyle-induced health EFfects In Children and infantS cohort (Ahrens et al., Int J Obes (Lond)., 2014) was determined as follows:

> zFMI+((zTG–zHDL)/2)+zHOMA-IR (FMI: fat mass index; TG: triglycerides; HDL cholesterol; HOMA-IR homeostasis model assessment for insulin resistance).

Multiple logistic regression analysis was used to study the association between high tertile 1st trimester plasma tHcy (RTBC ≥ 5.7, PRE-C ≥7.1µmol/L) and metabolic score above P90 in children (≥ 2.7 in girls; ≥1.9 in boys). The first model was adjusted for maternal characteristics (BMI, socioeconomic status, smoking during pregnancy: first trimester only versus never and throughout pregnancy versus never). This model was then further adjusted for child characteristics (birthweight z-score, breastfeeding and BMI z-score), model 2. The models were then repeated without the women who smoked throughout pregnancy. The association between prenatal tHcy and metabolic score in the child was also tested using multiple linear regression, followed by mediation analysis to see whether the tHcy-metabolic score association was mediated by birthweight z-score (PROCESS software).
Results
In women that did not smoke (n=167) or only smoked in the 1st trimester (n=15), high tertile tHcy was associated with a higher probability of elevated MetSco in the child (OR: 3.94, CI: 1.3, 12.1 [model 1]; OR: 4.5, CI: 1.0, 21.7 [model 2]). The association was not mediated by birthweight z-score. When women that smoked for the duration of pregnancy (n=29) were included in the analysis, the significance of the association between prenatal tHcy and metabolic score in the children was lost in both models.
Conclusions
High tHcy maternal status in the 1st trimester was associated with increased probability of high MetSco in children but the association was not mediated by birthweight.

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Associations between pregnancy homocysteine and cobalamin status and metabolic score in the offspring.

Alejandra Rojas-Gómez[1,2], Carla Ramos-Rodríguez[1,2], Pere Cavallé-Busquets[2,3,4], Monica Ballesteros[2,5], Per M Ueland[6], Luis Masana[2,7], Mercedes Heras[2,7], Joan D Fernandez-Ballart[1,2,4], and Michelle M Murphy [1,2,4].

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Background

Moderately elevated plasma total homocysteine (tHcy) during pregnancy has been associated with low birth weight but studies investigating its association with child health are scarce.

Aims

To study the association between pregnancy 1st and 3rd trimester tHcy, plasma B12, holoTC and metabolic score in the offspring.

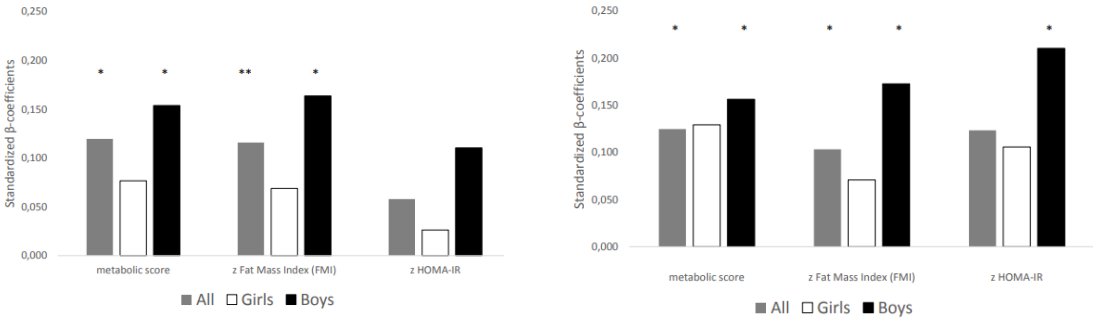
Methods

Mother-child dyads from the Preconception (PreC, n = 79) and Reus-Tarragona Birth (RTBC, n = 210) cohorts were followed from early pregnancy until the children were aged 6-8 years. A modified metabolic score (MetSco) from the IDEFICS cohort was calculated: zFMI+((zTG–zHDL)/2)+zHOMA-IR (FMI: fat mass index; TG: triglycerides; HDL cholesterol; HOMA-IR homeostasis model assessment for insulin resistance). A higher MetSco suggests higher metabolic risk.

Multiple linear regression analysis (MLRA) was used to study the association between maternal tHcy, B12 and HoloTC status and child MetSco, adjusting for maternal and child characteristics. High tHcy tertile (1st trimester, ≥7.1µmol/L in PreC, ≥ 5.7,in RTBC and 3rd trimester, ≥ 5.7 umol/L, both cohorts). Low B12 tertile (1st trimester, <286.8 pmol/L; 3rd trimester, <194.6 pmol/L). Low holoTC tertile (1st trimester, <53.3 pmol/L; 3rd trimester, <47.6 pmol/L).

Results

Pregnancy tHcy was positively associated with child MetSco as shown in the figure.



*Association between 1st (left) and 3rd (right) trimester tHcy (high vs mid-low tertiles), and child outcomes. Standardized B-coefficients are reported. *p <0.05, **p <0.01. Model for ((zTG–zHDL)/2) was not significant (not shown).*

Maternal 1st trimester lowest vs mid-high holoTC tertiles was associated with MetSco in boys (p=0.005), zFMI (p=0.017) and zHOMA-IR (p=0.044). Maternal B12 was not associated with child outcomes.

Conclusions

High tHcy and low holoTC during pregnancy were associated with higher MetSco in children.

Funding

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