PRENATAL ONE CARBON METABOLISM AND IN UTERO PROGRAMMING OF GROWTH AND ADIPOSITY IN THE OFFSPRING

Pol Solé Navais

Pol Solé Navais

Prenatal one carbon metabolism and *in utero* programming of growth and adiposity in the offspring

Doctoral thesis

Thesis supervised by Dr. Michelle Murphy,

and co-supervised by Prof. Joan D Fernández-Ballart.

Department of Basic Medical Sciences

UNIVERSITAT ROVIRA i VIRGILI

Reus

2016

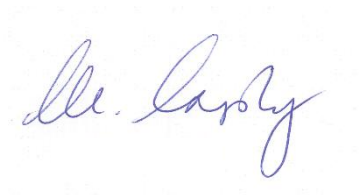
UNIVERSITAT ROVIRA i VIRGILI

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

Michelle Murphy, "Professora Agregat", at the Departament of Basic Medical Sciences of the Universitat Rovira i Virgili,

CERTIFIES

That the thesis, entitled "*Prenatal one carbon metabolism and in utero programming of growth and adiposity in the offspring*" by Pol Solé Navais, was carried out under my supervision in the Department of Basic Medical Sciences of the Universitat Rovira i Virgili, and fullfils the requirements to obtain the title of Doctor.



Reus, 5th May 2016

Dr. Michelle Murphy

UNIVERSITAT ROVIRA i VIRGILI

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

Joan D Fernández-Ballart, "Catedràtic d'Universitat", Departament of Basic Medical Sciences of the Universitat Rovira i Virgili,

CERTIFY

That the thesis, entitled "*Prenatal one carbon metabolism and in utero programming of growth and adiposity in the offspring*" by Pol Solé Navais, was carried out under my supervision in the Department of Basic Medical Sciences of the Universitat Rovira i Virgili, and fullfils the requirements to obtain the title of Doctor.

Reus, 5th May 2016

Prof. Joan D Fernández-Ballart

A vosaltres, Mama, Papa i Francesc,

per haver estat sempre amb mi.

I a tu, Fiona,

perquè vull que sempre hi siguis.

Acknowledgements

Primer de tot, volia agraïr al Joan i a la Michelle la oportunitat d'endinsar-me en el món de la investigació i la confiança dipositada per a contribuir en el seu projecte. Quan al 2012 em van dir que volien que aparegués a la foto de grup, no era realment conscient de tot el que representa aquest món. Avui en dia, ha esdevingut una passió que m'ha portat a escriure aquestes línies. Podría destacar les virtuts d'ambdós directors, però tan sols els agrairé l'impuls i les eines que m'han donat per poder seguir creixent, haver-me ensenyat (o intentar-ho almenys) a tenir perspectiva i haver-me demostrat que seguir endavant no sempre és el camí senzill, però si el més gratificant.

A més a més, resulta impossible no agraïr la tasca de tots els membres del Servei d'Obstetrícia i Ginecologia dels hospitals universitaris Sant Joan de Reus i Joan XXIII de Tarragona, especialment als responsables: Dr. Pere Cavallé Busquets i Dr. Ramón Miralles.

Al Prof. Jordi Salas i al Dr. Ricardo Closa per fer possible la realització del treball de camp de la fase de seguiment dels nens als hospitals universitaris Sant Joan de Reus i Joan XXIII de Tarragona respectivament.

Al personal del laboratori central i les ATS i infermeres d'extraccions de l'Hospital Sant Joan de Reus, al personal del laboratori d'investigació de l'Hospital Universitari Joan XXIII i del Biobanc – Institut d'Investigació Sanitària Pere Virgili.

Al finançament de:

- Instituto de Salud Carlos III. FIS: PI 10/00335
- Instituto de Salud Carlos III/ Fondo FEDER. FIS: 13/02500
- CIBER Fisiopatología de la Obesidad y Nutrición (CIBERobn)
- Comisión interministerial de Ciencia y Tecnología de España. SAF: 2005-05096

- Italfarmaco (2005-2008)
- AGAUR: 2009 SGR 1237
- Institu d'Investigació Sanitària Pere i Virgili: 2010/IISPV/21

En concret cal fer menció particular a la Isa, la qual recordaré sempre per la seva empenta, i que es mereix tota la simpatía i l'estima de tots els membres de l'equip de la nostra unitat. També destacar la feina dels membres del biobanc IISPV, aixi com la seva implicació en la recollida de mostres. I no podría faltar una menció a totes les infermeres que han anat punxant els nens de l'estudi.

Vull agraïr també, en aquestes línies, el paper imprescindible de cadascuna de les mares que han participat durant l'embaràs, i especialment a totes aquelles que també ho han fet a la fase de seguiment, a les quals ja he pogut agraïr individualment la seva implicació en aquest modest estudi. No podría faltar aquí, l'agraïment a tots els seus nens i nenes que han passat per la nostra consulta i aguantar totes les bromes que fem, així com aguantar (amb més o menys esforç) les extraccions. Tots ells han sigut capaços de treure'm un somriure fins i tot els dies més grisos.

També vull agraïr a totes persones que de manera (in)directa m'han estat acompanyant aquests gairebé quatre anys de doctorat.

Primer començaré agraïnt a tot el personal de la facultat de medicina que dia a dia batalla per seguir endavant i que sempre tenen alguna paraula amable: Amparo, Yoli, Sílvia, Juan, Espe, Montse, Inma i un llarg etc.

A la Núria, que més que la millor infermera que ha punxat a la majoria de nens, ha estat una confident i amiga a l'Hospital de Reus.

A la Joana, perquè ella és part responsable de que el treball de camp hagi anat bé, i per compartir tota aquesta feinada que està fent (fins i tot quan no es presenen!).

A la unitat de Pediatría que després de molts anys segueixen donant-me suport: Vero, Natàlia, Marta, Mariona, Carme, Dr. Closa i Escribano. Gràcies.

Als becàris amb els quals vaig començar treballant el primer dia, com amb els que comparteixo despatx en l'actualitat: Carlos, Maribel, Olalla, Sílvia, Jose i Gemma.

En quatre anys no tot és feina. Així doncs, vull recordar especialment en aquests moments totes les persones que han acabat formant part de la meva vida, no només com a companys de feina, sinó també com a amics. Primer de tot, als ja Drs. Martí, Andrés, Olalla, Sílvia, Marta, Jordi amb els quals vaig compartir dels moments més divertits dins i fora de la universitat.

A tots els Randoms, que poc a poc s'han anat convertint en un grup de gent imprescindible. Gràcies per tots els riures i les copes de més... Perquè formar part d'un grup de gent amb ocupacions i preocupacions tan diverses et força a canviar el punt de vista i la forma de ser. Sempre, a millor. Nohora, Jordi, Pia, Laia, Victor, Erica, Laura, Cintia, Simona, Fadua, Anna, Lucía, Jesús, Núria, Rocío, Teresa i Pablo. En especial, al Dr. Patraca que ara ho deu estar llegint des de Mèjic (en fin pinche pendejo eres cuate cabrón!). Sobretot, a qui ha tingut la paciencia d'aguantar les meves "bromes" no només 10h al dia, sinó de trobar-nos després per sopar junts i seguir aguantat-les. Amb vosaltres he entès que alguns companys de feina no són només això. Gràcies Gemma i Jose, per tots els alt-i-baixos que hem anat sortejant.

Amb els que ahir em reunía per recordar cada dos anys i entre riures, partides de cartes al bar, viatges a Tenerife, i com vam tirar endavant la carrera plegats. També per no oblidar una amistat que ens segueix perseguint fins i tot 6 anys després de veure'ns a diari. Gràcies Aurea, Ingrid, Victor i Eric.

Estar aqui seria impossible si no recordés als nois. Si, els de sempre. Perquè des dels primers moments, heu estat els de sempre, part de la família, dels meus fonaments. En aquests quatre anys us he abandonat, si. Per això tinc sort de que sigueu els de sempre, i és

que res no canvia. Passi el temps que passi. Vosotros os mereceis estas líneas, y muchas más. Ya sabéis, con vuestra amistad me sobra para sobrevivir. Dídac, Javi, Carlos, Om, Jorge (no me corrijas demasiado). Os debo las gracias por demasiado, así que gracias por ser quienes siempre he conocido, y os prometo que en cuanto termine esto... Yo me tomo la penúltima con vosotros.

A la família valenciana, per fer-me sentir com un més en tot moment. Tiets, cosins, germans de la Fiona, gràcies per acollir al catalanet tan bé. Especialment a Empar i Enric, per cuidar-nos sempre com ho han fet, i per deixar-me tastar el millor de València: vosaltres.

I a tu, Santi, perquè des del primer dia que et vaig conèixer (amb el pel llarg) fins avui, no has fet més que ser cada dia més part de la familia.

Ara s'apropa el moment delicat. El moment en el que les mans tremolen i es gelen, i els ulls s'omplen de llàgrimes. Ho escriuré un sol cop i no hi haurà correccions, com a la nostra història. Amb les primeres copes de vi vam començar a construir allò que cap dels dos hagués pogut arribar a imaginar. Les de la darrera setmana, em confirmen que de les poques decisions que he pres en aquesta vida, compartir-la amb tu ha estat la única encertada. Perquè ets incansable a l'hora de fer-me feliç, d'estimar-me i en treure la millor versió de mi mateix. I perquè aconsegueixes que jo sigui incansable al teu costat. Tu em fas fort, i m'empenys a diari per ser la persona que vull ser. Agafats de la mà, em guíes pels millors camins, i de ben segur, així és com aconseguirem tot allò que desitjem. Els meus millors records, ara trocets de mi, son al teu costat. Vull seguir aprenent dia a dia amb tu. Ets inigualable, Fiona. Per tot el que no ha vingut, però acabarà venint de la millor forma. Perquè la meva millor sort és tenir-te aprop. T'estim.

A vosaltres Papa, Mama i Francesc. Perquè sé que tot allò que pugui ser algún dia, amb total franquesa i seguretat serà gràcies a vosaltres. Per saber-me fer diferenciar el que és just del que és injust, per ensenyar-me que les arrels no s'han de perdre mai. Us he dedicat aquest treball, però no és el llibre el que us vull dedicar, sinó el camí que m'ha portat fins

aquí. Els resultats no son immediats, i son fruit d'anys de sacrificis. I vosaltres n'heu fet molts. La dedicació, la constància i el mèrit de l'esforç no s'ensenyen a les aules. Tot això (i molt més) ho he après a casa. Sóc un reflex del que sou vosaltres, i és el que em fa sentir més orgullós. Això que començo ara és una petita etapa d'un llarg camí, i sé que vosaltres estareu al meu costat. I jo seré al vostre. Sempre heu estat els meus pilars i sempre ho sereu. Sé que mai us podré retornar tot el que m'heu donat, per això només us puc dir, gràcies.

Abstract

Prenatal folate and cobalamin, as well as other one carbon (1C) metabolism nutrients , have been linked with foetal development. In the USA, mandatory fortification with folic acid has proven to be successful in the prevention of neural tube defects and the reduction of homocysteine concentrations in the adult population and has been proposed to be linked with the reduction in mortality from stroke. Therefore, 1C metabolism appears to positively influence health both in early and late stages of life. However, whether the effects of *in utero* 1C metabolism extend beyond foetal development is unknown. Widespread exposure to folic acid from fortified foods and supplements in numerous countries has led to concern regarding potentially harmful effects of very high intakes, in particular in subjects with cobalamin deficiency. In Europe, beneficial and deleterious effects of folic acid fortification are being considered before implementing mandatory fortification of flour with folic acid.

In this thesis, we evaluated the interactions between prenatal folate and cobalamin and their effects on 1C metabolism during pregnancy and subsequently on mid-childhood growth and adiposity.

Five hundred and sixty three pregnant women from the Reus-Tarragona Birth Cohort were followed-up throughout pregnancy and 162 of their offspring until 7.5 years of age. Extensive lifestyle, biological and obstetrical data was collected from the mothers from the first trimester throughout pregnancy and the offspring at mid-childhood. Fasting blood samples were collected at each trimester, labour and from the cord, and from the children aged 7.5 years. Child weight, height, BMI (age- and sex-standardised z-scores according to World Health Organisation) and fat mass (by bioelectrical impedance) were measured. Maternal and child red blood cell (RBC) and plasma folate, plasma cobalamin, total homocysteine (tHcy), methylmalonic acid (MMA), as well as maternal holotranscobalamin (holoTC) were determined in fasting blood samples. Maternal and child *MTHFR* 677C>T,

Abstract

TCN2 776C>G, *MTRR* 66A>G and 524C>T genetic variants were also determined. First trimester overweight was associated with lower plasma folate, despite higher folic acid intake in the mothers and in those with high folate intake, RBC folate was higher in overweight women (p= 0.011). First trimester cobalamin status was associated with that of the offspring at mid-childhood. The associations between cobalamin inadequacy and plasma tHcy, MMA or haemoglobin were not modified by elevated folate. However, in women with elevated first trimester folate, mean cell volume was 4.8 (95% CI: 2.0, 7.7) and 5.5 (95% CI: 1.9, 9.1) fL lower in cobalamin inadequate compared with adequate at early and late pregnancy respectively. Early pregnancy inadequate RBC folate, plasma cobalamin or elevated tHcy was associated with lower offspring linear growth. Folic acid supplement use at preconception, but not in the first or second trimesters was associated with higher offspring linear growth at mid-childhood. In addition, inadequate holoTC at early or mid-pregnancy or late pregnancy RBC folate inadequacy was associated with higher adiposity in the offspring. Child metabolic markers of folate and cobalamin status were not associated with growth or adiposity. Children born to variant homozygous mothers for the *MTHFR* 677C>T genetic variant had persistently higher BMI from birth to mid-childhood (p= 0.033). The maternal *TCN2* 776C>G polymorphism was associated with lower height from birth to mid-childhood (p= 0.029) and with a faster adiposity rebound after infancy. Among child genetic variants, only the *MTRR* 66A>G was associated with lower linear and ponderal growth.

In conclusion, we found no evidence of a harmful effect of elevated folate status in women with cobalamin inadequacy during pregnancy. Pregnancy 1C metabolism influences offspring growth and adiposity from birth to mid-childhood.

Keywords: folate – cobalamin – genetic variants – pregnancy – *in utero* programming – child – adiposity – growth

	What is known	What is unknown	What this thesis adds	Future research
Chapter 1	Harmful effects of combined elevated folate and low cobalamin status have been reported in countries with folic acid mandatory fortification.	Whether periconception folic acid supplement use, exacerbates metabolic or haematological indicators of cobalamin inadequacy during pregnancy.	First trimester folate status does not modify the association between cobalamin inadequacy and its metabolic or haematological markers during pregnancy.	Monitoring of the effects of elevated folate should be further assessed in a population with high prevalence of cobalamin deficiency.
	The effect of BMI on RBC or plasma folate varies according to fortification with folic acid.	Whether BMI is associated with RBC or plasma folate in a pregnant population, and whether folate intake modifies the association.	BMI is negatively associated with plasma folate, and its association with RBC folate depends on total folate intake.	To assess this association in controlled randomised clinical trials.
Chapter 2	Maternal cobalamin and folate status and breastfeeding determine child vitamin status up to the age of 2 years.	Whether the effects of breastfeeding and maternal B-vitamin status on child 1C metabolism persist at mid-childhood.	Early pregnancy cobalamin is the major determinant of cobalamin status in the offspring. Breastfeeding is not associated with child vitamin status at 7.5 years.	To investigate whether the effects of maternal cobalamin on status in the offspring are mediated by shared genetic variants.

Abstract

	What is known	What is unknown	What this thesis adds	Future investigations
Chapter 3	Preconception folic acid supplement use and adequate cobalamin status reduce NTD prevalence, and are positively associated with birth weight.	Whether prenatal folate or cobalamin influence growth at 7.5 years.	Inadequate 1st trimester RBC folate, cobalamin or elevated tHcy are associated with lower offspring linear growth at mid-childhood.	Replication studies are needed in different populations.
	Maternal *MTHFR* 677C>T and other genetic variants related to folate or cobalamin transport or metabolism have been linked with foetal development in human and experimental studies.	Whether maternal or child genetic variants affecting 1C metabolism or transport are associated with postnatal offspring growth.	Maternal *MTHFR* 677C>T and *TCN2* 776C>G were associated with higher BMI and lower height in the offspring from birth to 7.5 years respectively. Child *MTRR* 66A>G was linked with lower growth.	Other maternal genetic variants affecting 1C metabolism should be investigated in relation to offspring growth and adiposity.
	Inconsistent effects of prenatal folate status on offspring BMI have been reported.	Whether prenatal folate effects on offspring adiposity vary according to timing of exposure or population characteristics.	Inadequate late but not early pregnancy folate status is associated with lower offspring BMI.	Pregnancy folate effects on offspring adiposity are different in developing and developed countries.
	Cobalamin inadequacy during pregnancy is not associated with offspring adiposity, but it is associated with higher insulin resistance.	Are markers of active cobalamin status or SNPs affecting cobalamin transport linked with offspring adiposity?	Inadequate pregnancy holoTC and maternal *TCN2* 776C>G are associated with higher BMI and a faster offspring adiposity rebound after infancy in the offspring respectively.	Other cardiometabolic risk factors such as insulin resistance, or markers of endothelial function and inflammation should be investigated.
	IGF1 axis mediates *in utero* effects of 1C metabolism on mice postnatal growth.	Child IGF1 axis has not been assessed in relation to prenatal 1C metabolism.	-	-

Abbreviations

Abbreviations	Definition
1C	One carbon
ANCOVA	Analysis of covariance
BMI	Body mass index
CI	Confidence interval
DFE	Dietary folate equivalents
DHF	Dihydrofolate
dTMP	Deoxythymidylate monophosphate
EAR	Estimated average requirements
FR	Folate receptor
GH	Growth hormone
GW	Gestational weeks
Hcy	Homocysteine
HoloTC	Holotranscobalamin
IGF	Insulin-like growth factor
IOM	Institute of Medicine
MCV	Mean cell volume
MMA	Methylmalonic acid
PBS	Phosphate buffered solution
PCFT	Proton coupled folate transporter
PGC1-α	Peroxisome proliferator-activated receptor gamma coactivator 1-α
PPAR	Peroxisome proliferator-activated receptor
RBC	Red blood cell
RDA	Recommended dietary allowance
RFC1	Reduced folate carrier
RTBC	Reus-Tarragona Birth Cohort
SAM	S-adenosylmethionine
SNP	Single nucleotide polymorphism
tHcy	Fasting plasma total homocysteine
THF	Tetrahydrofolate
NHANES	National Health and Nutritional Examination Survey
NTD	Neural tube defect
WHO	World Health Organization

Enzyme and genetic variant nomenclature

Abbreviation	International nomenclature	Definition
BHMT	EC 2.1.1.5	Betaine-homocysteine s-methyltransferase
CBS	EC 4.2.1.22	Cystathionine-β-synthase
DHFR	EC 1.5.1.3	Dihydrofolate reductase
FPGS	EC 6.3.2.17	Folylpoly-gamma-glutamate synthetase
FOLH1	EC 3.4.17.21	Folate hydrolase
MAT I & III	EC 2.5.1.6	Methionine adenosylmethyltransferases I & III
MMA-CoA mutase	EC 5.4.99.2	Methylmalonyl-CoA mutase
MTHFD1	EC 1.5.1.5 EC 6.3.4.3 EC 3.5.4.9	Methylenetetrahydrofolate dehydrogenase
MTHFR	EC 1.5.1.20	Methylenetetrahydrofolate reductase
MTR	EC 2.1.1.13	Methionine synthase reductase
MTRR	EC 2.1.1.13	5-methyltetrahydrfolate-homocysteine methyltransferase reductase
PCC	EC 6.4.1.3	Propionyl CoA carboxylase
SHMT	EC 2.1.2.1	Serine hydroxymethyltransferase
TYMS	EC 2.1.1.45	Thymidylate synthetase

Abbreviation	Reference SNP	Definition
MTHFR 677C>T	rs1801133	*Methylenetetrahydrofolate reductase* 677C>T
TCN2 776C>G	rs1801198	*Transcobalamin 2* 776C>G
MTRR 66A>G	rs1801394	*5-methyltetrahydrfolate-homocysteine methyltransferase reductase* 66A>G
MTRR 524C>T	rs1532268	*5-methyltetrahydrfolate-homocysteine methyltransferase reductase* 524C>T

Table of contents

List of figures

List of tables

INTRODUCTION

Introduction

Ageing is emerging as a determining factor of global health at the population level, as the proportion and absolute number of elderly people are increasing dramatically around the world. In 2015, there were 901 million people aged 60 or over, comprising 12% of the world's population. These values are predicted to increase to 2.1 billion people by 2050, and to account for 21.3% of the global population (1). In high-income settings, longer survival in older age groups has contributed significantly to the rise in life expectancy at birth, which is a key driver of population ageing. However, whether these added years in life expectancy are accompanied by good or unfavourable health remains unclear (2). Non-communicable diseases are the worldwide leading cause of death, and were responsible for 38 million deaths (68%) in 2012 (3). Moreover, between 2005 and 2013, disability-adjusted life years (productive years of life lost due to morbidity/disability as well as to premature death) increased globally by 10%, exclusively as a result of non-communicable diseases (4). In Spain, the percentage of premature deaths (<70 years) attributed to non-communicable diseases was 26.9% and 13.4% in men and women respectively in 2012 (3). So far, epidemiological studies of non-communicable diseases have provided essential knowledge on which the strategies targeting the reduction of impact and treatment of the diseases are based. Most research is focused on the study of environmental or genetic stimuli in preclinical or clinical stages of the disease during adulthood, after the effects of the underlying biological lesion are manifested. There is less information available regarding the underlying pathophysiological mechanisms from which the biological lesions, leading to non-communicable diseases, develop. The World Health Organization (WHO) has already recognised that healthy ageing trajectories may be influenced by experiences in the prenatal period (5). Information regarding the effects of early life exposures on subsequent health is needed for the development of population-based primary prevention strategies aimed at producing healthy ageing trajectories. One of the most outstanding public health

strategies in terms of health- and cost-effectiveness is the fortification of cereal grains with folic acid in the USA and Canada since 1998. This mandatory policy was implemented to prevent the first occurrence and the recurrence of neural tube defects (NTD). It was based in evidence from two clinical trials in which a reduction in NTD risk of 70% and 100% was achieved after the daily preconceptional folic acid supplementation with 4 mg and 400 µg respectively (6,7). NTD-affected pregnancies in the USA and Canada, have been reduced by approximately 50% has since mandatory folic acid fortification was introduced (8,9). Other beneficial health effects have also been attributed to folic acid fortification. The average increase of 100 µg/day in folic acid intake in the USA (10) was followed by a 10% reduction in fasting plasma total homocysteine (tHcy) (11). It has also been suggested that this policy was successful in the reduction of stroke mortality (12). Therefore, mandatory fortification with folic acid has proven to be of value in the prevention of developmental disorders and diseases in both early and late stages of life. However, whether early life exposure leads to the effects observed in late life remains unknown.

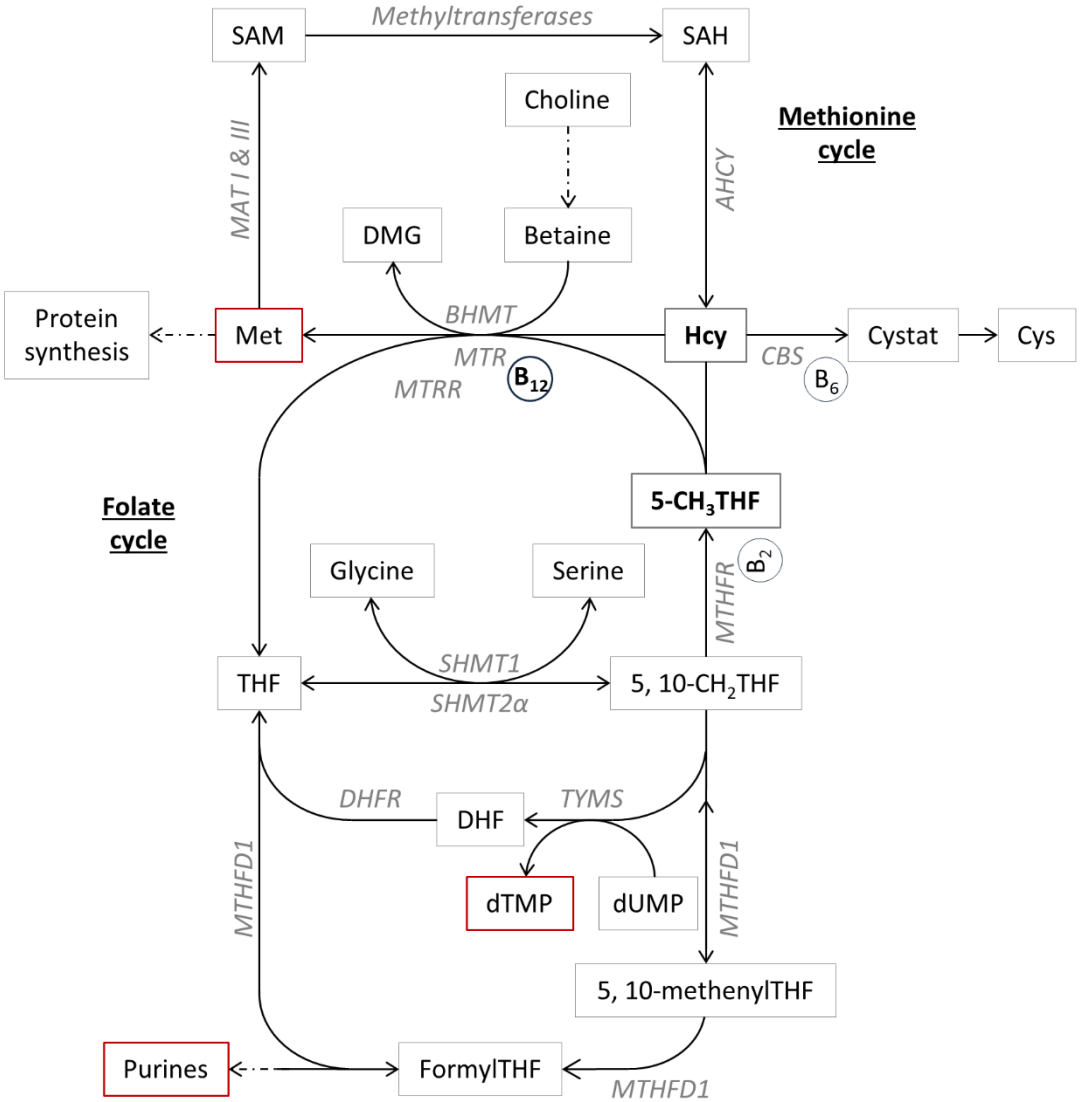
1. One carbon metabolism: general insights.

One carbon (1C) metabolism involves a complex metabolic network containing the interconnected folate and methionine cycles, among other metabolic cycles and pathways. This system integrates numerous amino acids and other essential nutrients, such as B vitamins that act as substrates, cofactors or coenzymes in numerous reactions, into an array of metabolic cycles and pathways with both inter-dependent and disparate functions. The folate cycle, an integral part of the 1C metabolic network, is responsible for the conversion of dietary folate into active forms leading to the supply and ultimate transport of carbon units to metabolic reactions within the network and methyl groups to methylation reactions that participate in the epigenetic regulation of gene function. In the cytoplasm, 1C metabolism includes three different biosynthetic pathways: the *de novo*

synthesis of DNA and RNA nucleotides and the remethylation of homocysteine (Hcy) to methionine. This essential amino acid is involved in protein biosynthesis and is a precursor for the generation of s-adenosylmethionine (SAM), which in turn is a cofactor and methyl group donor for a number of methylation reactions.

Each particular biosynthetic pathway uses specific forms of tetrahydrofolate (THF), which are interconverted enzymatically. Methionine synthase reductase (MTR, EC 2.1.1.13) is the enzyme responsible for the remethylation of Hcy to methionine (13), and requires the carbon unit from 5-methylTHF and cobalamin as coenzyme (**Figure 1**). Hcy has an alternative pathway (transsulfuration) in which it is used to produce cystathionine, which is further converted to cysteine. The folate cycle is also involved in the formation of nucleotides necessary for DNA and RNA synthesis. 10-formylTHF is used as a cofactor for *de novo* purine biosynthesis. Thymidylate synthetase (TYMS, EC 2.1.1.45) catalyses the transfer of the carbon unit from 5, 10-methyleneTHF to deoxyuridine monophosphate forming deoxythymidylate monophosphate (dTMP) and dihydrofolate (DHF). This biosynthetic pathway contributes to DNA stabilisation (14). In the methionine cycle, phospholipids can be partly generated involving SAM (15). Moreover, phosphatidylcholine, which is essential in cell structure (16), lipid transport (17) and neurotransmission (18), can be synthesised through the transfer of three methyl groups from SAM to phosphatidylethanolamine (19). Moreover, 1C metabolism is a major source of methyl groups (SAM) for post-translation modifications that are independent of genomic changes including histone, DNA and RNA methylation (20–22), that may affect many physiological functions (23). Accordingly, 1C metabolism status contributes to DNA stability, cellular biosynthesis and to the regulation of gene transcription and expression through nucleic acid and protein methylation. These essential functions highlight the importance of 1C metabolism in other crucial metabolic systems. Considering that both the folate and methionine cycles are ubiquitously present in every human cell, derangements in 1C metabolism can have profound effects on cell function, metabolism, growth and proliferation.

Figure 1. Folate and cobalamin mediated one carbon metabolism.



AHCY, adenosylhomocysteinase; BHMT, betaine-homocysteine s-methyltransferase; Cys, cysteine; Cystat, cystathionine; DHFR, dihydrofolate reductase; DMG, dimethylglycine; MAT I & III, methionine adenosylmethyltransferases I & III; Met, methionine; MTHFD1, methylenetetrahydrofolate dehydrogenase; MTRR, 5-methyltetrahydrfolate-homocysteine methyltransferase reductase; SAH, s-adenosylhomocysteine; SHMT, serine hydroxymethyltransferase. Adapted from (483).

The classical clinical picture of severe folate or cobalamin deficiency includes megaloblastic anaemia, which is caused by a decrease in DNA synthesis and a subsequent abnormal maturation of erythropoietic precursors. Unavailability of dTMP leads to the misincorporation of deoxyuridine monophosphate in place of dTMP during DNA base addition, slowing down DNA replication (24). The pathophysiology of folate and cobalamin deficiency may also be accompanied by neuropathy, encephalopathy or myelopathy among other neurological complications (25,26).

The pathways involved in the 1C network are regulated by complex interactions between nutrients and enzymes that regulate the reactions. Various genetic polymorphisms, mainly affecting enzyme functions, have been described. The network is sensitive to status and availability of various amino acids (cysteine, serine and methionine) and B vitamins (folate, cobalamin, vitamin B_2, vitamin B_6) and lifestyle factors such as smoking, alcohol consumption or age. THcy is a global biomarker of 1C metabolism status. This sulphur-containing amino acid is an intermediary in the methionine cycle and the transulphuration pathway. When either of these is impaired, intracellular Hcy rises. Numerous environmental, nutritional and genetic factors have been studied in relation to Hcy. In this regard, age, male sex, smoking, alcohol consumption and impaired renal function increase its plasma concentrations (27,28). With regard to genetic variants, the *methylene tetrahydrofolate reductase* (*MTHFR*) 677C>T polymorphism (see *Introduction: Folate* for more information) has been consistently associated with higher tHcy (29). Folate and cobalamin statuses are major modifiable determinants of tHcy (27,30,31), reflected by the responsiveness of tHcy to folic acid or cobalamin supplementation (32–34). Indeed, inadequate folate or cobalamin status at different stages of the life course may contribute to a broad range of clinical consequences. Imbalances in the 1C metabolic network have been linked with developmental anomalies such as NTDs (6,35) and congenital heart defects (36) as well as pregnancy complications such as stillbirth, preeclampsia (37) or low birth weight (38,39) and pathologies such as cardiovascular diseases (40,41) and cancer

(42,43). Cobalamin status and the *MTHFR* 677C>T genetic variant, among other single-nucleotide polymorphisms, have also been linked with NTD affected pregnancies (44,45). However, the effects of the 1C metabolic network during early stages of development on subsequent health may not be limited to NTDs and foetal development.

This thesis is focused on the interactions between cobalamin and folate status and their effects on maternal 1C metabolism during pregnancy and subsequently on early growth and development in the child.

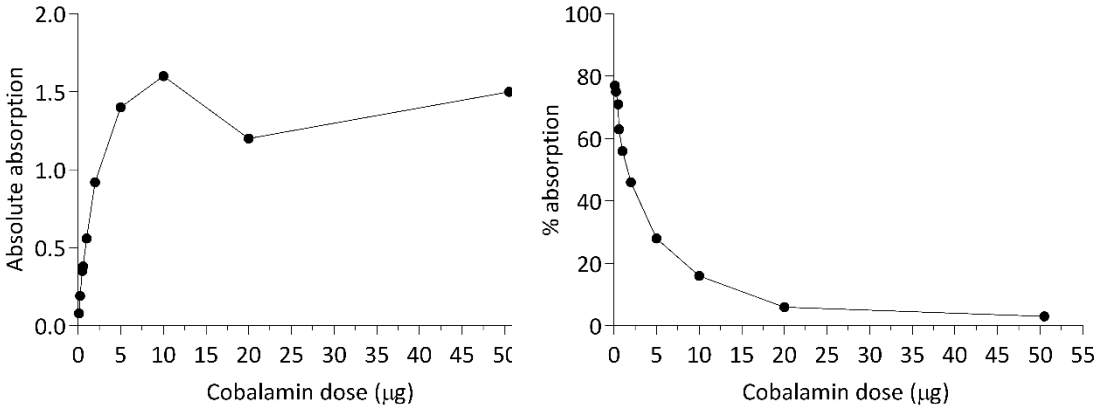
2. Cobalamin

Characteristics, functions, food sources, bioavailability and requirements from preconception to mid-childhood

Cobalamin is an essential water-soluble vitamin exclusively synthesised by microorganisms. The site of cobalamin synthesis by human intestinal flora in the colon is far removed from the ileum where absorption occurs. Ruminant gastrointestinal tract bacteria produces cobalamin, which accumulates in animal tissues or is secreted via the animal's milk after its absorption (46). Monogastric animals such as pigs and poultry are also sources of cobalamin, albeit its content is lower compared to that found in ruminant tissues. Humans must obtain cobalamin through the intake of animal foods or synthetic forms used in multivitamin supplements or fortified foods (e.g. ready-to-eat breakfast cereals). The term cobalamin, in scientific publications, refers to compounds containing a cobalt atom in the centre of a corrin ring. "Vitamin B_{12}", refers to synthetically produced stable cobalamin, which is not found in nature. Different forms of cobalamin have been identified, however only four forms are metabolised by humans. Methylcobalamin and adenosylcobalamin are the only ones directly used as coenzymes, but are the most light sensitives. On the other hand, hydroxycobalamin and cyanocobalamin must be first converted into an active cobalamin form before its use in 1C metabolism and these are the forms used in supplements and fortified foods. In general, adenosylcobalamin and hydroxycobalamin are the predominant forms found in foods, however methylcobalamin is also very common in dairy products (47). In the USA, the intake of cobalamin containing supplements, fortified cereals and milk contributes to a great extent to adequate plasma cobalamin concentrations (48). In contrast, dairy products and fish intake are the major nutritional determinants of plasma cobalamin concentrations in Norway (49). In Spanish children, milk and dairy products provide 69.5% of cobalamin required to meet the Recommended Dietary Allowance (RDA) (50).

Data on cobalamin bioavailability in humans is scarce. However, it is generally assumed to be 40 or 50% for healthy adults with normal gastrointestinal function (51,52). Absolute absorption of vitamin B_{12} supplements rises with increasing dose, however the relative absorption diminishes as the consumed dose increases (**Figure 2**). This is due to the absorption capacity of intrinsic factor, which is thought to reach saturation at a rate of 1.5-2.0 µg of cobalamin. Once this dose is exceeded, cobalamin can be absorbed through passive diffusion at very low rates (1%) (53). In healthy humans, the absorption of food-bound cobalamin has been shown to vary according to the quantity and type of protein consumed (54). For example, cobalamin from meat is more bioavailable than that from fish or eggs (55–58).

Figure 2. Relative and absolute absorption of vitamin B_{12} supplements according to dose.



Adapted from (484).

In order to estimate the cobalamin RDA, the Food and Nutrition Board of the Institute of Medicine (IOM) considers bioavailability of food-bound cobalamin to be 50% (59). The preconception RDA for cobalamin is not defined by IOM or Spanish guidelines (60), however inadequate cobalamin status has been consistently linked with NTD affected pregnancies (44,61). **Table 1** describes IOM and Spanish RDAs during pregnancy and from birth until 9 years of age. For pregnant women, the estimated IOM RDA is 2.6 µg/day. The

IOM recommendation resulted from the addition of 0.2 to the estimated average requirements of adult women (2.2 µg/day), based on the assumption that foetal cobalamin deposition during pregnancy is 0.1-0.2 µg/day, and that maternal cobalamin absorption is more efficient during pregnancy. The Spanish RDA is lower (Table 1).

However, the recommendations may be inadequate because some of the assumptions made when estimating cobalamin requirements during pregnancy remain to be confirmed. The assumed increased efficiency in cobalamin absorption was based on studies performed in mice (62) or rats (63), but supporting evidence in humans is limited (64). Currently there is insufficient available evidence on which to base pregnancy requirements, but it appears that greater cobalamin intakes than those currently recommended may be required to achieve optimal cobalamin status (65).

Table 1. RDA for cobalamin (µg/day) from preconception until mid-childhood.

	IOM (59)	**Spanish** (60)
Pregnancy	2.6	2.2[1]
0-6 months	0.4[2]	0.4
7-12 months	0.5[2]	0.5
1-3 years	0.9	0.7
4-5 years	1.2	1.1
6-9 years	1.2	1.2

[1] Second half of pregnancy.
[2] Adequate intake.

From birth to 12 months, the IOM estimated average intake of cobalamin reflects the observed intake of cobalamin in breastfed infants born to omnivorous mothers with adequate cobalamin intake in a study by Schneede and coworkers from 1994 (66). Hence, the estimated average intake was set at 0.4 µg/day. Moreover, lower intakes were associated with increased urinary methylmalonic acid (MMA), a biomarker of low cobalamin status. However, infant and maternal cobalamin statuses were not assessed, and cobalamin bioavailability from human breast milk remains unknown. Its determination is

complicated by methodological artefacts and the necessity to remove unsaturated haptocorrin. This increases the detected cobalamin content in human milk (67), but the technique was not yet developed at the time of the studies on which the recommended average intakes are based. This suggests that the estimated average intakes set by the IOM for cobalamin are lower than required. For children up to 1 year, the average intake was estimated to be 0.5 µg/day. Spanish RDAs (60) coincide with the average intake from the IOM. Bearing in mind the methodological limitations that might have underestimated IOM infant requirements; the Spanish approach seems to underestimate cobalamin requirements to a higher degree. Data on cobalamin status in children from 1 to 9 years is limited, and thus the IOM estimated the RDA by extrapolating from adult values. Spanish recommendations are very similar to those from the IOM.

Absorption, transport and metabolism

Active and passive mechanisms exist for cobalamin absorption. The active process is complex, and involves a vast number of anatomical areas within the gastrointestinal tract, as well as specific proteins that bind a single cobalamin molecule (68). Animal proteins are severed in the stomach by pepsin and chlorhydric acid. Then, free cobalamin rapidly binds to haptocorrin, released by salivary and parietal cells. Intrinsic factor, which is one of the three cobalamin binding proteins found in humans, is also secreted in the stomach, but its affinity for cobalamin is low in the acidic environment media and in the presence of haptocorrin. Haptocorrin is digested by pancreatic proteases in the duodenum thereby liberating cobalamin and allowing it to bind to intrinsic factor. The intrinsic factor-cobalamin complex enters the mucosal cells in the distal ileum by receptor-mediated endocytosis through the cubilin receptor. When it leaves the enterocyte, cobalamin enters the circulation. Intrinsic factor does not enter the bloodstream, and free cobalamin binds to either haptocorrin or transcobalamin. Plasma haptocorrin binds between 70-80% of

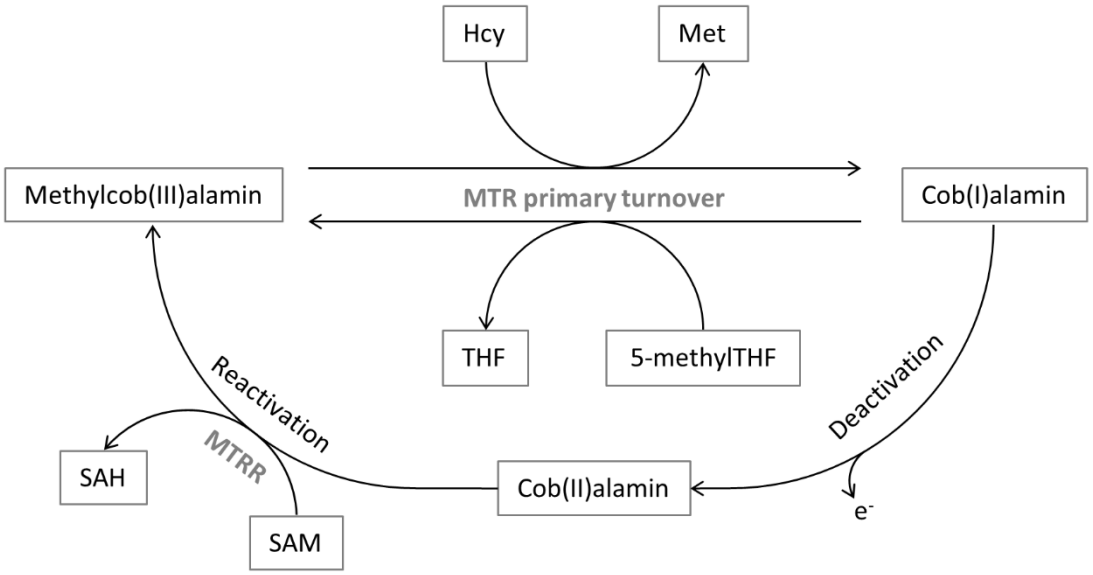
cobalamin (holohaptocorrin), however it is not readily available for cellular uptake (69), and its functions are not yet established. Transcobalamin protein binds the remaining cobalamin fraction (named the active cobalamin form), and transports the newly absorbed cobalamin to tissue cells, where it is internalised through mediated cellular uptake. The transcobalamin receptor, encoded by the *CD320* gene, mediates the intracellular uptake of the transcobalamin-cobalamin complex (holotranscobalamin, holoTC). Once in the cell, transcobalamin is degraded in the lysosome and the free cobalamin released is converted into cobalamin cofactors (70,71). Foetal cobalamin requirements must be met by the transport system that provides all nutrients across the placental barrier. Transplacental cobalamin transport is also mediated through the transcobalamin receptor (72), however, an alternative holotranscobalamin receptor must function, because transcobalamin receptor knock-out is not lethal to embryos (73).

In mammalian systems, only two enzymes are known to require cobalamin as a cofactor: MTR in the cytoplasm and methylmalonyl CoA mutase (EC 5.4.99.2) in the mitochondria. In the folate cycle, MTR depends on methylcobalamin as an intermediate methyl carrier to transfer the carbon group from 5-methylTHF to homocysteine (74). In contrast, the mutase reaction is unrelated to the folate pathway. Because cobalamin is essential for these two enzymes, elevated plasma tHcy and MMA are metabolic manifestations of cobalamin deficiency (75). However, plasma tHcy is not a specific marker for cobalamin deficiency because it is also increased in situations of folate deficiency and altered 1C metabolism. Mammalian cytoplasmic and mitochondrial cobalamin-dependent reactions are detailed below.

In the cytosol, and in the first turnover, the methyl group is transferred from 5-methylTHF to cob(I)alamin generating methylcob(III)alamin and THF. In the second turnover, the methyl group is transferred to hcy forming methionine and cob(I)alamin (**Figure 3**). The oxidation of cob(I)alamin accidentally occurs once in every 200-2000 turnovers, leading to the inactivation of MTR. Then, the cofactor requires a reductive methylation in which SAM

(and not 5-methylTHF) supplies a methyl group, and the electron is supplied via the 5-methyltetrahydrofolate-homocysteine methyltransferase reductase enzyme (MTRR, EC 1.16.1.8).

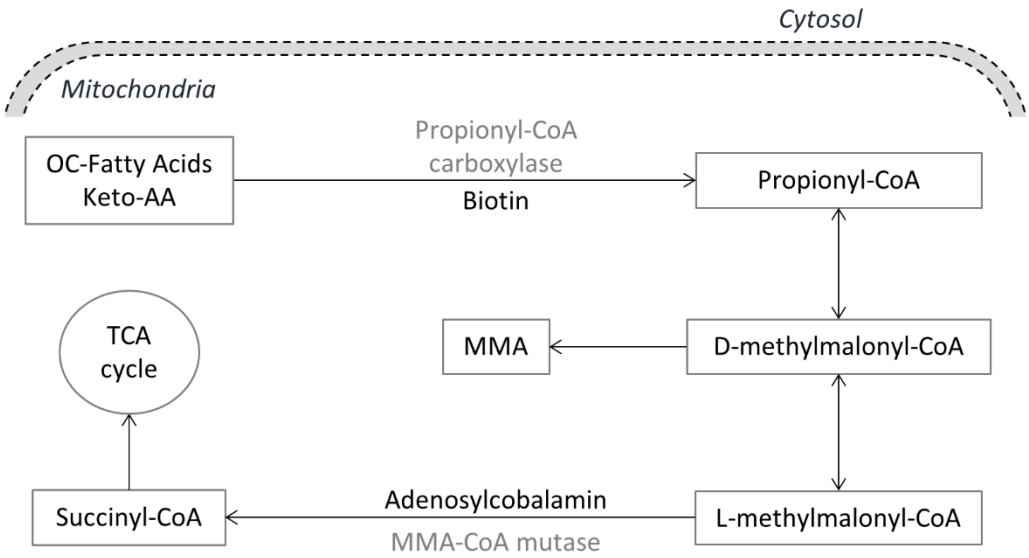
Figure 3. Cobalamin dependent remethylation of homocysteine to methionine via MTR and MTRR.



Adapted from (485).

In the mitochondria, methylmalonyl CoA mutase depends on adenosylcobalamin to catalyse the conversion of methylmalonyl CoA to sucinyl CoA. This is an intermediate step in the conversion of propionate to succinate during the oxidation of odd-chain fatty acids and in the catabolism of ketogenic amino acids such as isoleucine, lysine or tryptophan (Figure 4). Low adenosylcobalamin concentrations in this pathway lead to elevated MMA.

Figure 4. Cobalamin-dependent isomerisation of methylmalonyl-CoA to succinyl-CoA via methylmalonyl-CoA mutase.



Keto-AA, ketogenic amino acids; MMA-CoA mutase, methylmalonyl CoA mutase; OC-Fatty acids, odd chain fatty acids. Catabolism of odd chain fatty acids and ketogenic amino acids. Adapted from (486)

Cobalamin homeostasis during pregnancy and childhood

During the normal course of pregnancy, plasma cobalamin concentrations decline by 25% from 10-12 weeks prior to gestation (76). Plasma holoTC drops from preconception until 8 gestational weeks (GW), and then remains constant throughout the rest of the pregnancy (76,77). The decline in plasma cobalamin observed during pregnancy has been proposed to be due to a drop in holohaptocorrin (64). Unchanged cobalamin absorption following an oral cobalamin load at different stages of pregnancy, failed to explain stable holoTC concentrations during pregnancy (64). It is thought that this reflects a physiological mechanism to maintain an adequate cobalamin transfer to the foetus, given that, maternal cobalamin stores are depleted during pregnancy, and cobalamin is accumulated in the

placenta and foetus in mice (62). However, this hypothesis has not been tested in humans and available evidence suggests that only newly absorbed maternal cobalamin accumulates in the foetoplacental organs (63,65,78). In support of this theory, supplementation with 50 µg/day from 10 GW to 6 weeks postpartum did not reduce maternal MMA nor tHcy in Indian women, despite reducing child MMA and tHcy at 6 weeks postpartum (78). Cord blood cobalamin and holoTC concentrations are higher than at any point during pregnancy, suggesting a high foetal demand for these metabolites (76).

In normal pregnancy, plasma MMA increases as pregnancy progresses and despite haemodilution. This phenomenon may be explained by higher cobalamin transport to the foetus during pregnancy, as reflected by higher correlations between maternal cobalamin and cord blood MMA as pregnancy progresses. However, cobalamin status also affects late pregnancy MMA concentrations, which are higher in mothers with low (<67 pmol/L) than with normal preconception plasma holoTC (76).

After birth, child serum cobalamin decreases considerably and this is accompanied by an increase in plasma tHcy and MMA (79,80). The lowest serum cobalamin and highest tHcy and MMA occur in infants aged between 6 weeks and 6 months. Subsequently cobalamin increases, peaking at 3-7 years before decreasing again. Plasma tHcy remains low until the age of 7 years when it starts to rise until reaching young adult concentrations at puberty. MMA concentrations remain low throughout childhood (81). The apparently impaired cobalamin metabolism observed in infants has been suggested to be harmless, and related to developmental, physiological or nutritional factors inherent to this age group. Moreover, elevated MMA may be caused by increased propionate production by intestinal bacteria (82), rather than by liver or kidney immaturity (83). Thus, metabolic but not clinical cobalamin deficiency might be common among apparently healthy infants in developed countries, and may not be confined to sporadic cases related to exclusive breastfeeding combined with poor maternal cobalamin status.

Environmental and genetic determinants during pregnancy and childhood

Cobalamin deficiency (plasma cobalamin <148 pmol/L) is a public health concern worldwide, and has been the subject of detailed reviews (84,85). In developing countries, low socioeconomic status, low consumption of animal foods or derivatives and low educational level are associated with impaired cobalamin status (86). As cobalamin turnover rate is low, depletion is a lengthy process. Therefore, inadequate cobalamin intakes or malabsorption must be permanent in order to achieve cobalamin concentrations considered deficient. In India, an elevated prevalence of cobalamin deficiency has been observed (87,88), but is mainly due to vegetarianism and restricted access to animal food sources. These are not likely to be the causes of cobalamin deficiency (or marginal deficiency) in developed countries, where marginal deficiency is mainly due to food-bound cobalamin malabsorption (89,90). In these countries, despite low prevalence of cobalamin deficiency, it affects population subgroups such as the elderly and pregnant women (76,91–93). Currently, there is no clear agreement on the cut-off point for adequate cobalamin status either during pregnancy or in children. Thus the cut-offs for cobalamin, holoTC, tHcy and MMA applied in the general population, have been applied in pregnant women and children (65,94,95). Distribution based cut-offs have also been used (94,96,97). This complicates comparisons between studies and the study of cobalamin deficiency prevalence.

The prevalence of cobalamin deficiency at preconception was almost 10% in a Spanish population not taking cobalamin-containing supplements. More than 50% of women had subclinical deficiency (<221 pmol/L) by 32 GW (76). Higher rates of cobalamin deficiency during pregnancy were reported in studies carried out in the UK (93,98) and Canada (99). These reports of deficiency or marginal deficiency in cobalamin in countries with widespread access to animal food sources and comparatively little restriction of intake for

religious reasons, illustrate that deficiency is highly prevalent in pregnancy. Deficiency during pregnancy is not accompanied by metabolic abnormalities, as reported by unaltered MMA concentrations in these studies (76). It is important to collect the blood samples as early as possible during pregnancy, to avoid confounding by physiological phenomena that may affect intra-subject variability.

Several environmental factors have been identified as determinants of cobalamin status during pregnancy. Higher age, BMI, parity, smoking and alcohol consumption were associated with lower (91,92,100) and supplement use with higher cobalamin status (91,92). Cobalamin supplementation in doses ranging from 2.6 to 250 µg/day increases maternal cobalamin status or prevents its normal decline (65,78,101–103). However, only very high doses of cobalamin, exceeding almost 100 times the RDA, led to a reduction in plasma MMA or tHcy concentrations (102).

Maternal cobalamin status is the major determinant of cobalamin in the offspring up until the age of two years (80,95,104,105). Child cobalamin deficiency is considered to be secondary to maternal cobalamin deficiency. Physiological doses of cobalamin during pregnancy increase postnatal child cobalamin status, while reducing MMA and tHcy (65,78,101,102). Only one study, carried out in India has investigated whether the impact of cobalamin status during pregnancy on child cobalamin persists after two years of age (106). Exclusive breastfeeding for extended periods places greater nutritional demands on the mother. Therefore, longer exclusive breastfeeding, as well as breastfeeding *per se*, reduces child serum cobalamin (97,107). High concentrations of odd-chain fatty acids in breast milk might be responsible for increased MMA concentrations in breastfed children (108). In 2001, the WHO recommended that all infants should be exclusively breastfed until the age of 6 months, but several concerns have emerged given the insufficient available evidence to support this recommendation (109,110). Additionally, both parity (80), and maternal smoking during pregnancy (111) have been associated with reduced cobalamin status in the neonate. However, whether these effects persist after the neonatal period remains

unknown (105). In children, deficiency due to malabsorption increases with age (85), and limited access to animal food sources, and maternal depletion are the main causes of cobalamin deficiency at younger ages. Inborn errors of cobalamin absorption, transport and metabolism as causes of deficiency are very rare (112). Sex does not affect cobalamin status in children before puberty (81,113). Data from the National Health and Nutrition Examination Survey (NHANES) 1988-94 showed that less than 5% of children in the USA had cobalamin intakes below the RDA (59). According to this, the prevalence of cobalamin deficiency was lower than 1% (114). Fortified ready-to-eat breakfast cereals contribute significantly to reaching the RDA (115,116), however, data regarding their contribution to cobalamin status remains inconsistent (116,117). A meta-analysis of 76 studies including 1490 children published in 2001, reported that the mean cobalamin concentration in Spanish children aged 0-15 years old was 502 pmol/L (standard deviation, 93.7) (118). Data regarding child cobalamin status (or functional status) in developed countries is limited to few reports from Norway (81), USA (119), UK (120), Belgium (121) and Greece (122). In addition, none of these studies reported data regarding the association between maternal and child cobalamin status.

A vast number of genetic variants associated with cobalamin absorption, transport or metabolism have been described. In this thesis, emphasis is put on non-synonymous polymorphisms, with a sufficiently high frequency of the variant allele to explore its effects on cobalamin status.

The sequencing of the *TCN2* gene led to the identification of numerous single nucleotide polymorphisms (SNP) (123,124). Bearing in mind the function of transcobalamin in the cellular uptake of cobalamin, genetic variants affecting this protein could have subtle, but potentially important effects on the intracellular regulation of cobalamin functions. The *TCN2* 776C>G (rs1801198) genetic variant is one of the most studied SNPs in the *TCN2* gene. The prevalence of the variant allele is high, although marked differences between ethnic groups exist (125,126). The variant G allele has been reported to affect 35-45% of

Caucasians in convenience samples. The common cytosine to guanine substitution at base position 776, results in the replacement of proline (CCT) with arginine (GCT) at codon 259 (127). A higher transcription of *TCN2* has been associated with the C allele in comparison with the G allele (127,128). Consistent with these results, lower transcobalamin concentrations have been observed in variant homozygotes (127–130). These findings led to numerous reports in which the associations between this SNP and cobalamin metabolism biomarkers were reported, however, with different findings (125,128,131,132). It has been consistently reported to modify the effect of cobalamin status on plasma tHcy (125,133–135). The presence of the variant allele has been associated with higher tHcy in subjects with low cobalamin status. As for MMA, the effects remain inconclusive (132,134). The effects during pregnancy on MMA have not been studied yet, and the effects on tHcy are limited to case-control studies. However, this SNP has been associated with lower transcobalamin, holoTC and holoTC/ transcobalamin ratio (129). The only study performed in children reported higher tHcy in homozygous variant compared to common genotypes (136).

A key enzyme in the cobalamin-dependent remethylation of Hcy is MTR whose methylcobalamin cofactor cycles between different oxidation stages. During this process, MTRR maintains cobalamin in its active form, and transfers a methyl group from SAM to cobalamin. This enzyme does not only participate in the reductive activation of MTR (137,138), but also in its stabilisation (139). Two of the most studied non-synonymous polymorphisms of the gene encoding this enzyme are located at the 66 (rs1801394) and 524 (rs1532268) loci. Both genetic variants reduce the affinity of MTRR for MTR leading to lower reactivation of the enzyme (140). The genetic variant at locus 66 (adenosine to guanine substitution) is located within the flavin mononucleotide binding domain of the enzyme and may interfere with MTR (140). This polymorphism encodes a methionine in place of isoleucine at codon 22. The cytosine to thymidine substitution at base position 524, results in the replacement of serine with leucine at codon 175. The frequency of the variant

allele at the 66 loci is highest in Caucasian (20-38%), and much lower in Asian (7-10%) and African (6-8%) populations (141–143). The frequency of the variant allele of the *MTRR* 524C>T was 37% in a population based study carried out in Spain (144). Metabolic effects have been largely studied for the 66A>G, but not for the 524C>T SNP. However, conflicting effects of *MTRR* 66A>G on tHcy have been reported. Some authors have observed higher tHcy concentrations in GG carriers compared to AA (145–148), while others found no differences (149–155). It is important to highlight, that gene-nutrient and gene-gene interactions may partly explain these inconsistent findings. In a recent study carried out in a Spanish population, neither *MTRR* 66A>G nor 524C>T genetic variants were associated with plasma tHcy in unadjusted models, but these effects were modified when stratified by cobalamin and/or riboflavin status (only for *MTRR* 524C>T) (156). In contrast, analysing the same population data, the authors observed lower plasma tHcy in the presence of the *MTRR* 524 TT compared to CC genotype (144). This time, the associations were adjusted for various SNPs related to folate transport or metabolism, and to B vitamins status, among other environmental factors. This illustrates the importance of considering multiple nutrients and SNPs affecting 1C metabolism in analytical models rather than isolated enzymes or nutrients. The effects of the *MTHFR* 677C>T SNP were modified by both the *MTRR* 66A>G and 524C>T SNPs (144).

Cobalamin status and adverse pregnancy outcomes

Globally, clear recommendations to take folic acid-containing supplements from preconception throughout the first trimester to prevent NTDs are widespread (157). However, no recommendations are in place for cobalamin supplement use. In addition, most prenatal supplements contain, if any, such small amounts of cobalamin that they are unlikely to correct marginal deficiencies.

Introduction

Over the years, a significant number of authors have reported on the associations between inadequate cobalamin status during pregnancy and adverse pregnancy outcomes. **Table 2** summarises the published prospective birth cohort studies performed to analyse the associations between cobalamin from preconception and during pregnancy with miscarriages, preterm live births, small-for-gestational age (SGA) and birth weight. Elevated plasma tHcy has also been associated with various adverse pregnancy outcomes, but these are described in the following chapter.

The effect of cobalamin status on materno-foetal health is evident from the preconception period. Adequate preconception cobalamin concentrations (≥258 pmol/L) were associated with a 60% reduced risk of preterm birth (odds ratio: 0.4; 95% confidence interval [CI]: 0.2, 0.9) (158), but did not affect pregnancy loss or probability of conception in Chinese women (159). This is relevant because preterm births accounted for 29% of neonatal deaths worldwide in 2010 (160,161) and postnatal survival increases with increasing gestation length (162). Cobalamin deficiency, and markers of impaired cobalamin metabolism have been associated with increased risk of early or spontaneous and recurrent miscarriage (96,163). However, to date, these effects have not been confirmed in prospective cohort studies (88,100,158,159,164–166). In India, first and second trimester low cobalamin intakes have been associated with increased risk of SGA (166). In line with these results, low cobalamin status in the same stages of pregnancy was associated with increased SGA risk. Most of the available evidence supporting a deleterious effect of inadequate cobalamin status during pregnancy is limited to developing countries. In industrialised countries, there is no apparent effect of low maternal cobalamin on adverse pregnancy outcomes (91,100,164,165), even if cobalamin inadequacy during pregnancy is also highly prevalent (91,93,100). In addition, available evidence is restricted to a small number of publications, and some methodological flaws are apparent, such as the use of non-fasting blood samples and large inter-individual variation in the timing of blood sampling.

Table 2. Summary of prospective cohort studies assessing the link between cobalamin status and adverse pregnancy outcomes.

Country, Year	Outcome	n (%)	Stage	Cobalamin (pmol/L)[1]	Change or effect	RR or means difference (95% CI)
China, 2002 [2] (158)	Preterm live birth	29 (6.7)	Prec[3]	363 (122)	<258 vs. ≥258 pmol/L	0.4 (0.2; 0.9)
	SGA	65 (15.4)				0.9 (0.3; 2.3)
	LBW	33 (7.8)				1.1 (0.5; 2.3)
UK, 2006 (164)	Miscarriage	25 (5.2)	Egg recovery	-	1 unit increase	0.9 (0.6; 1.2)
India, 2006 (88)	SGA	108 (28.6)	12 GW	232 (87)	Tertiles (T1 vs. T3)	6.0 (1.7; 20.7)
			24 GW			9.3 (2.9; 29.7)
			34 GW			2.8 (1.0; 7.9)
Japan, 2007 [2] (165)	Birth weight	96	7-14 GW	405 (146)	1 unit increase (pmol/L)	-1.05 (p= 0.08)
			26-29 GW	301 (96)		-5.3 (p= 0.38)
			34-36 GW	265 (95)		0.78 (p= 0.44)
China, 2007 [2] (159)	Miscarriage	139 (29)	Prec[3]	363 (122)	<258 vs. ≥258 pmol/L	0.9 (0.5; 1.6)
	Conception[4]	486 (42)				0.9 (1.6; 1.3)
Netherlands, 2012 [2] (100)	Preterm live birth	175 (5.7)	13 GW	169	Quintiles	No association
	SGA	135 (4.4)				No association
	Preeclampsia	65 (2.1)				No association
India, 2013 [2] (166)	SGA	99 (31.6)	12 GW	186	Tertiles (T1 vs. T3)	1.4 (1.0; 2.2)
			24 GW	182		1.5 (0.9; 2.3)
Netherlands, 2015 [2] (91)	GA at birth	1950	13 GW	234 (94)	Quartiles (Q1 vs. Q5)	No association
	Birth weight					No association

GW, gestational weeks; LBW, low birth weight; Prec, preconception; Q, quartiles; RR, relative risk; SGA, short-for-gestational age; T, tertiles.

[1] Mean (SD). When units were pg/mL, mean and SD were multiplied by 0.738.

[2] Fasting conditions not reported or non-fasting confirmed.

[3] Preconception was, on average, two menstrual cycles earlier than conception.

[4] Conception was based on daily urinary human chorionic gonadotropin.

Cobalamin's crucial role in DNA synthesis and repair is established but evidence for an effect on pregnancy anaemia is limited to cross-sectional or case-control studies (167–170). In addition, NTDs, which are severe birth defects caused by failure of the neural tube to close during early foetal development, have been linked with cobalamin deficiency in populations with widespread low cobalamin or replete folate status. The prevalence of NTDs is 6.7 to 8.2 per thousand live births in some parts of India (171), where cobalamin deficiency is widespread (172,173). The role of folic acid in preventing NTDs is well established, however, its protective effect may be limited in cobalamin deficient subjects. In Canada, where folic acid fortification began in mid-1997, Ray and collaborators reported that in women with holoTC concentrations ≤55.3 pmol/L the risk of NTD-affected pregnancy was almost tripled compared to women with holoTC concentrations over 121 pmol/L (adjusted OR: 2.9; 95% CI: 1.2, 6.9) (61). The importance of cobalamin in the prevention of NTDs was also observed in an Irish case-control study (44). The authors recommended then, that all women should aim for plasma cobalamin concentrations >221 pmol/L at preconception in order to reduce the incidence of NTDs. Recently, the WHO has recommended more research on the added value of cobalamin in the prevention of NTDs (174).

The effect of the *TCN2* 776C>G genetic variant on NTDs remains inconsistent (175,176). However, a role in the aetiology of Down Syndrome (177,178) and miscarriage (179,180) has been suspected. Other adverse pregnancy outcomes in relation to the *TCN2* 776C>G SNP have not been investigated.

As is the case for the *MTRR* SNPs, the effect of the maternal *MTRR* 66A>G SNP on NTDs varies according to cobalamin status (181). Moreover, a meta-analysis on the potential association between this variant and NTD risk concluded that the maternal 66 GG genotype increased the risk by 55% (182). However, available evidence has shown inconclusive results, with some studies either reporting no effects (183–185) or increased risk of NTD (176,186,187). Congenital defects apart from NTDs, such as congenital heart defects or

Down Syndrome have been linked with the *MTRR* 66A>G genetic variant (188–190). A higher spontaneous preterm birth prevalence was reported in women with the homozygous variant compared to homozygous common genotype (191). Surprisingly, the variant allele was associated with reduced BMI in adolescents (192); however these findings have not been replicated yet.

3. Folates

Food folates and folic acid: sources, bioavailability and requirements

Folate is a generic term for a family of water-soluble vitamins, structurally related and required for intracellular DNA, RNA and amino acid synthesis. Folate-dependent enzymes exceed intracellular folate concentrations (193), and thus the diverse biosynthetic pathways compete for a limited pool of folate (194,195).

As in the case of cobalamin, mammals cannot synthesise folate and require preformed folates from the diet. Naturally occurring folates are a mixture of reduced forms of the vitamin (predominantly 5-methylTHF), and are usually found in the polyglutamylated form, containing a varied number of polyglutamate residues. In contrast, the synthetic form (folic acid), which is found in fortified foods or supplements, contains only one glutamate residue and is fully oxidised (196), and considerably more resistant to thermolabile destruction and more stable than naturally occurring food folates (197). Mandatory folic acid fortification of enriched cereal grain products began in January and November 1998 in the USA (198) and Canada(199) respectively, and has led to an increase in the folate content of staple foods such as bread, rice and pasta. In the NHANES 2003-06 from the USA, daily folic acid intake was estimated to be 117 µg for women consuming only enriched cereal-grain products, but reached 621 µg per day in women also consuming folic acid supplements and ready-to-eat breakfast cereals (14% of the population) (200). The polyglutamylated forms constitute two-thirds of the total folate intake in the non-fortified Norwegian population, and are mainly consumed through vegetables, bread and potatoes (201). In the USA, before mandatory fortification with folic acid, orange juice, white bread, dried beans, green salad and ready-to-eat breakfast cereals were the major sources of folate, contributing to 37% of the total daily intake (202).

Controlled long-term feeding trials have estimated that the relative bioavailability of food folates is lower than that of folic acid, but varies widely between studies, ranging from 30-

98% (203–207). Factors such as intestinal hydrolysis of polyglutamate residues, food folate conjugation, folate absorption in the small and large intestine, food matrix, instability of labile folates before ingestion or during digestion, and genetic variants that affect folate absorption or transport can potentially modulate the bioavailability of food folates (208). Other studies have estimated the bioavailability of food folates to be 50%, and that of folic acid (either in fortified food or as a supplement) 85% when taken with foods, or 100% when taken with water on an empty stomach (59,209). Thus, folic acid is 1.7 or twice as bioavailable as folates from food sources and bioavailability must be considered when assessing dietary intakes. Dietary Folate Equivalents (DFE) are defined as the quantity of food folates, plus the quantity of folic acid in the diet multiplied by 1.7 (210). This approach is used to calculate RDAs in some (59), but not all countries (60). RDAs calculated by the IOM and the Spanish recommendations for daily folate intake from preconception and until mid-childhood are described in **Table 3.**

In 1991, a study on the effects of daily preconceptional folic acid (4 mg) supplementation on the risk of recurrent NTDs, reported a 72% (relative risk: 0.28; 95%CI: 0.12-0.71) reduction in the risk of NTD recurrence (6). One year later, Czeizel and coworkers reported a reduction in the first occurrence of NTDs after preconceptional supplementation of multivitamins containing 0.8 mg of folic acid, other vitamins and trace elements (7). The IOM recommends that all women capable of becoming pregnant take 400 µg/dday of folic acid from supplements or fortified foods to prevent NTDs (59). These recommendations have also been adopted in Spain (60). During pregnancy, the RDA is set at 600 µg of DFE, independently of age. This recommendation stemmed from the fact that low dietary intake plus 100 µg is insufficient to maintain normal folate status, and so, 200 µg of DFE were added to the Estimated Average Requirement (EAR) of non-pregnant women. The Spanish RDA is set at 500 µg of folate per day. The adequate intake for infants from birth to 6 months is set at 65 µg/day and is derived from the average daily volume of breast milk (0.78 L) and the average folate concentration of human breast milk after 1 month of

lactation (85 µg/L). Adequate intakes for infants aged between 7 and 12 months, were extrapolated from the adequate intake for 0-6 month infants, and is set at 80 µg/day. The Spanish RDA equals the adequate intake for younger infants, and is lower than the IOM for infants aged 7-12 months (50 µg/day). The RDA for children aged 1-8 years was set by extrapolation from adult values.

Table 3. RDA for folates (µg/ day) from preconception until mid-childhood.

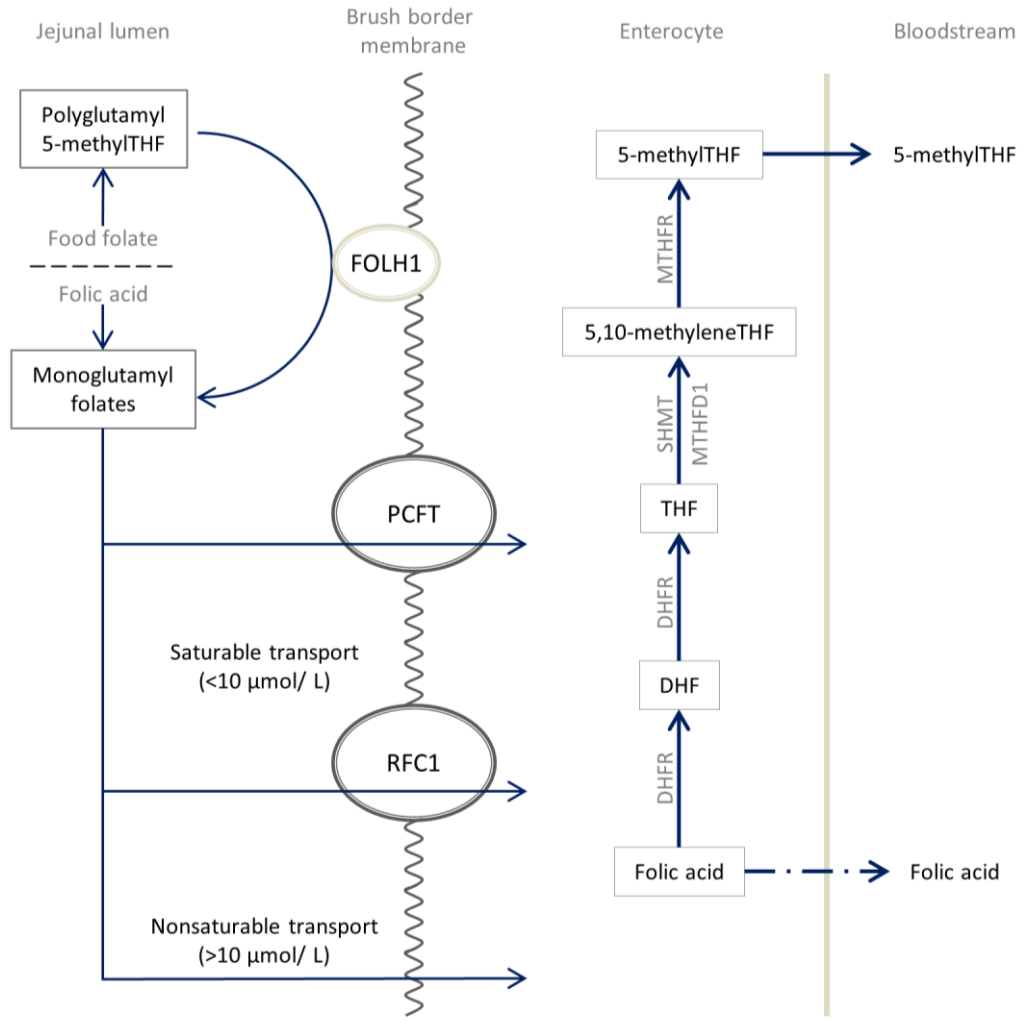
	IOM (59)[1]	**Spanish** (60)
Preconception	400[2]	400
Pregnancy	600	500[3]
0-6 months	65[4]	60
7-12 months	80[4]	50
1-3 years	120	100
4-5 years	160	150
6-9 years	160	200

[1] Dietary folate equivalents, otherwise indicated.
[2] Folic acid.
[3] Second half of pregnancy.
[4] Adequate Intakes.

Absorption, transport and metabolism

Folate absorption is a multistep process that primarily occurs in the duodenum and upper jejunum and involves a number of transport systems that operate efficiently within the acidic medium of the intestinal surface (211). The polyglutamylated forms (most of the naturally occurring food folates) must undergo deconjugation by brush border membrane folate hydrolase 1 (FOLH1, EC 3.4.17.21) in the small intestine before absorption. This is an obligatory step in folate absorption, given that only monoglutamyl forms cross cell membranes (**Figure 5**). After this step, folate monoglutamates are absorbed through saturable or nonsaturable mechanisms. Saturable mechanisms include a number of carrier-mediated transport proteins that include the reduced folate carrier (RFC1) and the proton

Figure 5. Absorption and hydrolysis of folic acid and food folates, and transport across the brush border membrane.



Abbreviations: DHF, dihydrofolate; DHFR, dihydrofolate reductase; FOLH1, ; MTHFD1, methylenetetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; PCFT, proton-coupled folate transporter; RFC1, reduced folate carrier; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate. Food folates are mainly 5-methylTHF, and thus can cross to the bloodstream readily after its deconjugation. Adapted from (487) and (208).

coupled folate transporter (PCFT) (212). The RFC1 has a low affinity for reduced folate and a highly reduced affinity for folic acid. In contrast, the PCFT has a similar affinity for both the reduced forms and folic acid, which is readily transported across the intestinal epithelium

by PCFT. Both transmembrane transporters are expressed on the apical membranes of the intestine, and their mRNAs are up-regulated under folate depletion conditions (213).

Given the acidic medium of the intestine, most of the absorbed folate is internalised to the enterocytes by PCFT, and not by the RCF1, which functions optimally in neutral mediums (212,213). Folic acid is metabolised to reduced folates, primarily 5-methyl-THF, during its passage through the enterocytes, however, low levels of unmetabolised folic acid (free folic acid) may be found in plasma from subjects consuming folic acid from supplements or fortified foods. DHFR mediates the reduction of folic acid, and its activity in humans is limited and varies considerably between persons (214). This limited ability to activate the synthetic form of folic acid may raise folic acid in circulation. Folic acid may appear in the circulation as unmetabolised folic acid in different degrees without being previously reduced. This form was detected in 94% of Irish elderly unexposed to folic acid fortification, however, it only accounted for 1.3% of total plasma folate (215). Other studies have reported that plasma free folic acid concentrations increase with folic acid supplementation (216). More recently, unmetabolised folic acid has been reported to be detectable across all age ranges in the USA population (217), but in a higher proportion (4%) than in Irish elderly adults (216). However, it is important to highlight that no differences in unmetabolised folic acid concentrations were observed between women randomly allocated to receive 400 µg of folic acid/day throughout the second and third trimester and placebo (218).

Cellular folates exit the basolateral membrane by a mechanism probably involving the multidrug-resistance-associated protein (219). Circulating folate, mainly 5-methylTHF, binds to low -affinity proteins, with 50% of it bound to albumin. A smaller amount is bound with high affinity to folate receptors (FR), a family of three closely related folate-binding proteins (FRα, FRβ and FRγ) that can mediate the internalisation of folates into cells. Membrane transport of folates from the portal system, where the pH is 7.4, into tissue cells is principally mediated by RFC1, and not PCFT. Transplacental transport may be mediated either by FRβ, RFC1 or PCFT because all are expressed in placental tissue (212,220–223).

Folate concentration in red blood cells (RBC) is much higher than in plasma, and almost all RBC folates are 5-methylTHF polyglutamates. The polyglutamate peptides must be re-established by folylpoly-γ-glutamate synthetase (FPGS, EC 6.3.2.17) before they can be retained in the cell for its normal metabolic functioning (224). Substrate competition for the FPGS limits the extent of polyglutamylation, and thus the degree in which folates are sequestered by the cell (225). Moreover, polyglutamylation increases folate affinity for folate-dependent enzymes (226). Monoglutamate forms not metabolised to longer-chain polyglutamates may be lost from the cell. As the catalysis of 5,10-methylTHF is irreversible *in vivo*, cobalamin deficient subjects experience a block in the cobalamin-dependent Hcy remethylation pathway. Given that 5-methylTHF cannot bypass the block imposed by the limited cobalamin, folates are unavailable for DNA synthesis and repair, mimicking folate deficiency which is known as the "methyl folate" trap hypothesis (227). In addition, 5-methylTHF has a low affinity for FPGS (228), and thus 5-methylTHF is not retained in the cell, causing decreased intracellular folate and it to be increased in the bloodstream in in vitro and in vivo models (229,230). However, to the best of our knowledge, this hypothesis has been scarcely investigated in humans.

The predominant form of folate found in foods (5-methylTHF) is readily used as a substrate for the remethylation of homocysteine to methionine, by MTR whose cofactor is cobalamin. This reaction also produces THF, which can be methylated by glycine, and thus form 5,10-methyleneTHF, or can be catalysed to generate formate and enter the purine synthesis pathway. The MTHFR enzyme (EC 1.5.1.20) catalyses the NADPH-dependent reduction of 5,10-methyleneTHF to 5-methylTHF, which carries the methyl group for MTR. The MTHFR is the only enzyme known to generate 5-methylTHF and is crucial in the folate-cobalamin metabolic axis within the cell because it regulates the balance between thymidylate (for DNA synthesis) and methionine synthesis (methylation reactions). This reaction is irreversible in vivo (231) and diverts one methyl group from thymidylate synthesis to Hcy remethylation.

Plasma folate and RBC folate have been widely used to assess folate status in population-based studies. In addition, plasma tHcy is used as a non-specific functional biomarker for folate status (232).

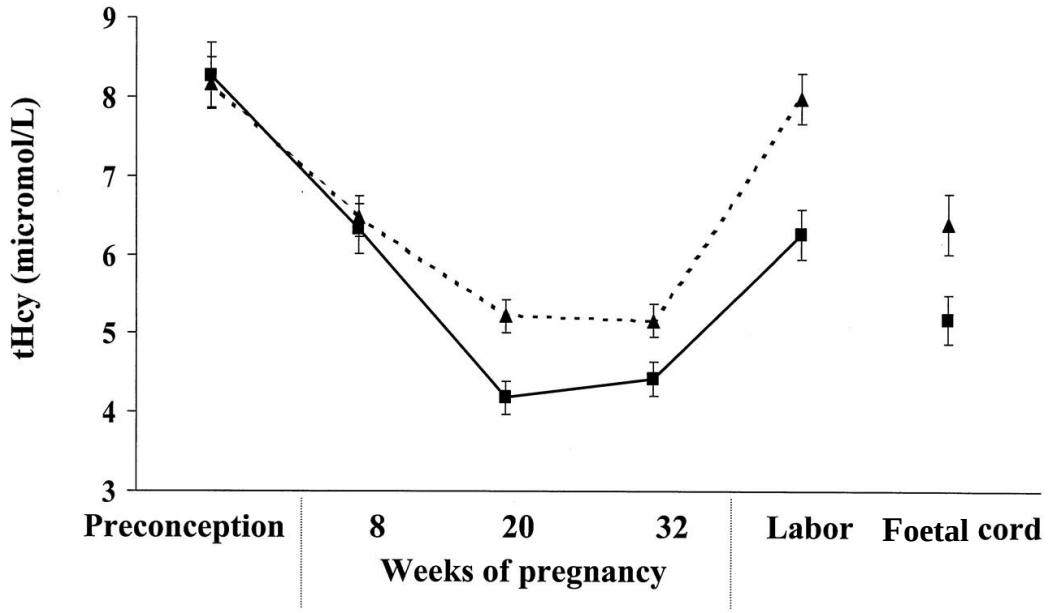
Folate status and its determinants from preconception to mid-childhood

The prevalence of folate deficiency is not widespread worldwide (233), but is highly prevalent in some countries such as France (234) or Spain (144). It is not associated with development level or geographical location, and may be widespread in well-nourished populations (233). The cut-off point for plasma folate deficiency was originally set at 7 nmol/L (235). Nowadays, plasma folate <10 nmol/L is considered deficient, based on increased plasma tHcy compared to at higher folate concentrations (236). This cut-off has been adopted by the WHO for the assessment of population folate status (237). However, serum folate is highly responsive to folic acid intakes, and is considered a measure of recent intake. RBC folate represents the amount of folate that accumulates in blood cells during erythropoiesis, and is a longer-term indicator of folate status, which is particularly critical in the early stages of development. The threshold for folate deficiency according to RBC was set at 340 nmol/L (237). Inadequate intake is the leading cause of folate deficiency, and increased requirements are also an important cause. Pregnancy has been recognised as a period when folate requirements are increased to support rapid cell replication and growth in foetal, placental and maternal tissues. In 2015, the WHO recommended an optimal threshold for RBC folate of 906 nmol/L (174) above which the greatest prevention of NTD would be achieved in women of reproductive age (238,239). In the NHANES 2007-12 survey, in the USA, Tinker and colleagues reported suboptimal RBC concentrations in 22.8% of women of childbearing age (240). These results suggested that a higher frequency might be expected in countries without mandatory fortification. The prevalence of folate deficiency (plasma folate <7 nmol/L) in women of childbearing age was 24.2% in a Spanish

population study that excluded B vitamin supplement users and in the absence of mandatory folic acid fortification (144). Moreover, folate deficiency during pregnancy has been shown to be highly prevalent in Western countries where fortification with folic acid is voluntary (91,93,100).

Substantial changes in folate biomarkers occur during pregnancy. Plasma folate decreases rapidly from the first trimester (241–243); with a larger reduction in women that cease regular folic acid supplement use (244) and in variant allele carriers of the *MTHFR* 677C>T polymorphism (245). Although RBC folate has been reported to follow the same pattern (244,246,247), a moderate increase was reported in another study (248). However, this study included only women with uneventful pregnancies and normal outcomes, and folic acid supplement regime was not reported. Serum folate does not decrease when folic acid supplementation is continued throughout pregnancy and in fact, RBC folate increases (249). **Figure 6** shows the fluctuations of plasma tHcy from preconception until labour and in cord blood according to folic acid supplementation. THcy decreases by 30% from preconception until the first half of pregnancy, and then increases, almost reaching preconception concentrations by labour (38,250). The decrease in early pregnancy is not explained by folic acid supplementation, serum albumin decline, nor haemodilution, and has been proposed to be endocrine-based (250).

Figure 6. Plasma tHcy concentrations at preconception and during pregnancy and cord blood according to folic acid supplement intake.



Geometric mean (bars define standard error). Continuous line: women taking folic acid supplements, dotted line: women not taking folic acid supplements. From (38). Unsupplemented group (▲): preconception and 8 and 20 weeks of pregnancy (n = 54); 32 weeks of pregnancy (n = 53); labour and foetal cord (n = 47). Group taking folic acid supplements during the second and/or third trimesters (▪): preconception (n = 39); 8 and 20 weeks of pregnancy (n = 35); 32 weeks of pregnancy (n = 38); labour (n = 37); foetal cord (n = 36). From (38).

Most of the surveillance on folate status focuses on adults, however, some publications report on folate status in UK, Norwegian and USA children. In infants, both serum and whole blood folate are high during the first year of life, and decrease to the median concentration found in early childhood (81). The low cobalamin status in the first year of life could explain the elevated serum/plasma folate status found in this age group, as a result of the "methyl folate" trap, which would lead to high plasmatic folate status (227). Moreover, the reduction of both tHcy and serum folate following cobalamin injections further supports this hypothesis. After this age, there is a decline in folate status from childhood to adolescence (81,119,120). The mechanisms underlying this decline, despite a

normal folate intake, are not clear. However, it might suggest increased folate requirements in order to sustain growth. Folate status in pre-school and school age children seem not to be compromised based in reports from developed and less developed countries (81,94,119,120,251–253).

In 1997, only one third of USA women of childbearing age consumed supplements containing the recommended daily amount of folic acid (254). As a result, mandatory folic acid fortification programmes were implemented in the USA and Canada in 1998. In 2015, 79 countries had implemented the mandatory fortification at least of industrialised milled wheat flour with folic acid (255). Such policies have notably increased folate status. In the USA, post-fortification RBC folate was 1.5 times greater than pre-fortification (11). Furthermore, tHcy was reduced by approximately 10% (11). Moreover, in the Framingham Offspring Study cohort, folate deficiency (<7 nmol/L) decreased from 22 to 1.7% and hyperhomocysteinemia (>13 μmol/L) decreased from 18.7 to 9.8% after fortification (256). The same effect on RBC folate was observed in Canadian women of reproductive age (257), and in Chilean women whose RBC folate increase was notably higher (250%) (258). Voluntary fortification of foods has also enhanced RBC folate, but only in subjects consuming more than 40 μg/day of folic acid from fortified foods (259). However, only 55% of the women that consumed ≥99 μg/day of folic acid from fortified foods had optimal RBC concentrations (>906 nmol/L).

Although folic acid intake is one of the major determinants of folate concentrations during pregnancy, other factors also affect folate status. In adults, several drugs, such as antiepileptic (260,261), folate antagonists (262) or antihypertensive drugs (263) have been associated with lower plasma folate and higher tHcy concentrations. Higher BMI (91,264–266), impaired renal function (267), age (100,268), smoking (100,268) and a low socioeconomic status (91,100,268) have also been linked with lower serum folate and higher tHcy both in adults and during pregnancy. High BMI could reflect low folate intake and a low quality-diet containing small amounts of green leafy vegetables and fruits.

However, the association between BMI and RBC folate is not consistent, with observations either reporting positive (264,266) or negative effects (269–271). These disparities between serum and RBC folate according to BMI, suggest that BMI affects cellular uptake and tissue distribution of folate. This hypothesis was confirmed by the observation of different pharmacokinetic responses to folic acid supplementation between obese and normal weight women of childbearing age (272). It is important to highlight that, the contraposed effects of BMI on serum and RBC folate were only found in countries where folic acid fortification is mandatory (264,266,272–274). In non-fortified populations (before the implementation of fortification, or never fortified), BMI is not associated with RBC folate (269–271,275). Whether this phenomenon occurs in a population exposed to voluntary fortification, but with an elevated consumption of folic acid supplements is still unknown, and may have relevant implications since both low folate and high BMI are risk factors for NTD affected pregnancies.

In children, BMI has also been negatively associated with serum folate and positively with both tHcy and RBC folate (265,274,276). In breastfed infants, human breast milk folate is maintained at the expense of maternal stores (277) and maternal and cord blood folate concentrations are the main determinants of folate status at the age of 6 months (105). In contrast to cobalamin concentrations, folate concentrations in neonates are positively associated with duration of exclusive breastfeeding (97). This may be due to the high bioavailability of folate from breast milk (278). In older children consuming ready-to-eat breakfast cereals, serum and RBC folate and tHcy concentrations were higher and lower respectively compared with non-consumers (120). It remains unclear whether the effect of maternal and exclusive breastfeeding on child folate biomarkers persist later than two years of age. No sex-differences in folate status biomarkers have been observed for children under 15 (83,113,120,279).

Genetic determinants of folate biomarkers have been widely studied. The genetic variant that has attracted most of the attention in the last decade is a nonsynonymous mutation at base position 677 of the gene encoding the MTHFR enzyme (rs1801133) (280). This cytosine to thymine substitution results in the replacement of an alanine with a valine at codon 222 and produces a thermolabile enzyme with reduced activity *in vivo* (280). MTHFR catalyses the irreversible reduction of 5,10-methyleneTHF to 5-methylTHF, and thus can have a wide range of metabolic effects. The worldwide frequency of this polymorphism has marked geographical and ethnic differences. The variant homozygous genotype is more common in northern China (20%), southern Italy (26%) and in Mexico (32%) according to a report from 2003, in which over 7000 newborns from 16 areas in the world were screened for the *MTHFR* 677C>T polymorphism (281). The lowest prevalence of the variant homozygous genotype was found in Canada, Finland and the USA and in newborns of African descent. Newborns of American Hispanic descent had the highest frequency of the variant allele. The metabolic phenotype of the variant allele of the *MTHFR* 677C>T polymorphism has been well established. A recent meta-analysis showed that variant homozygous subjects had, on average, 13% (95% CI: 7-18%) and 16% (95% CI: 12-20%) lower plasma/ serum and RBC folate, respectively, compared to common homozygous (282). Out of numerous SNPs affecting folate metabolism and transport, the *MTHFR* 677C>T had the greatest effect on folate status in a population that was unexposed to mandatory folic acid fortification or to folic acid supplement use (144). Moreover, *MTHFR* 677 TT is associated with higher plasma tHcy and lower RBC and plasma folates irrespective of the folic acid supplementation regime during pregnancy (29). Despite the well-established metabolic effects of this genetic variant in adults, its impact during childhood has been scarcely studied. However, available evidence suggests that the impact of this genetic variant on tHcy may be limited to older children (>10 years) (279,283,284), but increased tHcy in children with the homozygous variant genotype (2-17 years) has also been observed. The effects of the *MTHFR* 677 TT genotype on tHcy concentrations might be limited to boys (285) or to children with low folate status (284).

Folate status, homocysteine and adverse pregnancy outcomes

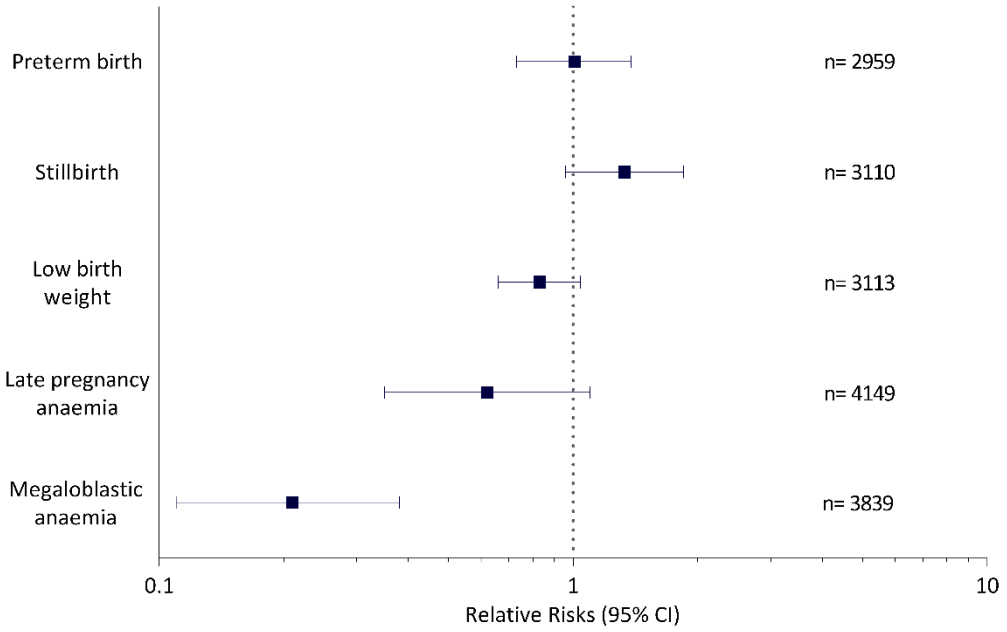
The critical and multiple cellular pathways, which depend on folate, highlight the decisive role this vitamin exerts in the establishment of a healthy ageing phenotype. Epidemiological studies have suggested that inadequate folate status is associated with increased risk for leukaemia, lymphoma and colorectal, breast and prostate cancers (286–290). A dual effect of folate on cancer risk has been suggested. Given the role of the folate cycle in DNA repair and synthesis, folate can either prevent, in non-cancerous cells, events that initiate mutation transformations, and may promote their proliferation in cancer cells. Mason et al. observed in the USA and Canada that absolute rates of colorectal cancer began to increase in 1996 (in the US) and 1998 (in Canada), peaking in 1998 and 2000 in USA and Canada respectively, continuing to exceed the pre-fortification trends by 4-6 additional cases per 100000 subjects (291). Although these observations do not provide causal evidence, the authors suggested that the initiation of folic acid fortification programmes could have induced the progression of lesions from adenomas or larger cancers that would hence become more evident at the time of routine screening. Moreover, the postfortification deviation from the downward trajectory was only temporary and with time returned to the original downward trend. It has been suggested that fortification uncovered neoplasms that would have been eventually recognised with time.

A recent meta-analysis that included 50000 participants from 13 placebo-controlled clinical trials (10 trials for the prevention of cardiovascular disease and 3 in patients with colorectal adenoma), concluded that folic acid supplementation (>0.5 mg/ day) does not significantly increase or decrease the incidence of overall (RR, 1.06; 95% CI: 0.99-1.13) nor site-specific cancer during the first 5 years of intervention (292). The lack of effects of homocysteine-lowering on the recurrence of cardiovascular events suggests that neither folic acid nor tHcy reduction prevent clinical events once the disease is already established (293).

Multiple B vitamin supplementation was reported to have beneficial effects against stroke, cognitive impairment and depression (40,294–296), however these results were not supported by a meta-analysis including 22000 participants. However, there was important heterogeneity in baseline participant conditions (healthy and severely demented or with excellent or poor B vitamin status were included) and intervention duration in the studies included in the analysis (297).

The importance of folates, and a major driver of much of the attention regarding this vitamin, stems from its link with specific developmental disorders associated with anomalies in neural tube closure. There is strong and consistent evidence suggesting a causal role for folate in the incidence of NTDs, however, the underlying mechanisms have not been fully elucidated. It is more likely that complex interactions within the 1C metabolism network underlie the development of NTDs attributed to folate inadequacy. Mandatory fortification with folic acid reduced the prevalence of NTDs by 30% in the USA (8) and almost 40% in Canada (9). In European countries, the prevalence of NTDs has not decreased despite the existence of a voluntary fortification policy and longstanding recommendations targeting the promotion of preconceptional folic acid supplementation (298). A recent meta-analysis including 2033 participants with a history, and 5358 with no history of NTD, found that preconception supplementation with folic acid reduced the risk of having an NTD affected pregnancy by 70% (RR, 0.31; 95% CI: 0.17-0.58) (299). However, there is insufficient evidence on the effect of preconception folic acid containing supplement use on the prevalence of other birth defects (299). Due to the relevance of folates in the prevention of NTDs, the effects of folic acid supplementation during pregnancy on adverse pregnancy outcomes have been extensively studied. **Figure 7** summarises the main results of a meta-analysis performed with data from randomised, cluster- randomised and cross-over controlled trials evaluating the supplementation of folic acid alone (or with other micronutrients) versus no folic acid (placebo, or same micronutrients but no folic acid) during pregnancy on adverse pregnancy outcomes (300).

Figure 7. Associations between folic acid supplementation during pregnancy and adverse pregnancy outcomes.



Relative risks and 95% confidence interval for the effect of folic acid supplementation during pregnancy versus no folic acid supplementation. Adapted from (300).

Supplementation with folic acid was only associated with a reduced risk of megaloblastic anaemia and with higher birth weight (mean birth weight difference: 135.8 g; 95% CI: 47.9, 223.7 g), although the risk of a birth weight under 2500 g was not reduced (300).

Impaired folate status has been associated with adverse pregnancy outcomes. Elevated tHcy has been consistently associated with different adverse pregnancy outcomes; birth weight is one of the most investigated. A negative association has been consistently found between maternal tHcy and birth weight in both industrialised and developing countries (38,39,100,301,302). In a previous thesis from our group, elevated first trimester tHcy (>7.4 µmol/L) was associated with lower birth weight (β-coefficient: 139.6 g; standard error: 54.3; p= 0.011), increased risk of IUGR (OR: 8.2; 95% CI: 1.1, 58.7; excluding smokers throughout pregnancy) and pathological foeto-placental Doppler function (OR: 3.1; 95% CI: 1.0, 9.2)

(303). Exposure timing is also essential in the association between tHcy and birth weight, which have been found as early as preconception, and until labour (38). In addition, the maternal variant allele of the *MTHFR* 677C>T SNP has also been associated with lower birth weight (39,302). **Table 4** summarises prospective cohort studies assessing the link between tHcy and adverse pregnancy outcomes other than low birth weight. Elevated tHcy increased by 27-90% the risk for miscarriage (or pregnancy loss) in three prospective cohort studies. However, the associations with preterm live birth (100,158) or preeclampsia (or pregnancy induced hypertension) remain inconsistent (100,301). It should be noted that blood samples were collected in fasting conditions only in one study (164),

Table 4. Summary of prospective cohort studies assessing the link between tHcy status and adverse pregnancy outcomes.

Country, Year	Outcome	n (%)	Stage	tHcy (µmol/ L)[1]	Analysis (µmol/ L)	Results (95% CI)
China, 2002 [2] (158)	Preterm live birth	29 (6.7)	Prec[3]	8.6 ± 3.4	>12.4 vs. ≤12.4	3.6 (1.3; 10.0)
UK, 2006 (164)	Miscarriage	25 (5.2)	Egg recovery	-	1 unit increase	1.3 (1.0; 1.6)
China, 2007 [2] (159)	Miscarriage	139 (29)	Prec[3]	8.6 ± 3.4	>12.4 vs. ≤12.4	1.5 (1.0; 2.5)
	Conception[4]	486 (42)				0.7 (0.3; 1.4)
Netherlands, 2012 [2] (100)	Preterm live birth	175 (5.7)	<18 GW	6.9	≤5.8 vs. ≥8.3	1.4 (0.9; 2.2)
	Preeclampsia	65 (2.1)				1.6 (0.9;2.9)
Canada, 2008 [2] (301)	Pregnancy loss	103 (4.9)	<20 GW	4.3-5.7	≥P_{90} vs. <P_{90} [5]	1.9 (1.1; 3.2)
	Hypertension	115 (5.7)				0.6 (0.3; 1.2)
	Preeclampsia	65 (3.2)				2.3 (1.3; 4.3)

GW, gestational weeks; Prec, preconception; tHcy, total homocysteine.
[1] Mean (or range) ± standard deviation (SD).
[2] Fasting conditions not reported or non-fasting confirmed.
[3] Conception was based on daily urinary human chorionic gonadotropin.
[4] Preconception was, on average, two menstrual cycles earlier than conception.
[5] Percentile 90 according to gestational age-specific tHcy deciles.

and those collected during pregnancy were drawn at mid-pregnancy, when tHcy has already started to decrease. This issue is very important because it adds inter-subject variability, and may account for many differences between observations of different studies. The *MTHFR* 677C>T genetic variant has also been linked with NTDs. A meta-analysis including 2000 subjects concluded that mothers with the *MTHFR* 677 TT genotype had doubled (odds ratio: 2.0; 95% CI: 1.5, 2.8) the risk of an NTD-affected pregnancy compared to the CC genotype (304). The effect of child genotype was almost the same as the maternal (odds ratio: 1.8; 95% CI: 1.4, 2.2). Studies investigating the associations between the *MTHFR* 677C>T genetic variant and other congenital defects have yielded inconclusive results (185,305). Other adverse pregnancy outcomes linked with the *MTHFR* 677C>T genetic variant include preeclampsia (TT vs CC: OR, 1.3; 95% CI: 1.1,1.5) (306) and unexplained recurrent pregnancy loss (TT vs CC and CT: OR 1.7; 95% CI: 1.3,2.1), but it was not associated with preterm live birth (307).

Folic acid safe use and its relationship with cobalamin status

The prevention of NTDs (8,9) and the reduction of folate deficiency and hyperhomocysteinaemia prevalence (256) are undeniable beneficial effects of folic acid fortification. Nevertheless, other unexpected health consequences attributed to folic acid fortification have been reported. Following folic acid fortification, haematocrit and haemoglobin increased (308) and anaemia attributed to folate deficiency was almost eliminated in the USA (309). However, other effects have raised some concerns regarding the safety of high doses folic acid use, despite relatively little evidence for causality. As described earlier in this chapter, a dual effect of folate on cancer risk has been suggested, and this is currently one of the major concerns with respect to folic acid fortification policies. In addition, higher prevalence of anaemia, cognitive impairment and an increase in MMA and tHcy concentrations has been observed in subjects with concomitant cobalamin

deficiency and elevated folate/folic acid use (310–312). This might be related to the presence of unmetabolised folic acid (313). These findings have never been reported in studies carried out in non-fortified countries (314,315), where the prevalence of subjects with elevated folate status is usually very low. Nevertheless, an Australian study conducted before the introduction of mandatory folic acid fortification, reported higher cognitive impairment in subjects with high RBC folate and low cobalamin status when compared to participants with normal status in both vitamins (316). Additionally, in subjects with normal cobalamin, elevated RBC folate was also associated with higher cognitive impairment. It should be highlighted that these studies were all observational, and thus causality cannot be ascertained. Several alternative hypotheses to a harmful effect of high folate status have been proposed. The severity of cobalamin deficiency is greater in subjects with elevated folate than in those with non-elevated folate status (317). The classification of subjects in the group of elevated folate and cobalamin deficiency artificially places individuals taking high amounts of supplements (or fortified foods) that contain both folic acid and cobalamin. Their cobalamin deficiency might thus have been caused by unrecognised cobalamin malabsorption, and hence be more severe. This was supported by a study by Miller and co-workers, in which the percentage of subjects taking B vitamins containing supplements was higher in cobalamin deficient subjects classified as having elevated folate (310). Furthermore, in cobalamin deficient patients, folates are released from the cell, leading to high circulating folate concentrations. This would indeed favour reverse causality (318,319).

Endorsing the safety of folic acid fortification, it is stated that the high folate status found in some subjects from the USA can only be achieved by a high intake of folic acid containing supplements and ready-to-eat breakfast cereals, and thus folic acid fortification is not the major contributor of high folate status (200). In Spain, modelled intake of fortified products as part of a regular breakfast meal would improve women's dietary folate intake, without exceeding the upper limit (1 mg/day) (320). However, the consumption of fortified products

in other meals was not explored and could potentially affect the achievement of the upper limit.

Given the current debate, no European country has implemented mandatory fortification of cereal grains with folic acid, and arguments both in favour and against fortification have emerged (321,322). In addition, neither voluntary fortification with folic acid nor various recommendations on folic acid supplementation in women of reproductive age have reduced the prevalence of NTDs in Europe (298,323). One of the population subgroups most exposed to high folic acid intakes are pregnant women. However, the metabolic or clinical effects of cobalamin deficiency in relation to high folate status or folic acid intakes have never been assessed in pregnant women. In Spain, folic acid supplements contain doses of folic acid ranging from 100 µg to 10 mg per tablet, and are recommended during pregnancy without taking cobalamin status into account.

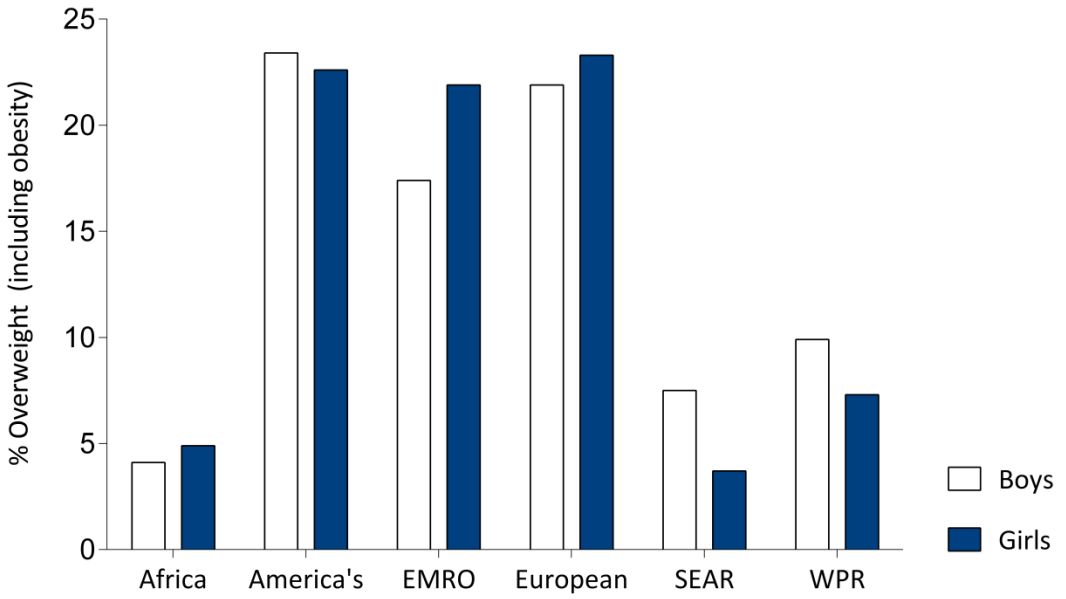
4. Growth and developmental programming

Childhood obesity: definition, prevalence and associated comorbidities

Obesity is defined by an excess of body weight in the form of fat, and today is recognised as the most important public health problem facing the world. The evolution in the number of publications arising from obesity research has doubled every 5 years since 1993 (324). It has been suggested that the ideal measurement of body adiposity should be accurate, precise, accessible, acceptable to the subject and well-documented (325). However, none of the currently used techniques meets all of these criteria. Among anthropometric measures, BMI, which is a measure of relative fatness, is the most documented due to its ease of measurement, acceptability and extended use. In adults, BMI cut-offs have been proposed according to an increase in the risk of mortality (326). Until early adulthood, BMI varies widely according to age and gender: it typically rises during the first months of life, falls until the age of 6 years, and then increases again (327–329). Thus, age- and sex-specific BMI cut-offs using either local or international data are needed in children and adolescents in order to define overweight and obesity.

The prevalence of child overweight including obesity is rising rapidly and is estimated to be 10% worldwide. Even though, marked geographical differences exist (**Figure 8**). In Africa, the prevalence is lower than 5%, but in Europe and the Americas more than one in five children are overweight (330). In Southern Europe, the prevalence is the highest among European countries, with a prevalence of 38.2% and 26.8% in Spanish girls and boys respectively (331). However, recent prevalence data in 5-7 year old European children reported that the prevalence is actually lower (332), a trend that has also been documented in most Southern European countries (332). Data from the USA does not suggest a decreasing trend, but rather a stabilisation in the prevalence of childhood overweight (333). In contrast, rates of severe forms of obesity show upward trends in the USA. In addition to the high prevalence rates of childhood overweight, comorbidities associated with elevated BMI such as dyslipidaemia, hypertension, impaired glucose

Figure 8. Worldwide prevalence of overweight in six WHO global regions according to the World Obesity Federation.



EMRO, Easter Mediterranean Region Office; SEAR, South East Asia Region; WPR, Western Pacific Region. Cut-offs for overweight were defined according to the World Obesity Federation (488).

tolerance and non-alcoholic fatty liver are some of the cardiometabolic complications observed in overweight and obese children (334–336). The effects of childhood obesity persist into adulthood as seen by the fact that 80% of obese children remain obese as adults (337). Overweight and obesity during childhood or adolescence has been linked with adult cardiovascular risk factors, cardiovascular heart disease and premature mortality (337–340). Risk of death from endogenous causes in children in the highest BMI quartile more than doubled (RR, 2.3; 95% CI: 1.5, 3.6) the risk observed for children in the lowest BMI quartile (338). However, the associations between childhood BMI and cardiovascular risk factors is lost after accounting for adult BMI (337,340). This suggests that cardiovascular risk factors associated with childhood overweight may be substantially reduced if childhood obesity is successfully treated. Taking into account both the prevalence and the increased mortality and morbidity associated with childhood obesity, it has been forecasted that the steady rise in life expectancy observed during the last two centuries may soon come to an end (341).

Nevertheless, public health experts have recognised the relevance of childhood obesity as a determinant of global health, and many secondary preventive strategies are being implemented such as school based educational programs (342,343). However, the effectiveness of interventions aimed at reducing child BMI either by school and/ or home-based programs has proven to be modest (344–348). Therefore, the identification of early life risk factors is crucial for the development of childhood obesity prevention strategies. Both genetic (349,350) and environmental (351–353) risk factors have been extensively documented. Given that 4-year-old obese children are already at increased risk of an adverse cardiometabolic profile (334), the interest in the study of the associations between early life risk factors (i.e. prenatal period) and cardiovascular health is rising.

Foetal origins of adult cardiovascular health

Introduction

The recognition by the WHO that healthy ageing trajectories can be modulated by prenatal exposures (5) supports the need to evaluate prenatal exposures that may lead to unfavourable health conditions. The metabolic programming of adult health and disease hypothesis proposed by Barker (354) plays a pivotal role. It has been consistently shown that low birth weight is associated with higher risk of cardiovascular disease, including ischaemic heart disease, type 2 diabetes and incident coronary artery disease (354–359). In addition, there is a 6% (95% CI: 3-8%) decrease in adult all-cause mortality for each additional kg in birth weight (360). In contrast, high birth weight has been associated with adult obesity (361). In these studies, birth weight has been used as a proxy of developmental exposures *in utero*. In line with this concept, other adverse pregnancy outcomes such as preterm delivery, gestational diabetes mellitus or excessive weight gain during pregnancy have been linked with offspring cardiometabolic risk factors both in children and adults (362–366). However, even though the treatment of mild gestational diabetes improves neonatal outcomes (367,368), it does not appear to prevent childhood obesity nor cardiovascular risk factors (369). This suggests that once the pathology is present, its treatment cannot reverse the mid-term impact on health. In low- and middle-income countries, per standard deviation increase in birth weight there is an increase in BMI by 0.5 kg/ m^2, in fat mass by 0.2 kg and in the risk of overweight by 28% (OR, 1.28; 95% CI: 1.21, 1.35) (370). In addition, faster relative weight gain at 2 years of age was associated with increased risk of adult overweight (OR, 1.51; 95% CI: 1.43, 1.60). These findings have also been reported in Western countries (361), where a positive association has been found between birth weight and rapid postnatal weight gain and adult central adiposity and BMI (371–374). In addition, early preterm (<34 GW) and late preterm (34-36 GW) birth increases the risk of adult obesity by 140 and 90% respectively, even after adjusting for birth weight (375).

Existing evidence proves a strong, positive and consistent effect of birth weight and preterm birth on subsequent BMI and central adiposity both during childhood and

adulthood. The above-mentioned examples show how the effect of environmental exposures in one generation shapes the development of the subsequent generation. The biological bases of this phenomenon are still unknown; however, two different mechanisms have been suggested: developmental plasticity and compensatory growth. Plasticity may be defined as the phenomenon by which different phenotypes arise from one genotype, in response to varied environmental conditions during development (376). This plasticity is understood to occur through epigenetic traits that do not modify the genomic sequence and include both DNA (377) and histone (378) methylation. It is in these pathways where 1C metabolism plays a crucial role in transporting and transferring methyl groups essential for methylation reactions. Moreover, different biomarkers, nutrients and genetic variants affecting enzymes from the 1C metabolism have been linked with a numerous pregnancy and neonatal complications, as highlighted in the previous chapters. It is possible that the mechanisms leading to adverse pregnancy outcomes may also lead to underlying pathological conditions in the foetus that eventually develop into chronic diseases in late life.

The role of one carbon metabolism in developmental programming: experimental evidence

The underlying biological mechanisms for the influence of developmental plasticity on the risk of chronic diseases are only beginning to be understood. Most of the evidence arises from experimental studies in which nutritional, endocrine, or physiological challenges during early development influence metabolic and cardiovascular functions in the offspring. A wide range of mechanisms have been proposed (379,380), however, the implication of the peroxisome proliferator-activated receptor (PPAR) family is one of the most studied in relation to 1C metabolism. This family of transcription factors regulates glucose and lipid metabolism, with PPAR-α involved in the catabolism of lipids, and PPAR-γ influencing the

storage of fatty acids in adipose tissue (381). Folate or cobalamin deprived female rats from preconception had lower placental expression of PPAR-γ than controls (382). A restricted diet in both folate and cobalamin during gestation and lactation led to a reduction in PPAR-α and increase in PPAR-γ expression in the myocardium in the offspring (379). Protein restriction in rat dams throughout gestation reduces PPAR-α methylation by 20.6% in their pups, resulting in a 10-fold increase in PPAR-α expression. These results have been supported by human randomised controlled clinical trials in which infant formula with a lower protein content reduces BMI and obesity risk (383) and preperitoneal fat (384) at school age. Supplementation with 5 mg/kg of folic acid during gestation in protein restricted rat dams prevented the effects observed in their pups (385). In addition to these metabolic effects, pups from cobalamin deficient dams had increased fat mass and decreased lean body mass (386). Decreased expression of protein arginine methyltransferase 1, of the deacetylase sirtuin 1 and increased SAH decreases methylation and/or increases acetylation of peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1-α) (379). This methylation/acetylation imbalance impairs the coactivation of PPAR described above (380). In contrast, *in vitro* studies in pre-adipocytes suggest that homocysteine downregulates PPAR-γ expression, thus suppressing adipogenesis (387). Overall, this evidence suggests that disturbances in 1C metabolism affect lipid and glucose metabolism, through PPARs methylation, increasing fat deposition and reducing lipid catabolism in pups born to 1C nutrient deprived animals.

The balance in growth hormone/ insulin-like growth factor 1 (GH/IGF-1) has also been identified as a mediator of the restricted growth observed in cobalamin deficient animals (388). Cobalamin deficiency in dams induces a decrease in liver taurine production in the offspring, which subsequently leads to the impairment of the GH/IGF-1 axis and to growth hormone resistance, with a dramatic decrease in osteoblast number and bone formation rate. This explained the impaired weight gain and reduced body size during weaning in pups born to cobalamin deficient dams.

Early life one carbon metabolism and offspring growth and adiposity in humans

Available evidence in humans of the effects of prenatal 1C metabolism on offspring growth and adiposity is limited. In addition, most of the evidence arises from studies carried out in developing countries, where cobalamin deficiency is widespread. In Western countries, folate and cobalamin deficiency during pregnancy are more prevalent than earlier recognised, and hence, these potential effects should also be monitored in industrialised countries. Table 5 summarises the available evidence assessing the role of pregnancy folate, cobalamin or tHcy on growth and adiposity in the offspring. In 2008, Yajnik and co-

Table 5. Summary of prospective cohort studies assessing the link between pregnancy folate, cobalamin or tHcy status and offspring growth or adiposity.

Country, year	Outcome	Child age[1] (n)	Exposure (mean)[2]	Stage (GW)	Analysis	Results (95% CI)
India, 2008 (389)	Height (cm)	6 (653)	RBC Folate (961)	28	Quartiles (Q1 vs Q4)	109 vs. 110
			Cobalamin (135)	18		110 vs. 110
	Fat mass (kg)		RBC Folate (961)	28		3.0 vs. 3.4*
			Cobalamin (135)	18		3.3 vs. 3.2
India, 2014 (39)[3]	Height (kg)	9.5 (533)	p-Folate (35.4)	30	1 SD increase	0.12 (0.02, 0.2)
			Cobalamin (163.5)			No association
			tHcy (6.0)			No association
	BMI (kg/ m^2)		RBC Folate (35.4)	30		0.05 (−0.0, 0.1)
			Cobalamin (163.5)			No association
			tHcy (6.0)			No association
Netherlands, 2015 (91)[3]	BMI (kg/ m^2)	5-6 (1950)	RBC Folate (29.7)	13	10 unit increase	-0.7 (-0.1, -0.0)
			Cobalamin (234)		100 unit increase	0.0 (-0.1, 0.1)

BMI, body mass index, CI, confidence interval; RBC folate, erythrocyte folate; GW, gestational weeks; p-folate, plasma folate; Q, quartile; tHcy, total homocysteine.
[1] Age in years.
[2] Folate in nmol/ L, cobalmin in pmol/ L and tHcy in µmol/ L.
[3] Fasting conditions not reported or non-fasting confirmed.
*p= 0.01.

workers reported no association between maternal plasma cobalamin at 18 GW and offspring height and fat mass at 6 years in 653 mother child-pairs from Pune, India (389).

However, maternal erythrocyte folate at 28 GW was positively associated with fat mass. Furthermore, pregnancy cobalamin and erythrocyte folate were negatively and positively associated with insulin resistance in the offspring respectively. In 2014, this study was replicated, but in offspring aged 5, 9.5 and 13.5 years (39). Pregnancy plasma folate concentrations were positively associated with insulin resistance and height in the offspring at 9.5 and 13.5 years after adjusting for child vitamin status and other potential confounders. Neither maternal plasma cobalamin nor tHcy concentrations were associated with insulin resistance nor growth or adiposity in the offspring. To date only one study has assessed the associations between maternal folate or cobalamin status and offspring growth or adiposity in a Western country. In contrast to the study by Yajnik, a negative effect of maternal folate status on offspring BMI (91) was found in the Netherlands. Maternal plasma cobalamin was not associated with offspring BMI.

A large UK pregnancy cohort (n= 5783), assessed the associations between the maternal *MTHFR* 677C>T SNP and body composition in the offspring. Neither maternal nor child *MTHFR* 677C>T SNP were associated with offspring body composition. In addition, maternal folic acid or folate intakes during pregnancy were not associated with body composition in the child at 9 years (390). It should be noted that folate and riboflavin status were not considered in this study and both of these modify the effect of the *MTHFR* 677C>T SNP on homocysteine (144,156).

Two randomised, placebo controlled clinical trials evaluated the effects of antenatal supplementation with folic acid, or folic acid plus other vitamins on offspring growth at 6-8 years (391,392). These studies conducted in Nepal provided daily supplements to 4926 women from enrolment (first trimester) through three months postpartum and 3524 offspring were followed-up at 6-8 years. Mothers were allocated either to: vitamin A

supplements alone (controls); folic acid; folic acid and iron; folic acid with iron and zinc; or multiple micronutrient supplements containing folic acid, zinc, iron and 11 other vitamins and minerals. All supplements contained vitamin A, and provided the recommended dietary allowance for pregnant or lactating women for all micronutrients except for iron and zinc. Maternal supplementation with folic acid alone reduced the risk of metabolic syndrome in the offspring by 37% compared to the control group at 6-8 years of age, but had no effect on any of its components (plasma glucose, HDL cholesterol, triglycerides, blood pressure or waist circumference) (391). Other intervention arms in the same trial included supplements containing folic acid, but were not associated with metabolic syndrome in the child. Maternal supplementation with folic acid, iron and zinc resulted in an increase in mean height (0.6 cm; 95% CI: 0.0, 1.3) and a reduction in mean triceps (-0.3 mm; 95% CI: -0.4, -0.1) and subscapular (-0.2 mm; 95% CI: -0.3, -0.1) skinfold thickness. Other micronutrient combinations containing folic acid failed to show a growth benefit, so the effects were attributed to zinc (392). In the same study, prenatal supplementation was not associated with insulin resistance in the offspring (393). However, maternal cobalamin deficiency (<148 pmol/ L) in the first but not in the third trimester, was associated with 26.7% higher insulin resistance in the offspring compared to children born to women with normal cobalamin status.

Different approaches have been used to test the association between 1C metabolism and bone development. Overall, maternal folate intake or folate status was positively associated in a dose-dependent fashion with offspring total bone mineral density in the UK ALSPAC cohort (394) and with bone mineral content in Indian children (395). In addition, UK children with the *MTHFR* 677TT genotype had an average of 0.91 (CI: -1.62, -0.20) g/ cm^2 less spinal bone mass density compared to the other genotypes for this polymorphism (396). In line with these results, there was also a consistent but rather small effect of the maternal *MTHFR* 677 TT genotype on child's spinal bone mass density (397).

HYPOTHESIS AND OBJECTIVES

Hypothesis and objectives

Hypothesis

In utero exposure to low folate or cobalamin concentrations impairs linear and ponderal growth and adiposity in the offspring at mid-childhood.

Objectives

Main objective

To determine the interactions and effects of folate and cobalamin on one carbon metabolism during pregnancy and investigate their association with mid-childhood growth and adiposity.

Specific objectives

- To describe cobalamin and folate metabolic indicators in the first trimester of pregnancy.

- To determine the association between genetic, nutritional and other lifestyle factors and first trimester folate and cobalamin status.

- To investigate cobalamin and folate status interactions and their association with metabolic and haematological markers of low cobalamin status during pregnancy.

- To describe cobalamin and folate metabolic indicators in children at mid-childhood (7.5 years).

- To determine the association between genetic, nutritional and other lifestyle factors and mid-childhood folate and cobalamin status.

- To investigate the association between prenatal folate and cobalamin status and mid-childhood one carbon metabolism status indicators.

Hypothesis and objectives

- To assess the associations of folate and cobalamin status during pregnancy on mid-childhood linear and ponderal growth and adiposity.

- To investigate the associations between maternal and child genetic variants affecting folate and cobalamin metabolism or transport and mid-childhood linear and ponderal growth and adiposity.

MATERIAL AND METHODS

Material and methods

1. Design and study population.

The work reported in this thesis is from The Reus-Tarragona Birth Cohort (RTBC). The RTBC is an ongoing population based longitudinal cohort study from early pregnancy until mid-childhood carried out by the Area of Preventive Medicine and Public Health from the Universitat Rovira i Virgili. This study was designed to identify early pregnancy nutritional and maternal genetic factors influencing foetal growth and pregnancy outcomes, as well as growth, development and mental performance in their offspring at 7.5 years. The study, to date, consists of two different phases, described below: the pregnancy phase and the follow-up phase in the children aged 7.5 years.

Both phases were carried out according to the Declaration of Helsinki, and were approved by the Ethical Committees from each of the two participating University Hospitals (Sant Joan, Reus and Joan XXIII, Tarragona). Signed informed consent was obtained from all study participants in the pregnancy phase and from the childrens' legal representatives in the follow-up phase.

This study was registered at www.clinicaltrials.gov as NCT01778205.

2. Pregnancy Phase

Women attending their first antenatal check-up, before 12 GW, with a confirmed viable foetus and singleton pregnancy were eligible to participate and were recruited by the Areas of Obstetrics and Gynaecology from the two University Hospitals. Exclusion criteria were chronic illness or surgical interventions affecting nutritional status, or use of any medication that affects folate or vitamin B_{12} metabolism.

In accordance with the Spanish Obstetrics and Gynaecology Society guidelines (398,399), pregnant women with low obstetrical risk were recommended to take daily supplements containing 400 µg of folic acid until the end of the first trimester. The recommended prenatal supplement also contained 2 µg of cyanocobalamin. Women with a history of a previous pregnancy affected by NTDs/ other congenital defects or complications or with a first-degree relative with NTDs were recommended to take 4-5 mg of folic acid per day until the end of the first trimester. Daily iron supplements (40 mg) were also prescribed to all women from 12 GW throughout the pregnancy.

Participant recruitment started in January 2005 and 2006 in the Sant Joan, Reus and Joan XXIII, Tarragona University Hospitals respectively. By October 2014, 626 pregnant women had been recruited.

Fasting blood samples were drawn from mothers at <12, 24-27, and 34 GW, and non-fasting blood samples on admission to hospital with confirmed labour and from the umbilical cord vein. Blood samples were collected together with samples routinely collected during the prenatal care of pregnancy according to the regional health authorities protocol (398).

Medical and obstetrical history, lifestyle and dietary intake data collection

Maternal age and BMI were recorded at the first antenatal check-up. Participants were interviewed by the study team at 20 and 32 GW on supplement use, lifestyle and dietary habits. Two interviews were performed to account for changes in toxic habits and supplement use that might occur during pregnancy. The information covering the six months preconception throughout the first trimester of pregnancy was collected at 20 GW and from the first trimester onwards, at 32 GW. A validated food frequency questionnaire (400) was used to estimate dietary intake during pregnancy (administered the day after labour).

Even though the obstetricians recommended folic acid and cyanocobalamin supplements to be taken during the first trimester of pregnancy, timing of initiation and supplement intake pattern varied widely. As a preventive measure against anaemia, low dose iron supplements (40 mg/d) were recommended during the second and third trimesters of pregnancy to all participants. Anaemic women were treated with iron supplements containing 80 or 120 mg/d, as the obstetricians saw fit. Questionnaires that specifically targeted folic acid, cobalamin and iron use were designed to obtain detailed information regarding supplement brand, composition, timing of initiation, frequency and duration of use. Other multivitamin/ mineral supplement use was also recorded with the same detail. Flash cards with images of all available supplements on the market were used to help the participants to recall the supplements that they used so that the investigators could clarify the doses contained. We used this information to estimate the daily intake of folic acid, cobalamin and iron from supplements during the first trimester and between the 4^{th} and 7^{th} months of pregnancy.

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

Participants were classified according to socioeconomic status in three different categories (low, mid and high) based on household income and maternal and paternal occupation (401).

Information on parity, previous adverse obstetrical outcomes, gestational age at labour and offspring's sex and birthweight were obtained after birth from the obstetrical records. Gestational hypertension was also registered and was defined as a first recognised systolic blood pressure >140 mmHg and/or a diastolic blood pressure >90 mmHg at least in two check-ups (6 hours apart) after the 20th GW (398).

Complete blood count was routinely performed in all maternal blood samples at each of the University Hospitals. Late pregnancy anaemia was defined as a haemoglobin <11 g/dL at 34 GW or at labour.

Gestational diabetes was diagnosed following the pregnancy monitoring protocol established in both University Hospitals (402) by employing a two-step approach. The first screening was performed with a 50 g oral glucose challenge test (GCT) at 24-27 GW in low risk pregnancies. If positive (glucose concentration >7.8 mmol/L), a 100 g Oral Glucose Tolerance Test was performed (403). Blood samples for glucose determination were taken at baseline (fasting) and at 1, 2 and 3 hours after glucose administration. Gestational diabetes was diagnosed if at least two glucose determinations were above 5.8, 10.6, 9.2 or 8.1 mmol/L at the respective sample collections. In pregnancies with previous gestational diabetes, a glucose challenge test was performed at the first obstetrical visit and, if negative, at 24-27 GW and again at 30 GW. In the case of a positive glucose challenge test, the protocol described above for low risk pregnancies was then applied.

Preterm birth was defined according to WHO criteria (birth at less than 37 GW) (404). SGA, hereinafter referred to as intrauterine growth restriction, was defined as a birth weight below the 10th percentile according to sex and gestational age standardised references from the Spanish Obstetrics and Gynaecology Society (405). Large-for-gestational age was defined as a birth weight above the 90th percentile according to sex and gestational age, by using the same references as above. We used WHO 2006 Child Growth Standards to determine z-scores for birth weight (406).

Blood sample collection

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

All samples were processed at the Institut d'Investigació Sanitària Pere Virgili Biobank (http://www.iispv.cat/plataformes_de_suport/en_biobanc_iispv.html) and in the Joan XXIII University Hospital research laboratory and the central hospital laboratories of both hospitals (cord and labour samples) according to a strict standardised protocol. This ensured that samples were kept at 4º C until processed within less than an hour of collection to separate whole blood, plasma, serum, washed red cells and leukocyte fractions for storage in the biobank at -80º C. Times of sample collection and separation of plasma were recorded as a quality control measure to ensure that this protocol was complied with.

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

Plasma was separated from the EDTA vacutainer at 1500 g for 15 minutes at 4º C and stored in aliquots of 1 ml -80º C. Serum was separated after coagulation of blood at room temperature (approximately 30 minutes after its collection) and aliquoted and stored at -80º C.

In order to isolate leukocytes, phosphate buffered solution was added to the remaining buffy coat and erythrocytes in the EDTA tube, and was thoroughly mixed by inversion. The mixture was transferred to a Falcon tube containing 30 ml of haemolysis solution, mixed by inversion and then kept at room temperature for 20 minutes to lyse the remaining erythrocytes. The Falcon tube was centrifuged for 5 minutes at 2000 g at room temperature, the supernatant discarded and the pellet resuspended in 20 ml of haemolysis solution. The tube was centrifuged under the same conditions as described above and as a result, the leukocytes were isolated from the other cell types. The supernatant was discarded again and 450 µl of PBS and 10 ml of Cell Lysis Solution (Qiagen GmbH, Hilden, Germany) were added to the leukocyte aliquot and preserved at -80º C. This tube was kept at room temperature and protected from light for at least 1 month and a maximum of 2 years.

Leukocyte DNA was extracted and quantified in preparation for maternal and cord genetic polymorphism determinations. Autopure Protein Precipitation Solution (3.33 mL) was added and mixed with vortex to obtain a homogenous mix before incubation in iced water for 15 minutes. The mixture was centrifuged at 2000 g at 4º C for 12 minutes. The supernatant containing the suspended DNA was removed, and added to a 50 ml Falcon tube with 10 ml of cold 100% isopropanol. The tube was gently mixed by inversion until the precipitated DNA was visible. After centrifuging at 2000 g at 4º C for 5 minutes, the supernatant was discarded (pellet contained the DNA). The tube was dried for 30-40 minutes on absorbent paper, and 1200 µl of DNA Hydration Solution were added to the Falcon tube, mixed and further incubated at room temperature for 3-4 days in a mixer incubator. DNA quantification was performed using a spectrophotometer Nanodrop 1000 at 260 nm wavelength using 2 µl of hydrated DNA.

Biochemical and genetic determinations

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

Red blood cell and plasma folate

Folate concentrations in plasma and whole blood were determined by an automated microbiological assay using a microtiter plate and a chloramphenicol-resistant strain of *Lactobacillus casei* (407). Performances for inter- and intra-assay coefficient of variation were 4 and 5% respectively and the lower detection limit was 2 nmol/L (408).

Plasma cobalamin

Total cobalamin concentrations were determined by an automated microbiological assay using a microtiter plate and a colistin sulphate-resistant strain of *Lactobacillus leichmannii* (409). The lower detection limit was 30 pmol/L, and inter- and intra-assay coefficients of variation 4 and 5% respectively (408).

Plasma total homocysteine, methylmalonic acid and cotinine

Plasma total homocysteine, methylmalonic acid and cotinine were determined by liquid chromatography–tandem mass spectrometry (410). Cotinine, an oxidised metabolite of nicotine, is a marker of recent tobacco exposure. Participants with plasma cotinine concentration >10 ng/mL were considered smokers, and ≤10 ng/mL non-smokers (411). The lower limits of detection were 0.1 and 0.03 µmol/L and 1 nmol/L, inter-assay coefficient of variation 2, 3-8 and 6% and intra-assay coefficient 1-2, 1-4 and 2-3% respectively for total homocysteine, methylmalonic acid and cotinine determinations .

Plasma holotranscobalamin

Plasma holotranscobalamin concentrations were determined by the AxSYM active-B_{12} immunoassay (Abbott Laboratories, Abbott Park, IL, USA), with a lower limit of detection of 1 pmol/L. The inter-assay coefficient of variation was lower than 10%.

Plasma creatinine

Creatinine concentrations were determined by Jaffé reaction (Química Clínica Aplicada, SA, Amposta, Spain) and were used as an estimation of renal function (412). Inter- and intra-assay coefficients of variation were 2.1 and 1.7% respectively.

Genetic polymorphisms

DNA isolated from both mother and cord blood leukocytes were used to determine genetic polymorphisms affecting folate metabolism and cobalamin metabolism and transport. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was used to analyse the *methylenetretrahydrofolate reductase* 677C>T, *transcobalamin 2* 776C>G, *5-methyltetrahydrofolate-homocysteine methyltransferase reductase* 66A>G and *5-methyltetrahydrofolate-homocysteine methyltransferase reductase* 524C>T genotypes (413).

3. Follow-up phase at 7.5 years

When the offspring of mothers included in the pregnancy phase of the study were 7.5 years old, a letter was sent to their parents/legal guardians briefly describing the aims and protocols of the child development phase. Two weeks later, the letter was followed up with a telephone call to offer any further information required and to establish whether the child agreed to participate or not. In the case of agreement, two visits were programmed in the aforementioned University Hospitals within four months of acceptance. The first visit, early in the morning following an overnight fast, consisted of anthropometrical, body composition and blood pressure measurements, and blood sample collection. The second

visit, that did not require the chid to be fasting, was performed at an agreeable time with the parents to encourage participation. This time, the child's legal guardian (usually one of the parents) was interviewed by a member of the study team and three questionnaires on physical activity, clinical history and food intake were completed. All recruited participants born before September 2008 were included in the analysis (n= 162).

Clinical, dietary intake and physical activity data collection

Parents were interviewed by members of the study team to obtain data on previous illnesses and chronic diseases that was categorised according to the International Classification of Diseases-10. Information on food allergies, medication and exposure to tobacco smoke was also recorded. The parents were asked whether children were exposed to passive smoking. They were also asked to assess whether precocious puberty had started in their children with the aid of the Tanner scales for child physical development. Pubertal stage was classified according to breast development in girls and genital development in boys. Information regarding feeding mode during the first years of life, including exclusive and total breastfeeding lengths, as well as age (months) at introduction of complementary feeding. Information on household income and on parental occupation was obtained to reassess socioeconomic status.

Dietary intake was assessed with a previously validated (400), 45-item food frequency questionnaire proxy administered by the parents for the child. This questionnaire included information on dietary habits and supplement intake. Total daily energy, macro- and micronutrient intakes were derived using an adapted version of a French food composition database (414). Information on brand, dose and frequency of vitamin or mineral containing supplements use was registered.

Physical activity was assessed using the Physical Activity Questionnaire for Children (415). This is a 7-day recall questionnaire to estimate physical activity in school age children

considering their participation in different activities and sports, the physical exertion during the declared activity and activity throughout the whole day (during school time, recess after meal times and after school or during extra-curricular activities and at weekends/during holidays). A final value accounting for total physical activity during the previous week was then calculated. The higher the value the more active the child was. This questionnaire has acceptable reliability and convergent validity (416,417) and discriminates the known sex- and age-differences in physical activity correctly (418). Physical inactivity was also taken into account. Interviewers asked for the time spent in front of a screen (either playing video games or watching TV) was recorded and a daily average time was calculated (hours/day).

Information on growth trajectories from birth and until the age of 6 years was obtained from the paediatrician's growth records provided by the parents. Height, weight and check-up date were recorded. Whether children were up to date on their programmed vaccinations or not was also recorded.

Anthropometric and body composition measurements

Anthropometric measures were taken by a trained member of the study team following the WHO guidelines (419). Children were asked to come fasting to the check-up and to pass urine before assessment. Measurements were taken at a similar time of the day to minimise biological variation and were recorded by observers working in pairs. One observer took the measurements and the other one recorded the data immediately to minimise mistakes during data entry. Hospital technicians ensured that the equipment was calibrated regularly. The same team member carried out measurements for every child.

Participants were asked to take off their shoes, clothes and hair ornaments and undo braids, when necessary. Child stayed in their underwear during the whole visit and room temperature was kept comfortable throughout the check-up. Before measurements were

undertaken, all procedures were explained to the parents and child, reassuring them of the harmless nature of the measurements. All measurements were taken to the last completed number.

Height was measured in duplicate with University Hospital stadiometers with an accuracy of 0.1 cm. Children were asked to stand 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 recorded in duplicate using an electronic scale (Tanita BC-420MA, Tanita Corporation, Tokyo, Japan) and a precision of 0.100 g. Children were asked to stand still on the centre of the weighing platform with their feet slightly apart.

All circumferences were measured in duplicate with a non-stretchable measuring tape (Seca GmBH & Co Kg, Hamburg, Germany) with an accuracy of 0.1 cm. To measure head circumference the observer stood on one side of the child. The tape was passed around the head and held just above the eyebrows and over the occiput protuberance and parallel to the floor. Mid-upper-arm circumference was measured at half the distance between the tip of the acromion and the olecranon process, marked previously with eyeliner. The tape was wrapped around the relaxed right arm lying flat. To measure waist circumference (420), the child was asked to stand still, with its feet together, weight uniformly distributed and breathing normally. The measurement was taken at the mid-point between the iliac crest top and the lower margin of the last palpable rib and hip circumference was measured at the widest portion of the buttocks (420). Both waist and hip circumferences were measured after exhaling normally and with the tape lying parallel to the floor.

Right subscapular, tricipital, bicipital and suprailiac skinfold thicknesses were measured in triplicate using a Harpenden skinfold calliper (Holtain Ltd, Crymych, Wales), with an accuracy of 0.2 mm. Bicipital and tricipital skinfold thicknesses were measured at the mid-point between the tip of the acromion and the olecranon process, in the anterior and

posterior arm respectively. Subscapular skinfold thickness was measured below the inferior angle of the scapula and the suprailiac skinfold was measured just over the iliac crest top. All skinfold thickness measurements were taken grasping the skinfold gently to reduce discomfort and following the natural lines of skin. To prevent skin contraction, skinfold thickness measurements were taken consecutively at different places in a row three times, hence avoiding successive application of pressure to the skin.

Study members encouraged children to cooperate. In the rare cases where a child was uncooperative, measurements became increasingly difficult to assess and hence, only one set of measurements was undertaken (<1% of children).

Body composition was estimated using tetrapolar bioelectrical impedance analysis (Tanita BC-420MA, Tanita Corporation, Tokyo, Japan). Bioelectrical impedance is based on the existing relationship between resistance to a known current and total body water (which is calculated), and hence fat mass is estimated (421). The estimations of two body compartments (fat mass and fat-free mass) are deduced using equations not supplied by the manufacturer. Two measurements were undertaken at 50 kHz. Total fat mass, fat-free mass and total impedance were recorded.

Calculated variables

The mean of the repeated measurements of anthropometry and bioelectrical impedance variables was calculated and used for the analysis. BMI was calculated as weight (kg) divided by height squared (m^2). Weight (kg), height (m) and BMI (kg/m^2) were transformed to age- and sex-specific z-scores according to WHO references (422). These references were used to account for age and sex differences in growth. The Lambda-Mu-Sigma (LMS) method was used to derive the z-scores to account for asymmetry of the distributions. This

method summarises the distribution with three parameters: the median (M), the coefficient of variation (S) and the skewness of the distribution (L).

For any given x value, the z-score was then calculated as: $z = \frac{\left[\left(\frac{x}{M}\right)^{L} - 1\right]}{L*S}$.

Height, weight and calculated BMI from paediatric records of child growth and development were also transformed to age- and sex-specific z-scores according to the WHO references for 0-60 months and for school-age children (406,422) for each measurement from birth to 6 years.

Overweight and obesity were defined according to the International Obesity Task Force criteria (327). These cut-offs were employed because values are based on internationally based percentiles passing through BMI 25 and 30 kg/ m^2 at age 18, which are linked to mortality rates.

Mean skinfold thickness was calculated as the mean of subscapular, tricipital, bicipital and suprailiac skinfold thicknesses (423). The waist-to-height and waist-to-hip ratios were also calculated as the ratio between waist circumference (cm) and height (cm) and waist (cm) and hip (cm) circumferences respectively.

As fat mass and fat-free mass are strongly correlated with height, they are usually expressed as a percentage of weight, but this is not the most informative way to express body composition data. Hence, adjusting fat-free mass and fat mass for height squared generates the fat-free mass index and the fat mass index, which provides indices of relative fat and fat-free mass expressed in the same units as BMI (424).

Blood sample collection

A trained nurse collected children's blood samples while they were in the supine position. Two vacutainers of 10 ml were collected: one containing EDTA-K_2 and another with a clot activation factor. Sample processing was initiated at the hospital with a portable centrifuge in order to ensure that plasma was separated in <30 minutes and serum fraction in <1 hour. Samples were then transported to the Faculty of Medicine URV laboratory in coolers, where the aliquots were frozen at -80º C and the sample processing protocol was continued.

Sample collection was carried out using exactly the same protocols as in the pregnancy phase. Whole blood, plasma and leukocytes from the EDTA vacutainer and serum from the clot activator vacutainer were separated into different aliquots and frozen at -80º C.

Biochemical and genetic determinations

Aliquots of 0.5 ml were transported on dry ice to BeVital A/S (Bergen, Norway) and analysed in batches within <18 months of sample collection. Plasma aliquots of 0.5 ml and 25 µL of whole blood were shipped from every sample and 120 ng of lyophilised DNA from those children whose SNPs were not genotyped because cord blood was unavailable.

Red blood cell and plasma folates, cobalamin, tHcy and methylmalonic acid were determined using the same techniques as for the pregnancy phase. The same polymorphisms as for the mother were genotyped in the children.

4. Statistical analysis

Every chapter has a detailed description of the statistical analysis used. All statistical analysis were performed using SPSS (SPSS Inc, Chicago, IL, USA) for Windows, version 22.0, and all graphs were drawn with GraphPad Prism version 6.0 (GraphPad Software, San Diego, CL, USA).

RESULTS CHAPTER 1

Chapter 1: Determinants of early pregnancy folate and cobalamin status

Introduction

Mandatory folic acid fortification of flour has been highly effective in the prevention of NTDs (425,426). However, the safety of folic acid fortification has been questioned (310–312). Harmful effects have been attributed to elevated plasma folate concentrations, particularly in subjects with cobalamin deficiency. Studies carried out in the USA where mandatory fortification is in place, have reported that elevated folate status exacerbates metabolic and clinical signs of cobalamin deficiency (310–313,427). Increased MMA and tHcy (310,311) and risk of anaemia and cognitive impairment (312,313) were reported in elderly subjects with elevated folate status combined with cobalamin deficiency, compared to those with normal status in both vitamins. It has been speculated that, the effects on cognitive impairment and anaemia were mediated to some extent by unmetabolised folic acid (313). In contrast, in elderly subjects from the UK, Ireland and Norway, countries where folic acid fortification is voluntary, no significant associations were observed between folate status and metabolic and clinical indicators of cobalamin deficiency (314,315,428). In these studies, the prevalence of elevated folate combined with cobalamin deficiency was lower than in studies performed in the USA, and the criteria for defining cobalamin deficiency was different. Concerns regarding possible adverse effects in sectors of the population that are susceptible to low cobalamin status, such as the elderly, has enhanced the debate on the safety of folic acid fortification, and delayed the implementation of mandatory fortification with folic acid policies in Europe (321–323), while NTD prevalence has remained stable over the past 25 years (298).

In Western countries, inadequate cobalamin status during pregnancy is more prevalent than previously recognised, affecting 35% of pregnant Canadian women, (93,99) and has been linked with adverse pregnancy outcomes such as intrauterine growth restriction

(88,166), prematurity (158) and NTDs (44) among others. On the other hand, higher rates of NTDs (238), preterm birth (429) and SGA (430) have been reported in pregnant women with inadequate compared to normal folate status. Moreover, moderately elevated early pregnancy tHcy, a nonspecific functional metabolic marker of both cobalamin and folate status, has also been related with several adverse pregnancy outcomes such as preeclampsia (301,431,432), preterm birth (158) and miscarriage (159,164). A negative association has been consistently found between early prenatal tHcy concentrations and birth weight in both industrialised and developing countries (38,39,100,301,302). Whether elevated plasma folate exacerbates the effects of cobalamin inadequacy should be intensively monitored. Folic acid supplements are prescribed worldwide (157) without taking into account maternal cobalamin status. In India, elevated prenatal folate status in women with inadequate cobalamin status has been associated with gestational diabetes in the mother (433) and with increased risk of SGA and insulin resistance in the offspring (166,389).

On the other hand, low plasma folate has been consistently found in overweight and obese subjects (91,264–266), but the association between RBC folate and BMI is unclear. BMI was reported to be positively associated with RBC folate status in studies carried out in the presence of mandatory folic acid fortification (264,266,272–275), although no association was observed in one study (45) nor in studies carried out in countries where mandatory fortification is absent (269–271). Two studies have shown that obesity has a negative effect on short-term folate pharmacokinetics, lowering its uptake in obese subjects administered folic acid (272,434). These findings do not explain the opposite effects of BMI on plasma and RBC folate. Nevertheless, this issue may be of public health significance because the prevalence of NTDs is 75% higher in obese compared to non-obese women (435,436), which may be due to lower serum folate. Worldwide, 15% of adult women were obese in 2014 (437) and trends in overweight and obesity prevalence are not decreasing in the USA

nor in Europe (438,439). Folic acid recommendations in women of childbearing age and during pregnancy are the same regardless of BMI.

Metabolic and clinical effects of the *MTHFR* 677C>T SNP have been widely described in non-pregnant and pregnant populations. Out of multiple SNPs potentially affecting folate status, our group recently reported that the *MTHFR* 677C>T SNP most affected folate status (144). Studies of the effects of *TCN2* 776C>G and *MTRR* 66A>G and 524C>T on plasma cobalamin or its biomarkers, tHcy or MMA, are limited to non-pregnant populations, with the exception of *MTRR* 66A>G which has been studied once in pregnant women (152). The genetic variant at locus 66 has been more extensively studied than that at locus 524. In addition, conflicting effects of *MTRR* 66A>G on tHcy have been reported with some authors observing higher tHcy concentrations in subjects with the GG compared to AA genotype (145–148), while others found no differences (149–155).

It is not known whether early pregnancy BMI is associated with either RBC or plasma folate status in the absence of mandatory fortification in a population exposed to high folic acid supplement use, and whether folate intake modifies this relationship. Moreover, data regarding the safety of elevated early pregnancy folate in women with cobalamin inadequacy is lacking.

Specific objectives

The specific objectives of this chapter are:

- Objective 1: To describe cobalamin and folate metabolic indicators in the first trimester of pregnancy.

To determine the association between genetic, nutritional and other lifestyle factors and first trimester folate and cobalamin status.

- Objective 2: To investigate cobalamin and folate status interactions and their association with metabolic and haematological markers of low cobalamin status during pregnancy.

Material and methods

The materials and methods applicable to this chapter have been described in the general Material and methods section.

Statistical analysis

Descriptive statistics: Descriptive characteristics comprising means (95% CI) or median (P_{25}-P_{75}) and relative frequencies (95% CI), where applicable, are reported for all of the women that participated in the pregnancy study. Genotype and allele frequencies (95% CI) of the studied genetic variants are also reported. One sample or goodness of fit χ^2 was used to test deviations from Hardy-Weinberg equilibrium of the observed allele frequencies (440).

Objective 1: Maternal folate and cobalamin status (at <12 GW) are described. Folate deficiency was defined as: plasma folate ≤10 nmol/L (237) or RBC folate ≤340 nmol/L (237) and elevated plasma tHcy as tHcy ≥10 µmol/L (441). It should be noted that given the effects of pregnancy on tHcy, even in the first trimester, the use of this tHcy value as a reference in pregnant women is not ideal, but pregnancy reference values are not established. Marginal cobalamin deficiency was defined as: plasma cobalamin ≤221 pmol/L (442) or plasma MMA ≥0.210 µmol/L (443). RBC folate ≤906 nmol/L was defined as suboptimal for protecting against NTDs (238).

Plasma nutrient /biomarker (folate, cobalamin, holoTC, MMA and tHcy) distributions were skewed to the right so were natural log transformed. Demographic, lifestyle and genetic determinants of RBC and plasma folate and plasma cobalamin in early pregnancy were evaluated by univariate linear regression analysis. Stepwise multiple linear regression models were used to assess the associations between the previously identified explanatory variables and indicators of folate or cobalamin status. The same variables as for cobalamin were used to evaluate the determinants of plasma holoTC concentrations. Determinants of functional biomarkers of folate and cobalamin status (tHcy and MMA respectively) were identified by stepwise multiple linear regression analysis based on the determinants of folate and cobalamin status. The four studied genetic variants were included in all of the models as they were shown in a study of a similar local population to be associated with folate and cobalamin status (144,156).

The effect of maternal overweight (25≤ BMI <30 kg/m^2) or obesity (BMI ≥30 kg/m^2) compared with normal weight (18.5≤ BMI <25 kg/m^2) on first trimester RBC and plasma folate status was studied. Participants classified as underweight (BMI <18.5 kg/m^2; n=15), were excluded from these analyses. Analysis of covariance (ANCOVA) was used to assess whether differences in plasma folate according to BMI categories remained after adjusting for previously identified determining factors. Interactions between BMI categories and total folate intake (tertiles) on RBC folate were assessed by ANCOVA adjusting for the *MTHFR* 677C>T genetic variant in a co-dominant model. Results are stratified by total folate intake tertiles when significant interactions were observed between BMI and total folate intake on RBC folate.

Objective 2: The effects of early pregnancy low cobalamin status on metabolic and haematological parameters according to early pregnancy folate status were assessed throughout pregnancy. Early pregnancy marginal cobalamin deficiency was classified as defined in Objective 1. Women were also categorised as having elevated (≥45 nmol/L) or non-elevated (<45 nmol/L) plasma folate (310) at early pregnancy. Another cut-off has been

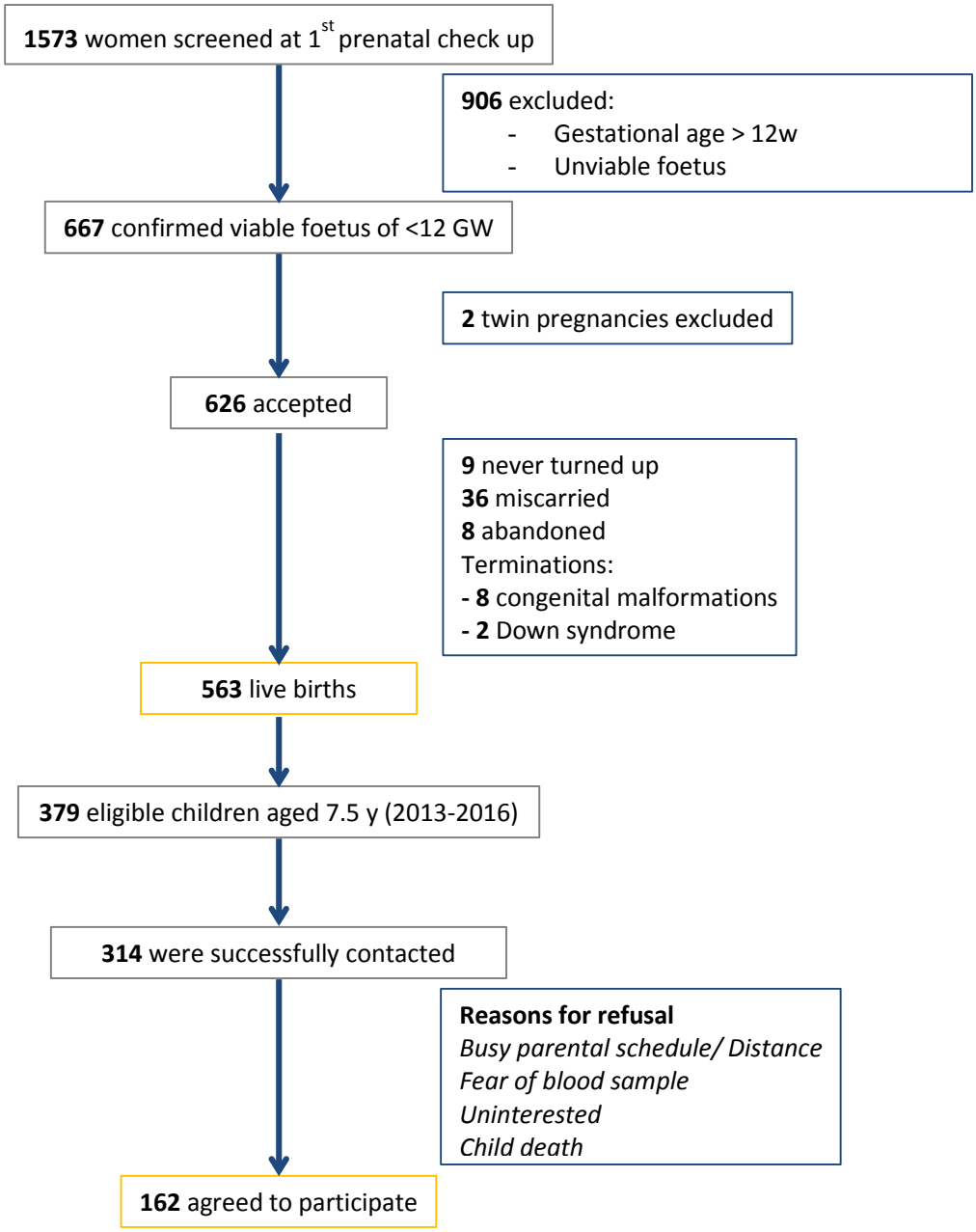
previously used for elevated folate (≥30 nmol/L) and all analyses were repeated using this cut-off (314,315). Cross-sectional and longitudinal interactions between folate and cobalamin statuses were assessed for plasma MMA and tHcy at <12, 12, 24-27 and 34 GW and at labour. The MMA model included plasma creatinine (at blood sample collection), first trimester BMI categories and smoking (for cross-sectional analysis) or smoking throughout pregnancy (longitudinal analysis). The plasma tHcy model included the same factors as MMA plus the maternal *MTHFR* 677C>T genetic variant in a co-dominant model. Interactions between marginal cobalamin deficiency and total folate intake (tertiles) on plasma and RBC folate were also assessed throughout pregnancy by ANCOVA analysis adjusting for the same factors as the tHcy models (except for plasma creatinine in RBC folate models).

The interactions between plasma folate and cobalamin status on haemoglobin and mean cell volume (MCV) were assessed in early (<12 GW) and late (34 GW) pregnancy by ANCOVA analysis. Logistic regression analysis was used to identify the interactions between folate and cobalamin statuses on early and late pregnancy (≥34 GW) anaemia (haemoglobin <11 g/dL). All models assessing haematological outcomes included first trimester BMI, age, socioeconomic status, plasma creatinine at time of blood sample collection, first trimester smoking or smoking throughout pregnancy and iron supplement use in the first or second trimester (for early or late pregnancy models respectively) as adjusting factors.

Results

One thousand five hundred and seventy three women were screened at their first antenatal check-up (**Figure 9**). 906 were excluded due to gestational age ≥12 weeks or unviable foetus, leaving 667 women eligible to participate. After inviting them to participate, a further 2 were excluded due to twin pregnancy and 626 women agreed to participate. Of these, 563 went on to have live births and were followed-up throughout pregnancy. By March 2016, 379 offspring had reached 7.5 years and were eligible to participate in the follow-up study. We successfully contacted 314 parents and 162 children (51.5%) were recruited.

Figure 9. Participant flow chart from recruitment until offspring follow-up at mid-childhood.



Reus-Tarragona Birth Cohort flow chart in the two phases: pregnancy and offspring follow-up at mid-childhood. Women recruited from January 2005 and until October 2014 were included in the pregnancy phase, and offspring born at or before September 2008 in the follow-up phase.

Selected descriptive characteristics of studied participants

Maternal demographic and lifestyle descriptive characteristics are shown in **Table 6**. Mean gestational age at recruitment was 9.1 (95% CI: 8.9, 9.3) weeks, mean first trimester BMI was 23.8 (95% CI: 23.5, 24.2) kg/m^2 and age was 31.9 (95% CI: 31.5, 32.3) years. Overweight, obesity and low socioeconomic status were observed in 21.8, 6.9 and 6.6% of the women respectively. Smoking throughout pregnancy was very prevalent (17.4%; 95% CI: 14.4, 20.7). Most women using illegal drugs stopped their use during pregnancy, and occasional or regular alcohol consumption was observed in 13.6% of women.

Table 6. Maternal demographic and lifestyle descriptive characteristics.[1]

	Value
Gestational age at recruitment, weeks [556]	9.2 (9.0, 9.4)
Early pregnancy age, y [560]	31.9 (31.5, 32.3)
Previous pregnancies, % [561]	52.9 (48.8, 57.0)
Previous miscarriages, % [561]	33.3 (29.6, 37.3)
Previous preterm birth, % [561]	5.3 (3.8, 7.5)
Planned pregnancy, % [533]	80.8 (77.2, 83.9)
1st trimester body mass index, kg/m^2 [550]	23.8 (23.5, 24.2)
Body mass index categories, % [550]	
Underweight	2.9 (1.8, 4.7)
Overweight	21.8 (18.6, 25.5)
Obesity	6.9 (5.1, 9.3)
Current smoking, %	
1st trimester [562]	28.4 (24.7, 32.2)
Throughout pregnancy [559]	17.4 (14.4, 20.7)
Socioeconomic status [558]	
Low	6.6 (4.8, 9.0)
Middle	49.8 (45.7, 54.0)
High	43.5 (39.5, 47.7)
Occasional alcohol consumption, %	
Preconception [518]	53.3 (49.0, 57.5)
Pregnancy [528]	13.6 (11.0, 16.8)
Illegal drug use, %	
Preconception [539]	2.4 (1.4, 4.1)
Pregnancy [539]	0.4 (0.1, 1.3)

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

The majority of women reported that their pregnancy was planned (80.8%). Folic acid supplement use was less frequent at preconception (34.5%) compared to during the first trimester (87.5%: p<0.05) (**Table 7**). Median total folate (638 µg/day) and cobalamin (6.0 µg/day) intakes throughout pregnancy were above the IOM RDA. However, folic acid from supplements contributed more to total folate intake (57.7%) than cobalamin supplement use to total cobalamin intake (15.1%).

Table 7. Maternal nutritional descriptive characteristics.[1]

	Value
Dietary intake	
Energy, kcal/day [467]	1953 (1905, 2001)
Total folate, µg/day[2] [441]	638 (517, 839)
Total cobalamin, µg/day[2] [441]	6.0 (4.7, 7.7)
Folic acid supplements, %	
Preconception [510]	34.5 (30.5, 38.9)
1st trimester [521]	87.5 (84.4, 90.1)
1st trimester folic acid intake, µg/day[2] [521]	400 (267, 534)
Cobalamin supplement use, %	
1st trimester [521]	63.7 (59.5, 67.7)
2nd trimester [538]	34.0 (30.1, 38.1)
1st trimester cobalamin supplement intake, µg/day[2] [529]	1.1 (0.0, 1.8)
Iron supplement use, %	
1st trimester [521]	15.5 (12.7, 18.9)
2nd trimester [538]	57.8 (53.6, 61.9)

[1] All data shown are means or percentages (95% confidence interval), unless otherwise indicated.
[2] Median (P_{25}, P_{75}).

Table 8 describes prenatal, obstetrical and perinatal characteristics. Mean gestational age at labour was 39 (95% CI: 38.9, 39.1) weeks, 4.6 (95% CI; 3.2, 6.7) % of births were premature and mean birth weight was 3218 (95% CI: 3180, 3255) g. There were 44 (7.8%) and 49 (8.7%) cases of intrauterine growth restriction and large-for-gestational age respectively. Gestational diabetes mellitus and pregnancy-induced hypertension affected

7.0% and 6.1% of the pregnancies respectively. Anaemia affected 2.8 (95% CI: 1.7, 4.6) % and 32.3 (95% CI: 28.6, 36.3) % of women during the first and third trimesters respectively.

Table 8. Prenatal, obstetrical and perinatal descriptive characteristics.[1]

	Value
GDM, % [558]	7.0 (5.2, 9.4)
PIH, % [479]	6.1 (4.2, 8.6)
Early pregnancy anaemia, %[2] [537]	2.8 (1.7, 4.6)
Late pregnancy anaemia, %[2] [536]	32.3 (28.6, 36.3)
GA at labour, weeks [563]	39.0 (38.9, 39.1)
Preterm delivery, %[3] [563]	4.6 (3.2, 6.7)
Offspring female sex, % [563]	51.2 (47.0, 55.3)
Birth weight, g [562]	3218 (3180, 3255)
Birth weight, z-score[4] [562]	-0.20 (-0.28, -0.11)
IUGR, %[5] [562]	7.8 (5.9, 10.3)
LGA, %[5] [562]	8.7 (6.7, 11.3)

Abbreviations: GA, gestational age; GDM, gestational diabetes mellitus; IUGR, intrauterine growth restriction; LGA, large-for-gestational age; PIH, pregnancy-induced hypertension;.
[1] All data shown are means or percentages (95% confidence interval).
[2] Defined as a haemoglobin <11 g/dL at ≤12 gestational weeks or >34 gestational weeks.
[3] Defined as a gestational age ≤37 weeks.
[4] According to the WHO age- and sex-standardised growth charts.
[5] According to the Spanish Obstetrics and Gynaecology guidelines.

Maternal genotypes and allele frequencies for the *MTHFR* 677C>T, *MTRR* 66A>G, *MTRR* 524C>T and *TCN2* 776C>G genetic variants are shown in **Table 9**. All the observed genotype frequencies were in Hardy-Weinberg equilibrium (p>0.05). The variant allele for the *MTRR* 66A>G and 524C>T SNPs were the most and least frequent respectively. However, the prevalence of variant homozygous genotypes was high for all of the studied SNPs (≥11.6%).

Table 9. Genotype and allele frequencies of studied polymorphisms.[1]

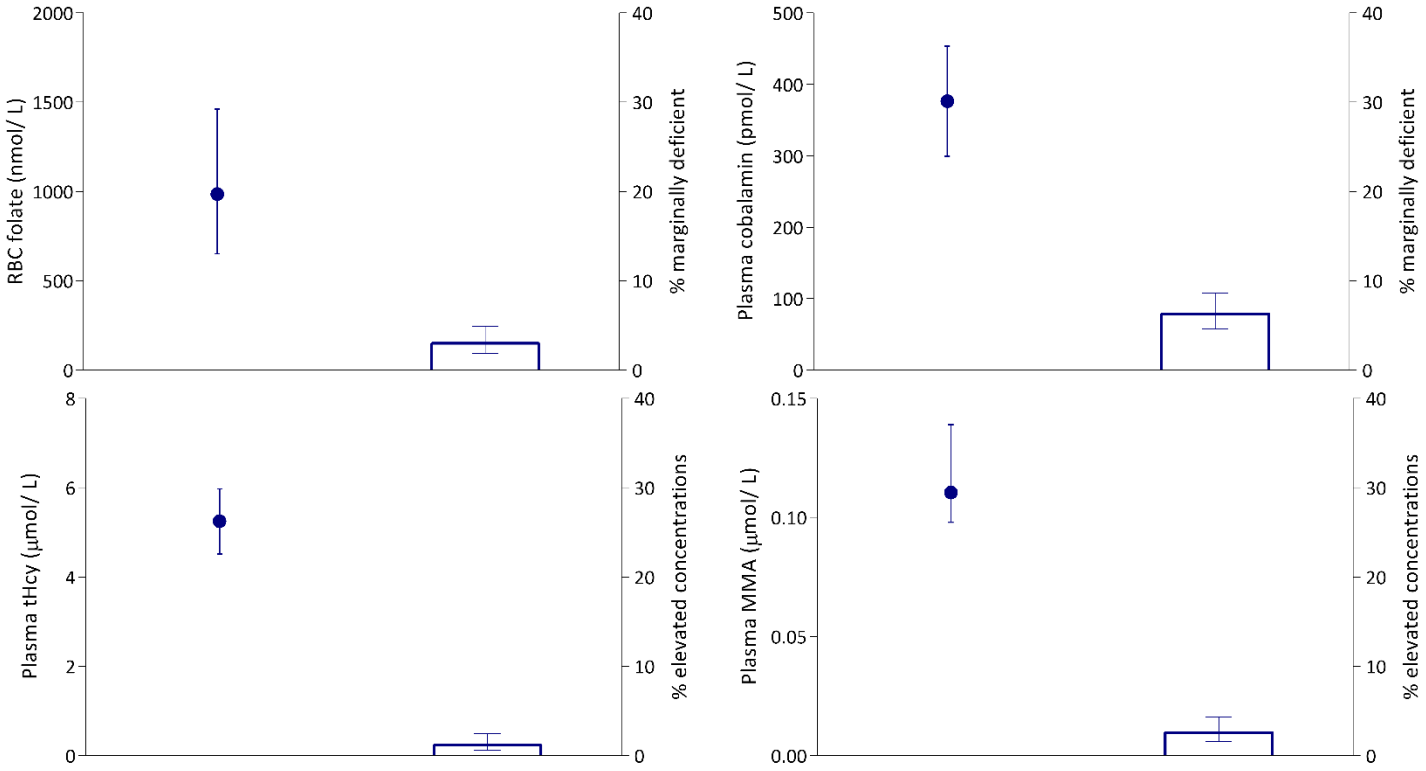
		Genotype frequency (%)			Variant allele frequency (%)
		Common homozygous	Heterozygous	Variant homozygous	
***MTHFR* 677C>T**	(n= 571)	34.3 (30.5, 38.3)	48.7 (44.6, 52.8)	17.0 (14.1, 20.3)	41.3 (38.5, 44.2)
***MTRR* 66A>G**	(n= 569)	29.5 (25.9, 33.4)	44.6 (40.6, 48.7)	25.8 (22.4, 29.6)	48.2 (45.3, 51.1)
***MTRR* 524C>T**	(n= 569)	41.3 (37.3, 45.4)	47.1 (43.0, 51.2)	11.6 (9.2, 14.5)	35.1 (32.4, 38.0)
***TCN2* 776C>G**	(n= 570)	33.2 (29.4, 37.1)	49.3 (45.2, 53.4)	17.5 (14.6, 20.9)	42.2 (39.4, 45.1)

MTHFR, methylenetetrahydrofolate reductase; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; TCN2, transcobalamin 2.
[1] Values are percentages (95% confidence interval).

Early pregnancy folate and cobalamin status

Folate and cobalamin status in early pregnancy were evaluated by assessing RBC and plasma folate and cobalamin, as well as their functional biomarkers tHcy and MMA (for cobalamin only). **Figure 10** shows the median (P_{25}, P_{75}) RBC folate, plasma cobalamin, tHcy and MMA and the prevalence of first trimester marginal deficiency or elevated plasma biomarker concentration. First trimester median plasma folate was 27.9 (P_{25}, P_{75}: 15.4, 41.6) nmol/L, and excess plasma folate (plasma folate ≥45 nmol/L) was present in 20.2% (95% CI: 17.1, 23.7%) of the women. First trimester RBC and plasma folate deficiency affected 3.1 and 11.4% of pregnancies respectively. Suboptimal RBC folate (≤906 nmol/L) was observed in 44.0 (95% CI: 39.1, 47.5) % of the women. The prevalence of suboptimal folate status was 3-fold lower in women that had taken preconception folic acid supplements compared to women that did not (58.3%; 95% CI: 52.9, 63.5; p< 0.001). The prevalence of folate deficiency in women not taking folic acid supplements either at preconception or during the first trimester was 33.3% (95% CI: 21.7, 47.5%). However, tHcy ≥10 µmol/L was only observed in seven women. Marginal cobalamin deficiency was found in 6.3 (95% CI: 8.6, 4.6%) % of women and elevated plasma MMA in 2.7 (95% CI: 1.6, 4.3%) %.

Figure 10. First trimester folate and cobalamin status in the Reus-Tarragona Birth Cohort.



Dots are median (P_{25}, P_{75}, Y left axis) and columns percentage of marginal deficiency (95% CI, Y right axis). Marginal deficiency was defined as: RBC folate ≤340 nmol/L, plasma cobalamin ≤221 pmol/L, tHcy ≥10 µmol/L and MMA ≥0.210 µmol/L. Sample size: plasma markers (n= 569) and RBC folate (n= 529).

Elevated first trimester plasma folate (≥45 nmol/L) was found in 20.2 (95% CI: 17.1, 23.7) % of the participants and concomitant elevated folate (≥45 nmol/L) and low cobalamin (≤221 pmol/L) in 1.1%. The use of a lower cut-off to define elevated plasma folate (≥30 nmol/L) yielded similar results (2.1%).

Determinants of early pregnancy folate and cobalamin status

We investigated whether demographic, lifestyle and genetic factors were associated with RBC and plasma folate and plasma cobalamin by univariate linear regression analysis (**Table 10**). In this table only the R^2*100 values are reported because we set out to identify which variables are most related with RBC and plasma folate and plasma cobalamin and to identify candidates for inclusion in subsequent multivariate analyses, rather than to specifically quantify the association. Pregnancy planning and preconception folic acid supplement use were positively associated with the three metabolic markers. Total folate intake, first trimester smoking and cobalamin supplement use were all significant determinants of both RBC and plasma folate. Cobalamin intake, plasma creatinine and socioeconomic status were all associated with the two plasma biomarkers. Folic acid supplement use during the first trimester was linked with RBC and plasma folate, BMI only with plasma folate and gestational age at blood sample collection and maternal age were associated with plasma cobalamin concentrations. In univariate models, the studied genetic variants were not associated with RBC and plasma folate or with plasma cobalamin concentrations.

Table 10. Univariate associations between demographic, lifestyle and genetic factors as determinants of RBC folate and plasma folate and cobalamin in early pregnancy.[1]

	RBC Folate	Plasma folate	Plasma cobalamin
Continuous variables			
Gestational age at recruitment, weeks	0.5 [503]	0.0 [539]	1.2† [539]
Maternal age, years	0.4 [515]	0.1 [551]	0.8* [551]
Total folate intake, µg/ day	4.2‡ [403]	9.9‡ [434]	0.0 [434]
Total cobalamin intake, µg/ day	0.6 [403]	3.5‡ [434]	2.5‡ [434]
Plasma creatinine, µmol/L	0.7[2] [512]	1.8† [548]	1.1* [548]
Categorical variables			
Pregnancy planned	6.1‡ [491]	4.8‡ [524]	1.1* [524]
BMI categories	0.5 [506]	1.5* [541]	0.7 [541]
First trimester smoker	2.2‡ [517]	2.1‡ [553]	0.3 [553]
Socioeconomic status	0.9 [513]	2.3‡ [549]	1.1* [549]
Preconception alcohol consumption	0.1 [477]	0.4 [509]	0.0 [509]
Preconception folic acid supplement use	17.9‡ [470]	8.3‡ [503]	1.1* [503]
First trimester folic acid supplement use	0.8* [480]	2.4‡ [512]	0.4 [512]
First trimester cyanocobalamin containing supplement use	6.8‡ [480]	6.7‡ [512]	0.7 [512]
MTHFR 677C>T polymorphism	0.9 [516]	0.5 [549]	0.8 [549]
MTRR 66A>G polymorphism	0.5 [515]	0.7 [548]	0.2 [548]
MTRR 524C>T polymorphism	0.9 [515]	0.4 [548]	0.5 [548]
TCN2 776C>G polymorphism	0.9 [516]	0.8 [549]	0.1 [549]

Abbreviations: BMI, body mass index; *MTHFR*, *methylenetetrahydrofolate reductase*; *MTRR*, *methylenetetrahydrofolate-homocysteine methyltransferase reductase*; *TCN2, transcobalamin 2*.

[1]Univariate linear regression analysis with demographic, lifestyle and genetic variants as independent variables and RBC folate and plasma folate and cobalamin as dependent variables. Plasma folate and cobalamin were natural log transformed before analysis. Values are univariate model R^2*100, symbols indicate model significance and square brackets sample size. Independent variables were regressed as continuous (with specified units), or categorical variables: Pregnancy planning [no vs. yes]; BMI categories [underweight BMI <18.5, overweight 25 ≤ BMI > 30 or obesity BMI ≥ 30 vs. normal weight 18.5 ≤ BMI <25 kg/ m^2]; first trimester smoker [no vs. yes]; socioeconomic status [medium or high vs. low]; preconception alcohol consumption [no vs. yes]; preconception folic acid supplement use [no vs. yes]; 1st trimester folic acid supplement use [no vs. yes]; 1st trimester cobalamin supplement use [no vs. yes]; maternal *MTHFR* 677C>T [CT vs. CC and TT vs. CC]; *MTRR* 66A>G [AG vs. AA and GG vs. AA]; *MTRR* 524C>T [CT vs. CC and TT vs. CC]; *TCN2* C>G [CG vs. CC and GG vs. CC]. *p< 0.05; †p< 0.01; ‡p< 0.001.

[2] p= 0.059.

We evaluated the effects of variables significantly associated in the univariate models with RBC and plasma folate and plasma cobalamin in multivariate analysis by stepwise multiple linear regression analysis, which are detailed below. Determinants of functional biomarkers of folate and cobalamin status were also assessed. Multi-collinearity was assessed by examining tolerance, which was lowest for plasma folate (0.69) in the model assessing tHcy concentrations.

RBC folate model

Folic acid supplement intake at preconception increased RBC folate (β= 586 nmol/L; 95% CI: 459, 713) and was its major determinant, explaining almost 17.3% of first trimester RBC folate variability. The factors that most contributed to RBC folate after preconceptional folic acid supplement use (in the model that explained in total 28.9% of its variability; n= 393; p < 0.001) were cobalamin supplements in the first trimester (β= -390 nmol/L; 95% CI: -513, -268), pregnancy planning (β= 172 nmol/L; 95% CI: 14, 329) and *MTHFR* 677 CT (β= -154 nmol/L; 95% CI: -282, -26) and TT (β= -307 nmol/L; 95% CI: -471, -142) compared to CC, in that order.

Plasma folate model

Plasma folate increased with increasing total folate intake (β= 1% per 100 µg/day; 95% CI: 0.7, 1.3) and was higher in women who had planned their pregnancy (β= 1% per 100 µg/day; 95% CI: 0.7, 1.3) and in those taking folic acid supplements before conception (β= 1% per 100 µg/day; 95% CI: 0.7, 1.3) or during the first trimester (β= 1% per 100 µg/day; 95% CI: 0.7, 1.3). These factors explained 28.8% of plasma folate variability (n= 408; p for model< 0.001). Total folate intake and folic acid supplement use at preconception, alone, accounted for 14.8% of plasma folate variability.

Plasma cobalamin model

Only 7.8% of total plasma cobalamin variability was explained in the fully adjusted model (n= 408; p for model< 0.001). Per µg/day of cobalamin ingested, early pregnancy cobalamin concentrations increased by 2.3% (95% CI: 1.0, 3.6%). Total cobalamin intake, plasma creatinine (β= 0.7%; 95% CI: 0.3, 1.2) and folic acid supplement use at preconception (β= 8.0%; 95% CI: 1.5, 14.9) were, in that order, the three major determinants of plasma cobalamin, and were positively associated with this B vitamin. The same model as for plasma cobalamin was used to identify the determinants of holoTC. Explained variability was lower than for cobalamin, and only plasma creatinine and gestational age at blood sampling were associated with holoTC (positive association).

THcy model

The same model as for plasma folate was fitted, but including RBC and plasma folate and plasma cobalamin. The model explained 25.2% of early pregnancy tHcy variability (n= 408; p for model< 0.001). RBC folate (β= -0.60%; 95% CI: -0.96, -0.24) and plasma cobalamin (β= -3.45%; 95% CI: -5.21, -1.70) were negatively associated with plasma tHcy, and plasma creatinine (β= 1.00%; 95% CI: 0.67, 1.32), *MTHFR* 677 TT vs. CC (β= 12.52%; 95% CI: 6.19, 19.23) and *MTRR* 66 AG vs. AA (β= 6.65%; 95% CI: 1.01, 1.11) and *MTRR* 66 GG vs. AA (β= 9.02%; 95% CI: 3.2, 15.19) were positively associated with tHcy concentrations.

Plasma MMA model

Plasma cobalamin (β= -0.08%; 95% CI: -0.10, -0.05) was the major determinant of MMA concentrations (R^2*100= 7.7%), and, along with first trimester smoking (β= -6.96%; 95% CI: -

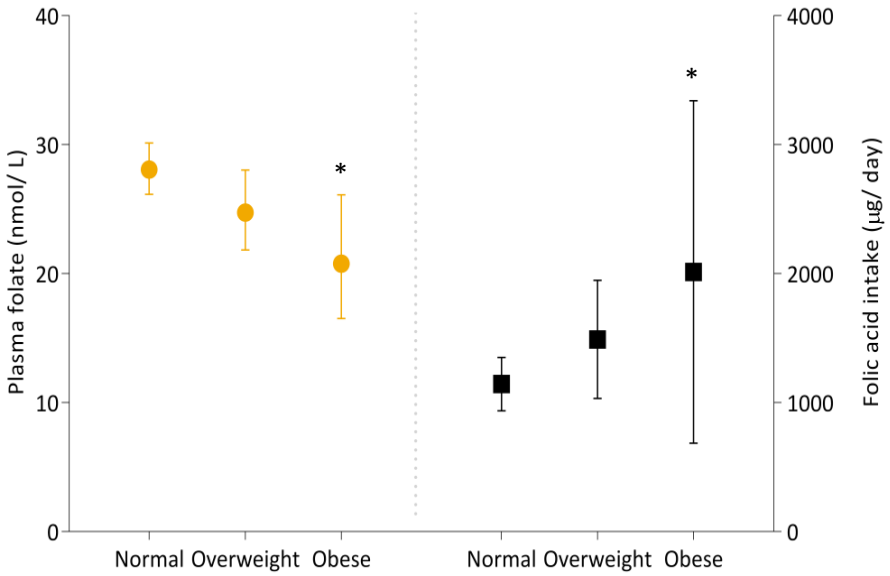
13.34, -0.12), was negatively associated with MMA. Creatinine (β= 0.69%; 95% CI: 0.28, 1.12) and the *MTRR* 524 TT vs. CC (β= 11.59%; 95% CI: 1.93, 22.16) were positively associated with MMA concentrations, and the final model accounted for 12.4% of MMA variability. All models were significant (p< 0.001).

Folate and BMI status interactions in early pregnancy

We further assessed the interactions between folate and BMI on plasma and RBC folate. Women with high BMI in the first trimester had lower plasma folate (**Figure 11**). However, we found no associations between RBC folate and BMI (p= 0.216).

Significant interactions between BMI categories and tertiles of total folate intake (p= 0.032) on RBC folate concentrations were found by ANCOVA and after adjusting for the maternal *MTHFR* 677C>T polymorphism in a co-dominant model. The associations between BMI categories and RBC folate were then assessed according to tertiles of total folate intake. There were no differences in RBC folate according to BMI categories in the first or second tertiles of total folate intake. In contrast, in the highest tertile, overweight women had significantly higher RBC folate (n= 32; mean: 1773; 95% CI: 1482, 2063 nmol/L) compared to women with normal weight (n= 85; mean: 1373; 95% CI: 1195, 1551 nmol/L; p= 0.011). These differences were found despite no differences in total folate intake between BMI categories within each tertile category of total folate intake. In addition, we found that the ratio RBC: plasma folate was higher in overweight (mean: 46.2; 95% CI: 41.8, 50.7; p= 0.036) and obese (mean: 50.0; 95% CI: 42.1, 57.7; p= 0.029) subjects compared to normal

Figure 11. Plasma folate and folic acid intake in early pregnancy according to BMI status.



Analysis of covariance was used to assess the effect of body mass index on plasma folate in early pregnancy adjusting for plasma creatinine, folic acid supplements intake at preconception, planned pregnancy and 1st trimester smoking. ANOVA was used to assess the effect of body mass index on folic acid intake. Dots are estimated means and bars 95% CI. Normal weight, 18.5 ≤BMI < 25 (n= 337); overweight, 25 ≤BMI <30 (n=109); obese, BMI ≥30 (n= 33) kg/m^2. Compared to normal weight women: *p< 0.05.

weight (mean: 40.8; 95% CI: 38.3, 43.3). Early pregnancy plasma tHcy was also higher in obese (mean: 5.9; 95% CI: 5.5, 6.3 µmol/L) compared to normal weight (mean: 5.3; 95% CI: 5.2, 5.5 µmol/L; p= 0.009) and overweight (mean: 5.4; 95% CI: 5.2, 5.6 µmol/L; p= 0.036) women. Adjusting for plasma cobalamin and creatinine concentrations did not change the results.

Folate and cobalamin interactions in early pregnancy on MMA and tHcy throughout the pregnancy

Women with elevated first trimester plasma folate (≥45 nmol/L) and marginal plasma cobalamin deficiency (≤221 pmol/L) accounted for a small proportion of the population (1.1%). After removing cases with a Cook's distance >1 (n= 1, Cook's distance= 2.99), elevated plasma folate did not modify the association between cobalamin status and plasma MMA concentrations in early pregnancy ($p_{interaction}$= 0.603, **Table 11**). No interactions between folate and cobalamin on MMA at 15, 24-27, 34 GW or at labour ($p_{interaction}$ always ≥ 0.333 throughout all of these time points) were observed. Models were adjusted for plasma creatinine, BMI categories and first trimester smoking (for <12 GW) or smoking throughout pregnancy (for all other time points). Folate status did not modify the association between cobalamin and tHcy concentration at any point ($p_{interaction}$≥ 0.178) after adjusting for plasma creatinine, BMI categories, *MTHFR* 677C>T (co-dominant model) and first trimester smoking (for <12 GW) or smoking throughout the pregnancy (for all other time points). A different cut-off for elevated plasma folate (≥30 nmol/L) has also been suggested, and therefore all analyses were repeated using this threshold. Subjects with a Cook's distance >1 were also excluded from these analyses (n= 2, Cook's distance ≥1.29). Again, no significant interactions were found between cobalamin and folate status on MMA ($p_{interaction}$> 0.143) nor on tHcy concentrations ($p_{interaction}$≥ 0.084) at any time of pregnancy.

Given the limitation of the low number of participants in the low cobalamin status sub-groups, we tested the same hypothesis by setting low cobalamin status at P_{25} (≤ 299 µmol/L). Folate and cobalamin interacted significantly on first trimester MMA concentrations ($p_{interaction}$= 0.030). In subjects with adequate cobalamin status, MMA did not differ according to plasma folate status (mean difference from non-elevated plasma folate: 0.004; 95% CI: -0.01, 0.02 µmol/L). However, in women with inadequate cobalamin status, MMA concentrations were higher in women with elevated plasma folate compared to non-elevated plasma folate (mean difference from non-elevated plasma folate: 0.02; 95% CI:

0.5, 0.00). The interaction on MMA was not observed at any other time point of the study ($p_{interaction} \geq 0.131$), nor on plasma tHcy at any point during pregnancy ($p_{interaction} \geq 0.066$).

Table 11. Biomarker concentrations according to early pregnancy cobalamin and folate status groups throughout pregnancy.[1]

Cobalamin status[2]	Folate status[2]	MMA (µmol/L)				
		<12 GW	**15 GW**	**24-27 GW**	**34 GW**	**Labour**
>221	<45	0.12 (0.12, 0.13) [405]	0.12 (0.11, 0.13) [307]	0.14 (0.13, 0.14) [360]	0.16 (0.15, 0.16) [360]	0.17 (0.17, 0.18) [355]
>221	≥45	0.12 (0.11, 0.13) [105]	0.12 (0.11, 0.14) [91]	0.13 (0.12, 15) [102]	0.17 (0.15, 0.18) [102]	0.17 (0.16, 0.19) [91]
≤221	<45	0.15 (0.13, 0.17) [29]	0.21 (0.17, 0.25) [21]	0.19 (0.17, 0.22) [27]	0.22 (0.19, 0.25) [27]	0.24 (0.21, 0.27) [26]
≤221	≥45	0.13 (0.10, 0.17) [5]	0.14 (0.05, 0.22) [4]	0.13 (0.07, 0.19) [4]	0.19 (0.12, 0.27) [4]	0.18 (0.11, 0.25) [3]
Cobalamin status	**Folate status**	**tHcy (µmol/L)**				
		<12 GW	**15 GW**	**24-27 GW**	**34 GW**	**Labour**
>221	<45	5.5 (5.4, 5.6) [405]	4.6 (4.5, 4.7) [305]	4.9 (4.8, 5.0) [358]	5.5 (5.4, 5.7) [357]	6.5 (6.3, 6.7) [342]
>221	≥45	4.8 (4.6, 5.0) [105]	4.3 (4.2, 4.5) [91]	4.3 (4.0, 4.5) [102]	5.1 (4.7, 5.5) [91]	5.9 (5.5, 6.3) [90]
≤221	<45	5.8 (5.4, 6.3) [29]	5.0 (4.6, 5.4) [21]	5.3 (4.9, 4.5) [27]	6.1 (5.4, 6.8) [26]	7.4 (6.6, 8.2) [24]
≤221	≥45	5.8 (4.9, 6.8) [6]	4.9 (4.1, 5.7) [5]	4.3 (3.2, 5.4) [5]	5.9 (4.1, 7.6) [4]	6.3 (4.5, 8.0) [5]

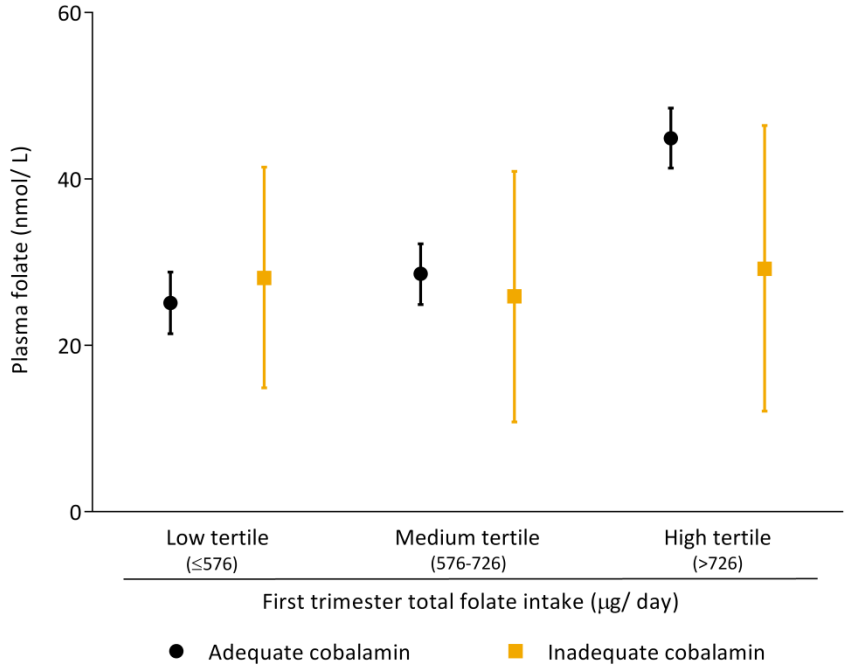
Abbreviations: GW, gestational weeks; MMA, methylmalonic acid; tHcy, total fasting plasma homocysteine.

[1] Means (95% CI) were derived by using ANCOVA analysis adjusting for plasma creatinine (at blood sample collection), first trimester BMI categories and 1st trimester smoking (at <12 GW) or throughout pregnancy (from 15 GW). Plasma tHcy model also included maternal *MTHFR* 677C>T (CT vs. CC and TT vs. CC). P-values for interaction term (cobalamin*folate status) were as follows for <12 gestational weeks until labour: for MMA, p= 0.603, p= 0.148, p= 0.077, p= 0.403 and p= 0.183; for tHcy, p= 0.178, p=0.583, p= 0.514, p= 0.851 and p= 0.614. Subjects with a Cook's distance >1 were excluded from the analyses.

[2] Cobalamin status (pmol/L) and folate status (nmol/L) at <12 GW.

In animal models, it has been suggested that cobalamin deficient animals with a high folate intake experience a rise in plasma (444) and a reduction in RBC folate concentrations, due to the inability to retain folate forms in tissue cells. We evaluated whether the effects of folate intake on plasma and RBC folate were modified by cobalamin status by ANCOVA analysis. **Figure 12** shows the first trimester effects of total folate intake on plasma folate concentrations according to cobalamin status. No differences in plasma folate were observed between cobalamin status groups within each tertile of total folate intake. No significant interactions were found between first trimester total folate intake tertiles and cobalamin status on plasma folate at any time of pregnancy ($p_{interaction} \geq 0.215$), following exclusion of participants with Cook's distance over 1 (n= 1; Cook's distance= 2.95). In addition, cobalamin status did not modify the association between total folate intake and RBC folate concentrations at any time of pregnancy ($p_{interaction} \geq 0.715$).

Figure 12. Plasma folate according to 1st trimester total folate intake and cobalamin status.



Analysis of covariance was used to assess the cobalamin status (> or ≤221 pmol/ L) on plasma folate in early pregnancy according to 1st trimester total folate intake and adjusting for plasma creatinine, 1st trimester smoking and *MTHFR* 677C>T. Sample size: Low tertile: adequate (n= 127), inadequate cobalamin (n= 10); medium tertile: adequate (n= 132), inadequate cobalamin (n= 8); high tertile: adequate (n=137), inadequate cobalamin (n= 7). P-value for interaction= 0.256.

The effect of first trimester folate and cobalamin interactions on haematological parameters

Haematological abnormalities have also been observed in subjects with concomitant cobalamin deficiency and elevated folate status. We used ANCOVA analysis to check for interactions between elevated plasma folate and marginal cobalamin deficiency, as previously defined, on haemoglobin concentrations and MCV in early (<12 GW) and late pregnancy (34 GW). Folate status modified the association between cobalamin and haemoglobin ($p_{interaction}$= 0.005) only at <12 GW. **Table 12** shows the effects of cobalamin

according to folate status on haemoglobin in early pregnancy. Women with non-elevated folate and cobalamin inadequacy had lower haemoglobin than women with adequate cobalamin (p= 0.04). However, in women with elevated folate status, inadequate cobalamin was borderline associated with higher haemoglobin concentration compared to women with adequate cobalamin status (p= 0.062). There were no interactions neither with MCV in early pregnancy ($p_{interaction}$= 0.136) nor with haemoglobin ($p_{interaction}$= 0.089) or MCV ($p_{interaction}$= 0.679) at 34 GW. All models were adjusted for BMI, socioeconomic status, creatinine concentrations, first trimester smoking (at <12 GW) or smoking throughout the pregnancy (from 15 GW) and iron supplement intake (first or second trimester intake according to the time of pregnancy studied).

Table 12. Early pregnancy haemoglobin concentrations (g/dL) according to cobalamin and folate status. [1]

	Cobalamin status	
	Adequate (>221 pmol/L)	**Inadequate (≤221 pmol/L)**
Non-elevated folate (<45 nmol/L)	12.7 (12.7, 12.8)	12.5 (12.3, 12.7)*
Elevated folate (≥45 nmol/L)	12.4 (12.1, 12.7)	13.3 (12.5, 14.0)†

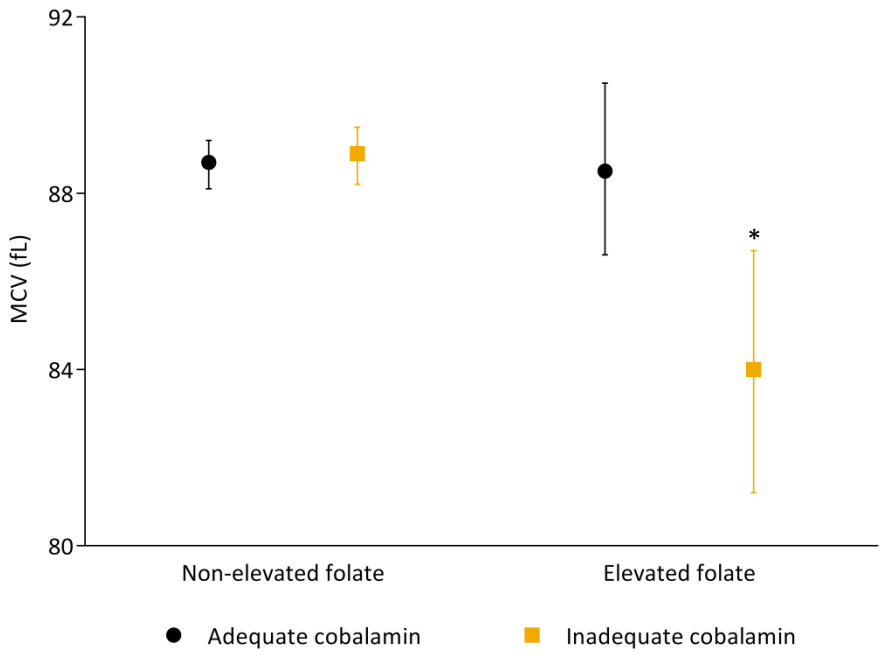
[1] Values are means (95% CI). Subjects were categorised according to cobalamin status and non-elevated plasma folate (<45 nmol/L) or elevated plasma folate (≥45 nmol/L). Means were derived by ANOVA after adjusting for first trimester BMI (kg/ m^2), age (years), socioeconomic status, plasma creatinine concentrations (µmol/L), 1st trimester smoker and iron supplement use. Different from adequate cobalamin status within each folate status group: *p= 0.040; †p= 0.062.

By using the lower cut-off for elevated plasma folate (≥30 nmol/L) no interactions were observed between folate and cobalamin statuses on haemoglobin concentrations at either <12 or at 34 GW ($p_{interaction}$= 0.177 and 0.666, respectively). However, significant interactions were observed on MCV at both <12 ($p_{interaction}$= 0.015) and 34 GW ($p_{interaction}$= 0.029). At <12 GW, there were no differences in MCV according to cobalamin status in subjects with non-

elevated folate status. However, in pregnant women with elevated folate status, MCV was 4.8 fL (95% CI: 2.0, 7.7 fL) lower in inadequate cobalamin status women than in those with adequate cobalamin status (**Figure 13**). The same results were obtained at 34 GW: in women with high first trimester folate status, MCV was lower if women also had inadequate cobalamin status (mean difference: 5.5; 95% CI: 1.9, 9.1). No differences were observed in subjects with non-elevated folate status (88.6 [95% CI: 89.9, 89.3] vs. 88.3 [95% CI: 85.9, 90.8] in adequate vs. inadequate cobalamin respectively).

Whether elevated folate status modified the associations between cobalamin and pregnancy anaemia (haemoglobin <11 g/ dL) was assessed at both early (<12 GW) and at late pregnancy (≥34 GW). No interaction between folate and cobalamin status was observed on the risk of early ($p_{interaction}$= 0.737) or late pregnancy ($p_{interaction}$= 0.999) anaemia. These observations were not different when a lower cut-off point for elevated folate status (≥30 nmol/L) was used.

Figure 13. Early pregnancy mean cell volume according to plasma folate and cobalamin status.



Analysis of covariance was used to assess the interactions between cobalamin status (> or ≤221 pmol/L) and plasma folate status (< or ≥30 nmol/L) on mean cell volume adjusting for BMI, socioeconomic status, creatinine concentrations, 1st trimester smoking and 1st trimester iron supplement intake. Sample size: Non-elevated folate: adequate (n= 233), inadequate cobalamin (n= 20); elevated folate: adequate (n= 221), inadequate cobalamin (n= 11). P-value for interaction= 0.015. Compared to adequate status within each folate status group. *p< 0.001

Discussion

In this chapter, we described folate and cobalamin status metabolic biomarkers and their determinants in early pregnancy. We also evaluated the interactions between elevated plasma folate and low cobalamin status on metabolic and haematological markers of cobalamin deficiency. Our results show no evidence of a deleterious effect of elevated plasma folate on the association between marginally low cobalamin and metabolic or

haematological markers during pregnancy in a population exposed to regular folic acid supplement use.

Marginal first trimester cobalamin and RBC folate deficiencies were found in 6.3% and 3.1% of women respectively. The prevalence of elevated plasma folate was 20.2% in early pregnancy. Only 1 in every 3 women used preconceptional folic acid supplements and, suboptimal RBC folate status was observed in 15.9% of the women. However, blood samples were not drawn at preconception and therefore first trimester folic acid supplement use could have reduced the prevalence of suboptimal folate status. This data and data from national birth cohorts suggests that folic acid supplement use at preconception in our sample is similar to Denmark (30%) (445), but higher than in Norway (446) and France (447) (20 and 15% respectively). There was no evidence of functional cobalamin or folate deficiency as indicated by plasma tHcy and MMA in early pregnancy.

In our sample, mean RBC folate concentrations were marginally lower and suboptimal RBC folate prevalence higher compared to those reported in Canadian pregnant women (mean RBC folate: 1280 nmol/L; suboptimal RBC folate: 26.3%) (448), where folic acid fortification was implemented in 1998. However, mean RBC folate and plasma tHcy concentrations were similar to those observed in women using folic acid supplements during the first trimester (400 µg/day) in Northern Ireland (249) and in The Netherlands (91). In a different Spanish cohort, plasma folate (35 nmol/L) and tHcy (4.4 µmol/L) were higher and lower respectively at 15 GW (243). Studies from India (389) and Nepal (103) reported slightly lower RBC and plasma folate status compared to our study. In a previous study of a representative sample of our local population, our group observed that a quarter of women of fertile age not using vitamin supplements were folate deficient (≤7 nmol/L) (144). In the present study, the prevalence of folate deficiency was lower in the whole sample, but was higher in women not taking folic acid supplements either at preconception or during the first trimester (33%; 95% CI: 21.7, 47.5). We observed that median cobalamin status was higher than in a different Spanish cohort (243), and almost doubled that of Northern Irish

(249) and Nepalese (103) women, while tripling that of Indian (389) women (at 18 GW). Plasma MMA was only assessed in the Indian study, in which the average concentrations were almost 8-fold higher than those from this study (389). Marginal cobalamin deficiency was observed in 35% of Canadian mothers at 12-16 GW (99). In the same Canadian study, tHcy was lower than in our study, probably because of the mandatory fortification with folic acid or timing of blood sampling. Cobalamin status in pregnant women in a previous study from our group was lower than in the present study, but MMA concentrations were similar (76). Lifestyle, nutritional, environmental and genetic factors may be responsible for the differences observed between different studies. However, pregnant women in our study may not be representative of fertile women in our population because it included women that had all of their prenatal care in the hospital. These were women with high-risk pregnancies or hospital workers. The majority of the women declared having planned their pregnancy, and the rate of planned pregnancies was higher than previously reported in many countries (449). It is possible that the frequency of folic acid supplement use at preconception was overestimated, because a recent multi-centre Spanish study reported it to be 22% (450).

At <12 GW, RBC folate is a good estimator of preconceptional folate status as seen by its association with preconceptional folic acid use. Unexpectedly, women using cobalamin-containing supplements during the first trimester had lower plasma and RBC folate compared to women not using cobalamin supplements. This is likely to be due to the fact that high dose folic acid supplements (>1 mg) on the Spanish market do not contain cobalamin, and supplements containing cobalamin usually have lower doses of folate (≤400 µg). Findings from previous studies suggesting a negative association between plasma folate and BMI (91,264–266) were confirmed in pregnant women despite a higher intake of folic acid from supplements in obese compared to normal weight women. However, the effects of BMI on RBC folate appear to be mediated by dietary folate intake. In pregnant women with medium-high folate intake, overweight was associated with higher RBC folate.

In contrast, we found no differences in RBC folate between obese and normal weight women. These findings support a previous report that obese compared to normal weight non-pregnant women consumers of at least 400 μg/dday of folic acid from supplements had higher RBC folate (264). Obesity may influence the intestinal uptake of folic acid if adiposity diminishes the uptake of orally administered folic acid (272,434). In addition, the up-regulation of transepithelial transport of folic acid in intestinal cells in folate deficiency (213) is a potential mechanism for a higher RBC folate when plasma folate is low (for example, in overweight or obese subjects). In addition, obese women had higher first trimester tHcy and RBC: plasma folate ratio than normal weight women.

In early pregnancy, the prevalence of concomitant elevated folate and low cobalamin status was low (1.1%). To the best of our knowledge, the interactions between folate and cobalamin status on metabolic and haematological indicators of low cobalamin have not been studied in pregnant women before. Our results do not replicate previous reports of a deleterious effect of elevated plasma folate status on plasma MMA and tHcy in cobalamin deficient old adults (310,311). On the other hand, the lack of interactions between early pregnancy elevated folate and low cobalamin status on haemoglobin and MCV, endorsed the metabolic findings as well as previous findings in old adults from the UK (314). However, when we applied a lower cut-off for elevated plasma folate, MCV was lower at early and late pregnancy in subjects with concomitant elevated plasma folate and low cobalamin than in subjects with adequate cobalamin and elevated folate. These differences were not observed in subjects with non-elevated plasma folate. In 2010, Morris and collaborators published similar results in relation to MCV (313). In noninstitutionalised USA adults with cobalamin deficiency, higher circulating folic acid was associated with lower MCV (β-coefficient: -1.44; 95% CI: -2.84, -0.03), potentially reducing the macrocytosis associated with cobalamin deficiency. However, the effects of elevated plasma folate (>75th percentile) on macrocytosis in low cobalamin subjects varied according to the method used

to determine plasma folate. This issue is unlikely to apply to our study since both plasma and RBC folate were determined by the gold standard (microbiological assay) (452). Initial concerns regarding folic fortification stemmed from the possible missed diagnosis of cobalamin deficiency by the MCV reduction by an elevated folate status. Given that folic acid must be first reduced to THF, and that THF may be used to synthesize purines and dTMP, elevated plasma folate from folic acid consumption may bypass the 5-methylTHF block in cobalamin deficient subjects. This would allow all cells in general, and rapidly dividing cells in particular, to mature properly. Proper maturation of erythrocytes is observed in parallel to a reduction in MCV following fortification with folic acid in the USA. The prevalence of low serum cobalamin in the absence of macrocytosis or anaemia did not increase. This suggests that there is no reason for concern regarding masking of cobalamin deficiency at the population level (453). We did not detect macrocytosis in our study and an effect on MCV does not necessarily indicate an effect on macrocytosis. The prevalence of women exposed to elevated plasma folate was higher than that reported in studies carried out in non-fortified countries but the prevalence of cobalamin deficiency was very low, and the use of a higher cut-off defining inadequate cobalamin status could have masked the potential negative effects of elevated folate. Furthermore, the exposure to high dose folic acid supplements was confined, at most, to some months before conception and until blood samples were collected in the first trimester. Therefore, a longer-term exposure, similar to that of folic acid fortification, may be needed to detect a deleterious effect of elevated folate status in subjects with low cobalamin concentrations.

As intervention studies with folic acid on cobalamin deficient populations would be unethical, the longitudinal design of our study offers the most suitable choice to investigate folate-cobalamin interactions. Relevant limitations must be highlighted, such as the lack of data regarding the underlying cause of low cobalamin status, which lessened our ability to predict early pregnancy cobalamin concentrations. In order to redress this point,

information on potential pregnancy confounders affecting plasma cobalamin, folate, MMA and tHcy such as renal function, supplement use or genetic polymorphisms was carefully recorded. In addition, and to ensure appropriate sample processing, optimized procedures were used to minimize sample processing artefacts in MMA, tHcy and cobalamin determinations (454). It is also important to highlight that, despite our relatively large sample size, the low cobalamin/ high folate group was small. Only a 1.1% of the total sample was classified in this group, however the prevalence in the population may be higher given the medium-high socioeconomic status of the studied sample.

The demonstration that the negative association between plasma folate and BMI is also observed in pregnant women during the first trimester gives some insight into the potential mechanisms by which BMI may influence NTD risk. The intake of folate appears to be responsible for the positive association between RBC folate and BMI.

Elevated plasma folate in pregnant women with inadequate cobalamin status has no harmful effects on metabolic or haematological markers of cobalamin deficiency in this non-fortified population exposed in the short term to folic acid supplement use. Despite high use of folic acid supplements in the first trimester, folate and cobalamin status are are still far from being optimal for the maximum prevention of NTD.

RESULTS CHAPTER 2

Chapter 2: Determinants of child folate and cobalamin status

Introduction

In Western countries, folate and cobalamin deficiencies are not prevalent during childhood. In the USA, cobalamin deficiency in children is estimated to be lower than 1% (114). A meta-analysis from 2001 including 1490 children, reported that the mean cobalamin concentration in Spanish children aged 0-15 years old was 502 pmol/L (standard deviation, 93.7) (118). Worldwide folate status in pre-school and school age children seems not to be compromised, according to existing reported data (81,94,119,120,251–253).

Breast and formula milk contain enough folate to maintain adequate status in newborns and protect them from folate deficiency. In breastfed infants, human milk folate is maintained at the expense of maternal stores (277). Maternal cobalamin during pregnancy is highly associated with breast milk cobalamin (455), and exclusive breastfeeding for extended periods places greater nutritional demands on the mother. Indeed, longer exclusive breastfeeding, as well as breastfeeding *per se*, is associated with reduced child serum cobalamin (97,107). In contrast to cobalamin, folate status in neonates is positively associated with exclusive breastfeeding duration (97). In 2001, the WHO recommended that all infants should be exclusively breastfed until the age of 6 months, but several concerns have emerged given the insufficient available evidence supporting this recommendation (109,110).

Maternal cobalamin status during pregnancy is the major determinant of offspring status up until two years of age (80,95,104,105). Child cobalamin deficiency is considered to be secondary to maternal cobalamin deficiency. Supplementation with physiological doses of cobalamin during pregnancy increases postnatal cobalamin in the baby, while reducing MMA and tHcy (65,78,101,102). Only one study, carried out in India has investigated

whether the impact of cobalamin status during pregnancy on child cobalamin persists after two years of age (106). Maternal and cord blood folate concentrations are the main determinants of folate status at the age of 6 months (105).

The effects of the *MTHFR* 677C>T polymorphism on tHcy and RBC and plasma folate status have been widely described in adults, but not in children. The limited body of evidence suggests that this genetic variant affects 1C metabolism in children older than 10 years (279,283,284), boys (285) or subjects with low folate status (284). Other genetic variants such as the *TCN2* 776C>G, *MTRR* 66A>G or 524C>G that have been studied in adults have attracted limited attention in paediatric settings. Only one study reported the effects of *TCN2* 776A>G on 1C metabolism in children, with higher tHcy in variant compared to common homozygotes (136).

Data regarding child folate or cobalamin status (or functional status) in developed countries is limited to a few reports from Norway (81), the USA (119), the UK (120), Belgium (121) and Greece (122) and none of them have reported the association between maternal and child cobalamin status. In addition, all studies reporting the association between pregnancy folate or cobalamin or breastfeeding and infant status in these vitamins are limited to children under two years of age. The effects of genetic variants affecting folate or cobalamin transport or metabolism have been scarcely studied. It is not known whether maternal folate and cobalamin status or breastfeeding affect offspring folate and cobalamin status at mid-childhood.

Specific objectives

The specific objectives of this chapter are:

- To describe cobalamin and folate metabolic indicators in children at mid-childhood (7.5 years).

- To determine the association between genetic, nutritional and other lifestyle factors and mid-childhood folate and cobalamin status.

- To investigate the association between prenatal folate and cobalamin status and mid-childhood one carbon metabolism status indicators.

Material and methods.

The materials and methods applicable to this chapter have been described in the general Material and methods section.

Statistical analysis.

We first examined the descriptive characteristics in the offspring at mid-childhood. Means (95% CI) or median (P_{25}, P_{75}) or relative frequencies (95% CI) are shown for mothers of children that participated in the follow-up check-up and those of non-participants. Descriptive characteristics are reported for all of the children and according to sex. Differences in maternal characteristics according to follow-up participation, and child characteristics according to sex were assessed by t-test or χ^2 for continuous or categorical variables respectively. Genotype and allele frequencies (95% CI) of the studied genetic variants are also reported. One sample or goodness of fit χ^2 was used to test deviations from Hardy-Weinberg equilibrium of the observed allele frequencies (440).

Folate deficiencies were defined as: plasma folate ≤10 nmol/L (237), RBC folate ≤340 nmol/L (237) and elevated plasma tHcy as tHcy ≥10 µmol/L (441). Marginal cobalamin deficiencies were defined as: plasma cobalamin ≤221 pmol/L (442) or plasma MMA ≥0.210 µmol/L (443). Suboptimal RBC folate was defined as ≤906 nmol/L (238).

The bivariate correlations between maternal folate and cobalamin status throughout pregnancy and cord and child vitamin statuses were assessed (Pearson correlation coefficients are reported). Plasma nutrient /biomarker (folate, cobalamin, holoTC, MMA and tHcy) distributions were skewed to the right so were natural log transformed.

Demographic, lifestyle and genetic determinants of RBC and plasma folate and plasma cobalamin at mid-childhood were evaluated by univariate linear regression analysis. All variables observed to be associated with child folate or cobalamin status were then included in the multiple linear regression models. Determinants of functional biomarkers of folate and cobalamin status (tHcy and MMA respectively) were identified by stepwise multiple linear regression analysis based on the determinants of folate and cobalamin status. The four studied genetic variants were included in all of the models.

Results

Comparison of selected descriptive characteristics of studied participants

Selected maternal demographic and lifestyle descriptive characteristics are shown in **Table 13** according to child participation in the follow-up phase. Mothers of child participants were more likely to have consumed alcohol before and during pregnancy than those of nonparticipants ($p< 0.001$). Daily energy intake and second trimester cobalamin supplement use were higher in mothers of participators compared to non-participators ($p< 0.05$) but no other significant differences in maternal lifestyle, demographic or nutritional descriptive characteristics were observed between the two groups. We assessed differences in obstetrical and perinatal characteristics between the two groups of mothers by t-test and χ^2 for continuous and categorical variables respectively, and no differences were detected.

Table 13. Maternal demographic, lifestyle and nutritional descriptive characteristics according to child participation in the follow-up phase.[1]

	Followed-up	Not followed-up
GA at recruitment, weeks	9.1 (8.8, 9.4) [161]	9.3 (9.1, 9.5) [395]
1st trim age, years	32.3 (31.7, 32.9) [162]	31.7 (31.2, 32.2) [398]
Previous pregnancies, %	49.4 (41.8, 57.0) [162]	54.4 (49.5, 59.2) [399]
Previous abortions, %	34.0 (27.1, 41.5) [162]	33.1 (28.6, 37.8) [399]
Previous preterm birth, %	3.7 (1.7, 7.8) [162]	6.0 (4.1, 8.8) [399]
Planned pregnancy, %	80.8 (73.8, 86.3) [151]	80.4 (76.1, 84.0) [382]
1st trimester BMI, kg/m^2	23.8 (23.1, 24.1) [162]	23.8 (23.4, 24.2) [388]
1st trimester smoking, %	22.2 (16.5, 29.2) [162]	18.5 (15.0, 22.6) [388]
Smoking throughout pregnancy, %	20.4 (14.9, 27.2) [162]	16.1 (12.8, 20.1) [397]
Socioeconomic status	[162]	[396]
Low	3.7 (1.7, 7.8)	7.8 (5.6, 10.9)
Middle	47.5 (40.0, 55.2)	50.8 (45.8, 55.7)
High	48.8 (41.2, 56.4)	42.0 (37.4, 46.8)
Alcohol consumption, %		
Preconception	70.7 (62.9, 77.5) [147]	46.4 (41.4, 51.4)† [371]
Pregnancy	24.2 (18.0, 31.6) [149]	9.5 (6.9, 12.9)† [379]
Preconception illegal drug use, %	2.0 (0.7, 5.6) [153]	2.6 (1.4, 4.7) [386]
Pregnancy illegal drug use, %	0.0 (0.0, 0.0) [153]	0.5 (0.1, 1.9) [386]
Dietary intake		
Energy, kcal/day	1869 (1773, 1965) [145]	1992 (1937, 2045)* [322]
Total folate, µg/day[2]	648 (535, 2399) [138]	636 (506, 774) [303]
Total cobalamin, µg/day[2]	5.9 (4.5, 7.1) [138]	6.2 (4.9, 8.0) [303]
Folic acid supplements, %		
Preconception	36.7 (29.4, 44.8) [162]	33.6 (28.9, 38.6) [392]
1st trimester	88.1 (81.9, 92.3) [151]	87.3 (83.5, 90.3) [370]
1st trimester folic acid intake, µg/day[2]	400 (267, 1936) [151]	400 (267, 501) [370]
Cobalamin supplement use, %		
1st trimester	58.3 (50.3, 65.8) [151]	65.9 (61.0, 70.6) [370]
2nd trimester	27.5 (21.0, 35.0) [153]	36.6 (32.0, 41.5)* [385]
1st trimester cobalamin supplement intake, µg/day[2]	1.1 (0.0, 1.8) [151]	1.3 (0.0, 1.8) [370]
Iron supplement use, %		
1st trimester	15.9 (10.9, 22.6) [151]	15.4 (12.1, 19.4) [370]
2nd trimester	64.1 (56.2, 71.2) [153]	55.3 (50.3, 60.2) [385]

Abbreviations: BMI, body mass index; GA, gestational age.

[1]All data shown are means or percentages (95% confidence interval), unless otherwise indicated. Differences between mothers of participators and non-participators were assessed by χ^2 and t-test for categorical and continuous variables respectively. *p< 0.05; †p< 0.001

[2]Median (P_{25}, P_{75}).

Selected characteristics of the 162 participating children are described in **Table 14**. Girls accounted for 52.5% of the sample, two out of three children were breastfed and exclusive breastfeeding duration was almost 3 months on average. Total folate and cobalamin intakes were above the IOM RDA. Overweight was observed in 32 children (19.8%) and obesity was prevalent in 8.0% of the sample, according to the World Obesity Foundation criteria. Girls were less physically active ($p< 0.01$) and had lower head circumference, higher fat mass, fat mass index ($p< 0.05$) and diastolic blood pressure compared to boys ($p< 0.001$).

Table 14. Offspring descriptive characteristics at follow-up (7.5 years) overall and according to sex.[1]

	Overall (n= 162)	Girls (n=85)	Boys (n= 77)
Age, months	90.1 (89.8, 90.3)	89.9 (89.5, 90.3)	90.3 (89.8, 90.7)
Breastfed, %	66.7 (59.1, 73.5)	68.2 (57.7, 77.2)	64.9 (53.8, 74.7)
Exclusive breastfeeding duration, months	2.9 (2.5, 3.3)	2.9 (2.4, 3.5)	2.8 (2.2, 3.4)
Screen viewing time, hours/day	1.7 (1.5, 1.8)	1.6 (1.4, 1.8)	1.7 (1.5, 1.9)
Physical activity, score	3.1 (3.0, 3.1)	2.9 (2.8, 3.0)	3.2 (3.1, 3.3)†
Energy intake, kcal/day	1836 (1716, 1955)	1792 (1690, 1893)	1884 (1657, 2111)
Total folate intake, µg/day	231 (217, 246)	236 (211, 261)	226 (213, 239)
Total cobalamin intake, µg/day	4.7 (4.4, 5.0)	4.6 (4.4, 4.8)	4.8 (4.1, 5.4)
Height, cm	127 (126, 128)	127 (126, 128)	126.9 (126, 128)
Weight, kg	26.9 (26.1, 26.7)	27.1 (25.9, 28.3)	26.6 (25.6, 27.7)
BMI, kg/m^2	16.6 (16.2, 17.0)	16.7 (16.1, 17.4)	16.5 (16.0, 16.9)
Height, z-scores[2]	0.52 (0.38, 0.66)	0.61 (0.43, 0.79)	0.41 (0.20, 0.62)
Weight, z-scores[2]	0.60 (0.43, 0.78)	0.66 (0.42, 0.90)	0.53 (0.29, 0.79)
BMI, z-scores[2]	0.39 (0.21, 0.58)	0.39 (0.12, 0.66)	0.39 (0.14, 0.65)
MUAC, cm	19.2 (18.8, 19.6)	19.1 (18.5, 19.6)	19.4 (18.8, 20.0)
Head circumference, cm	51.9 (51.6, 52.2)	51.5 (51.2, 21.8)	52.4 (51.9, 52.8)‡
Waist circumference, cm	56.9 (55.8, 57.9)	56.9 (55.6, 58.0)	55.6 (55.6, 58.0)
Fat mass, kg[3]	5.6 (5.1, 6.1)	6.1 (5.3, 6.8)	5.1 (4.6, 5.6)*
Fat mass index, kg/m^2 [3]	3.4 (3.2, 3.7)	3.7 (3.3, 4.2)	3.1 (2.8, 3.4)*
Skinfold thickness sum, mm[3]	33.8 (31.2, 36.3)	37.4 (33.8, 41.1)	29.8 (26.5, 33.1)
Overweight, %[4]	19.8 (14.4, 26.6)	23.5 (15.8, 33.6)	15.6 (9.1, 25.3)
Obesity, %[4]	8.0 (4.7, 13.2)	11.8 (6.5, 20.3)	3.9 (1.3, 10.8)
SBP, mmHg[5]	105 (103, 106)	105 (103, 107)	104 (103, 106)
DBP, mmHg[5]	63 (52, 65)	66 (64, 67)	61 (59, 62) ‡

Abbreviations: BMI, body mass index; MUAC, mid-upper arm circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure.

[1] All data shown are means or percentages (95% CI). Differences between sexes were assessed by χ^2 and T-test for categorical and continuous variables respectively.*p< 0.05; †p<0.01; ‡p< 0.001

[2] According to the WHO age- and sex-standardised growth charts.

[3] Valid measurements were not obtained for 2 girls and 2 boys.

[4] Defined according to the World Obesity Foundation (former International Obesity Task Force).

[5] Point blood pressure readings were not performed in 12 girls and 15 boys.

Child genotypes and allele frequencies for the *MTHFR* 677C>T, *MTRR* 66A>G, *MTRR* 524C>T and *TCN2* 776C>G genetic variants are shown in **Table 15**. No differences in genetic variant frequencies were observed among women or children followed-up compared to those not followed-up (data not shown). All the observed genotype frequencies were in Hardy-Weinberg equilibrium (p>0.05). The variant allele for the *MTRR* 66A>G and 524C>T polymorphisms were the most and least frequent genotypes respectively. However, the prevalence of variant homozygous genotypes was high for all of the studied polymorphisms.

Table 15. Child genotype and allele frequencies of studied polymorphisms.[1]

		Genotype frequency (%)			Variant allele frequency (%)
		Common homozygous	Heterozygous	Variant homozygous	
***MTHFR* 677C>T**	(n= 146)	36.7 (32.5, 41.2)	45.0 (40.5, 49.6)	18.3 (15.0, 22.0)	40.8 (35.4, 46.5)
***MTRR* 66A>G**	(n= 147)	28.2 (24.5, 32.5)	46.4 (41.9, 51.0)	25.4 (21.6, 29.6)	47.6 (42.0, 53.3)
***MTRR* 524C>T**	(n= 146)	45.8 (41.3, 50.4)	42.3 (37.9, 46.9)	11.8 (9.2, 12.1)	33.6 (28.4, 39.2)
***TCN2* 776C>G**	(n= 147)	32.4 (28.3, 36.8)	48.5 (43.9, 53.0)	19.1 (15.8, 23.0)	40.8 (35.4, 46.5)

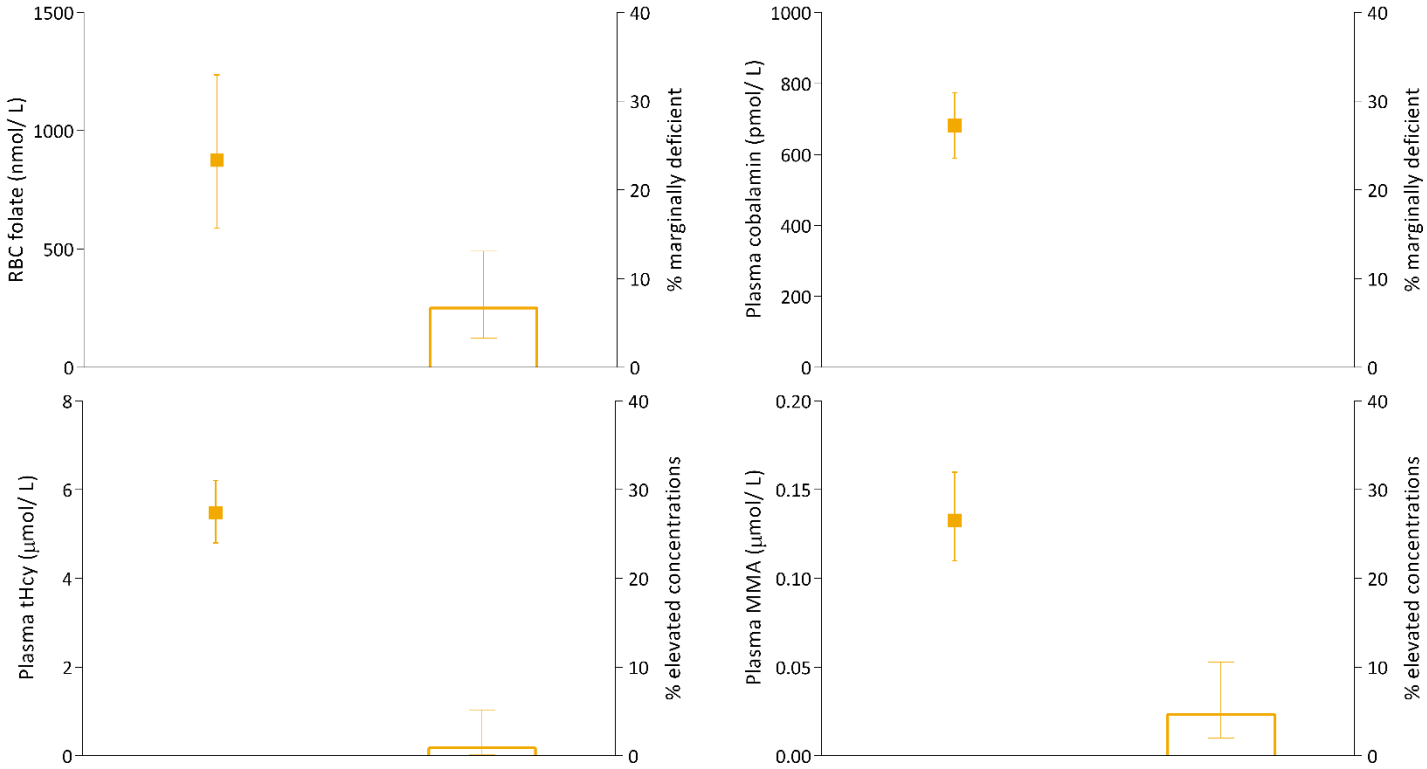
MTHFR, methylenetetrahydrofolate reductase; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; TCN2, transcobalamin 2.
[1] Values are percentages (95% confidence interval).

Child folate and cobalamin status

Folate and cobalamin status in the RTBC were evaluated by assessing RBC and plasma folate and cobalamin, as well as their functional biomarkers tHcy and MMA. **Figure 14** shows the median (P_{25}, P_{75}) RBC folate, plasma cobalamin, tHcy and MMA and the prevalence of deficiency or marginal deficiency in the offspring at mid-childhood. Median plasma folate in the offspring was 17.4 (P_{25}, P_{75}: 12.3, 26.9 nmol/L). The prevalence of mid-childhood RBC

folate deficiency was 6.7%, and plasma folate deficiency was 12.3% (95% CI: 9.2, 14.5%). Suboptimal RBC folate (≤906 nmol/L) was found in 53.3% (95% CI: 43.8, 62.6%) of children. Plasma folate excess (plasma folate ≥45 nmol/L) was observed in 5.7% (95% CI: 2.6, 11.8%) of the children. Elevated tHcy (≥10 µmol/L) was only observed in one child. Nevertheless, reference ranges have not been established for children and this is the proposed cut-off for elevated tHcy in adults. Plasma tHcy (µmol/L) was higher in children with low compared to adequate plasma folate (geometric mean [95% CI]: 6.4 [5.7, 7.4] versus 5.3 [5.1, 5.5]; $p< 0.001$) or low compared to adequate RBC folate (geometric mean [95% CI]: 6.5 [5.9, 7.2] versus 5.4 [5.1, 5.6], $p< 0.05$). We found no signs of marginal cobalamin deficiency in children, despite 5.7 (95% CI: 2.6, 11.8) % of them having elevated plasma MMA.

Figure 14. Mid-childhood folate and cobalamin status in in the Reus-Tarragona Birth Cohort.



Dots are median (P_{25}, P_{75}, Y left axis) and columns percentage of marginal deficiency (95% CI, Y right axis). Marginal deficiency was defined as: RBC folate ≤340 nmol/L, plasma cobalamin ≤221 pmol/L, tHcy ≥10 µmol/L and MMA ≥0.210 µmol/L. Sample size for plasma markers (n= 106) and RBC folate (n= 105).

Determinants of child folate and cobalamin status

The associations between maternal folate status (RBC and plasma folate and plasma tHcy) during pregnancy and status in the offspring were evaluated, and are shown in **Table 16**. Mid-pregnancy RBC folate was associated with offspring RBC folate ($p< 0.05$), but not with plasma folate nor tHcy at mid-childhood. On the other hand, early pregnancy plasma folate was associated with lower plasma tHcy in the offspring ($p< 0.05$), and at labour was positively associated with both child RBC and plasma folate ($p< 0.05$). Plasma tHcy at mid-pregnancy and at labour were associated with higher offspring plasma tHcy ($p< 0.05$). We found no associations between cord and mid-childhood measurements.

Table 16. Associations between maternal folate status during pregnancy and offspring RBC folate and plasma folate and tHcy at mid-childhood.[1]

		Child	
Pregnancy	**RBC folate**	**Plasma folate**	**Plasma tHcy**
RBC folate			
1st trimester	0.005 [103]	-0.041 [104]	-0.161 [104]
2nd trimester	**0.229*** [97]	0.033 [98]	-0.173 [98]
3rd trimester	0.172 [90]	0.047 [91]	-0.112 [91]
Plasma folate			
1st trimester	0.107 [103]	0.069 [104]	**-0.248*** [104]
2nd trimester	0.190 [97]	0.124 [98]	-0.106 [98]
3rd trimester	0.127 [92]	0.160 [93]	-0.173 [93]
Labour	**0.267*** [88]	**0.249*** [89]	-0.117 [89]
Cord	0.055 [86]	0.004 [87]	-0.023 [87]
Plasma tHcy			
1st trimester	-	-	0.158 [104]
2nd trimester	-	-	**0.215*** [98]
3rd trimester	-	-	0.174 [93]
Labour	-	-	**0.209*** [89]
Cord	-	-	0.178 [87]

Abbreviations: RBC, red blood cell.
[1] Pearson correlation coefficients are shown for the associations between maternal folate status indicators and offspring indexes at mid-childhood. *$p< 0.05$.

The associations between maternal plasma cobalamin and MMA throughout pregnancy and indicators in the offspring at mid-childhood are shown in **Table 17**. Maternal plasma cobalamin throughout pregnancy was strongly and positively associated with offspring cobalamin status and appeared to be stronger at mid-pregnancy. Cord cobalamin was not associated with child status. No associations were found between maternal cobalamin at any point during pregnancy or cord cobalamin and offspring plasma MMA or tHcy. Maternal and cord plasma MMA were strongly associated with offspring status at 7.5 years ($p < 0.01$).

Table 17. Associations between maternal cobalamin and MMA status during pregnancy and offspring plasma cobalamin, MMA and tHcy at mid-childhood.[1]

		Child	
Pregnancy	**Plasma cobalamin**	**Plasma MMA**	**Plasma tHcy**
Plasma cobalamin			
1st trimester	**0.258†** [103]	0.072 [104]	0.003 [104]
2nd trimester	**0.399‡** [97]	-0.015 [98]	-0.070 [98]
3rd trimester	**0.380‡** [92]	-0.078 [93]	-0.044 [93]
Labour	**0.332†** [88]	0.042 [89]	0.105 [89]
Cord	0.114 [86]	-0.092 [87]	0.020 [87]
Plasma MMA			
1st trimester	-	**0.295†** [104]	-
2nd trimester	-	**0.286†** [98]	-
3rd trimester	-	**0.286†** [93]	-
Labour	-	**0.322†** [89]	-
Cord	-	**0.287†** [87]	-

Abbreviations: RBC, red blood cell.
[1] Pearson correlation coefficients are shown for the associations between maternal folate status indicators and offspring indexes at mid-childhood. †p< 0.01; ‡p< 0.001.

We investigated whether early pregnancy or child demographic, lifestyle and genetic variants were associated with child RBC and plasma folate and plasma cobalamin using univariate linear regression analysis (**Table 18**). Only the R^2*100 values are reported

because we set out to identify which variables are most related with RBC and plasma folate and plasma cobalamin and are candidates for inclusion in subsequent multivariate analyses, rather than to specifically quantify the association. Tolerance was ≥0.59 in all models and hence there was no collinearity among the studied covariables.

Maternal folate status in early pregnancy, breastfeeding, exclusive breastfeeding duration and child BMI were not associated with child plasma or RBC folate status. Plasma cobalamin was also not associated with breastfeeding or with exclusive breastfeeding duration. Multivariate linear regression analysis was used to evaluate the determinants of folate and cobalamin status at mid-childhood. Given the relatively small sample size, we included all factors significantly (or borderline significantly) associated with the corresponding markers in the model. All genetic variants were included in the analysis.

Table 18. Univariate associations between demographic, lifestyle and genetic factors as determinants of RBC folate and plasma folate and cobalamin at mid-childhood.[1]

	RBC folate	Plasma folate	Plasma cobalamin
Continuous variables			
Maternal age, years	2.1 [104]	1.4 [105]	4.0* [104]
Child age, months	5.9* [104]	2.5 [105]	2.6 [104]
Total folate intake, µg/day	0.0 [104]	1.2 [105]	1.2 [104]
Total cobalamin intake, µg/day	0.0 [104]	0.4 [105]	0.3 [104]
RTEBC, portion/day	0.1 [104]	10.3‡ [105]	0.8 [104]
Creatinine, µmol/L	0.1 [104]	0.2 [105]	3.3[2] [104]
Exclusive breastfeeding, months	0.6 [104]	0.1 [105]	0.0 [104]
Categorical variables			
Sex	0.3 [104]	0.0 [105]	0.4 [104]
Child overweight	0.1 [104]	0.0 [105]	1.9 [104]
Breastfeeding	1.0 [104]	0.1 [105]	0.2 [104]
Socioeconomic status	6.5* [104]	12.3‡ [105]	0.5 [104]
Smoking throughout pregnancy	1.0 [104]	6.1* [105]	4.1* [104]
MTHFR 677C>T SNP	7.9* [102]	7.7* [103]	3.6 [102]
MTRR 66A>G SNP	2.1 [102]	0.7 [103]	3.1 [102]
MTRR 524C>T SNP	1.0 [101]	2.3 [102]	2.0 [101]
TCN2 776C>G SNP	3.4 [101]	0.6 [102]	4.1 [101]

Abbreviations: *MTHFR*, *methylenetetrahydrofolate reductase*; *MTRR*, *methylenetetrahydrofolate-homocysteine methyltransferase reductase*; RTEBC, ready-to-eat breakfast cereals; *TCN2, transcobalamin 2*.

[1]Univariate linear regression analysis with demographic, lifestyle and genetic variants as independent variables and RBC folate and plasma folate and cobalamin as dependent variables. Plasma folate and cobalamin were natural log transformed before analysis. Values are univariate model R^2*100, symbols indicate model significance and square brackets sample size. Independent variables were regressed as continuous (with specified units) or categorical (sex [male vs. female], child overweight [overweight vs. non-overweight], breastfeeding [≥1 month vs. <1month], 1st trimester smoker [no vs. yes], socioeconomic status [medium or high vs. low], *MTHFR* 677C>T [CT or TT vs. CC], *MTRR* 66A>G [AG or GG vs. AA], *MTRR* 524C>T [CT or TT vs. CC] and *TCN2* 776C>G [CG or GG vs. CC]) variables. *p< 0.05; †p< 0.01; ‡p< 0.001.

[2]p= 0.066.

RBC and plasma folate models

The *MTHFR* 677C>T variant homozygous genotype was negatively associated with both RBC (β= -251.7 nmol/L; 95% CI: -478.7, -24.7) and plasma folate (β= -28.1%; 95% CI: -45.5, -4.9%) compared to the common homozygous genotype. Child age (β= 58.0 nmol/L; 95% CI: 9.5, 106.6) and high versus low socioeconomic status were positively associated with RBC folate and ready-to-eat breakfast cereals with plasma folate (36.5% higher on average per portion increase; p= 0.007). Models predicted 25.7 and 29.1% of RBC and plasma folate variability (p for models = 0.005 and <0.001 for RBC and plasma folate respectively; n=101 and 102 respectively).

Plasma cobalamin model

Maternal plasma cobalamin concentration at early pregnancy was the major determinant of child cobalamin status: per every 100 pmol/L increase in first trimester cobalamin child cobalamin concentrations increased by on average 8.0% (95% CI: 3.5, 12.5%). Maternal smoking throughout pregnancy was borderline negatively associated with child cobalamin concentrations (p= 0.057). The model predicted 30.1% of cobalamin concentration variability (p for model <0.001; n= 99).

The determinants of functional metabolic markers of cobalamin and folate status were evaluated by stepwise multiple linear regression.

MMA model

This model was adjusted for the determinants associated with child cobalamin above, as well as child plasma cobalamin and early pregnancy plasma MMA. The effects of maternal MMA and cobalamin, and child cobalamin concentrations on child MMA were independent

of each other. The major determinant of child MMA was child cobalamin concentration (per 100 pmol/L, β= -4.7%; 95% CI: -0.7, -8.7%) and then first trimester plasma MMA (β= 252.4%; 95% CI: 52.1, 716.8%), cobalamin (per 100 pmol/L, β= 6.8%; 95% CI: 0.4, 13.2%) and smoking throughout pregnancy, in that order. After adjusting for the studied genetic variants, smoking was no longer associated with child MMA concentrations. The final model explained 20.8% of child MMA variability (p for model <0.001, n= 99).

THcy model

For tHcy, factors previously found to be associated with RBC and plasma folate were included in the model, as well as child cobalamin and RBC folate and maternal tHcy concentrations in early pregnancy. Child RBC folate alone predicted 23.8% of child tHcy, and per every 100 nmol/L increase in RBC folate, tHcy decreased by 1.8% (95% CI: -2.7, -0.9) in the fully adjusted model. Child plasma cobalamin was also negatively associated with child tHcy (model R^2*100= 30.1%; p< 0.001; n= 98). Child genetic variants were not associated with tHcy, however, there was a trend suggesting that children with the *MTHFR* 677 TT genotype had higher tHcy concentrations compared to the CC genotype (p= 0.064).

Discussion

In this chapter, we described folate and cobalamin status metabolic biomarkers and identified their major determinants at mid-childhood. We found no evidence of an effect of breastfeeding on long-term child folate or cobalamin status. Pregnancy cobalamin, as early as from the first trimester, persists as the strongest determinant of child cobalamin status at mid-childhood.

RBC folate deficiency was found in 6.3% of children but there was no evidence of cobalamin deficiency based on plasma cobalamin. In contrast, 5.7% of the children had elevated plasma MMA. Plasma folate deficiency was highly prevalent (12.3%), doubling that of RBC folate deficiency. However, there was no evidence of functional folate deficiency, based on elevated tHcy in the children. Cobalamin status (plasma cobalamin and MMA concentrations) and folate (plasma folate and tHcy) were similar to those reported in Norwegian (81) and Greek (456) children. All of our children were of a similar age, 7.5 years ± 2 months, and we did not observe any effect of age on plasma folate or tHcy. However, age was positively associated with RBC folate.

We confirmed that maternal cobalamin status during pregnancy is still a major determinant of offspring cobalamin concentrations at mid-childhood. Previously this association had been described for infants. To the best of our knowledge, this study is the first to identify this association from as early as the first trimester and throughout the rest of pregnancy until 7.5 years of age in the offspring. In addition, there seems to be a functional effect of maternal cobalamin status given that both maternal and child cobalamin status were associated with child MMA concentrations. Shared genetic and lifestyle factors between the mother and the child could explain the effects of maternal cobalamin on infant vitamin status. However, we found an effect of maternal cobalamin on infant MMA independently of the effect of child cobalamin and maternal MMA. Surprisingly, the associations between maternal cobalamin and child MMA were positive, where an inverse association was expected. However, data is lacking regarding reference ranges and the true biological meaning of MMA concentrations in growing children. It may be affected by cobalamin utilisation for growth.

Despite the well-established metabolic effects of the *MTHFR* 677C>T genetic variant on tHcy in adults, its effect during childhood has been scarcely studied. However, available evidence suggests that its effect on tHcy may be limited to older children (>10 years) (279,283,284), boys (285) or children with low folate status (284). We found that the

MTHFR 677 TT genotype was associated with lower RBC and plasma folate, and a borderline higher tHcy compared to the CC genotype. These results have never been reported before in children, but are very similar to those observed in adults from the same geographical region (144). Child *TCN2* 776C>G, *MTRR* 66A>G and 524C>T were not associated with either folate or cobalamin status markers. To the best of our knowledge, this study is the first to assess the effects of the *MTRR* 66A>G and 524C>T polymorphisms on folate metabolic markers in children.

Cobalamin transport and uptake mechanisms are less diverse than those of folate. Hence, cobalamin marginal deficiency is usually caused by food-bound cobalamin malabsorption (89,90), rather than by a low intake, as opposed to folate. We suggest that cobalamin status is more susceptible than folate to genetic variations affecting either cobalamin uptake or transport, which would explain the close and strong relationship between maternal and child cobalamin status. The case for MMA is relatively similar. Recently, a novel genetic variant (3-hydroxyisobutyryl-CoA hydrolase, rs291466; minor allele frequency: 0.43) affecting MMA has been reported, which is associated with elevated MMA, independently of cobalamin status (457).

One limitation of this study was the small sample size in relation to child metabolic parameters. The child recruitment phase of the study is ongoing and only biochemical determinations of the first 106 children have been carried out so far. However, we included functional biomarkers of folate and cobalamin status to provide more insight into intracellular folate and cobalamin status. We cannot rule out whether the association between maternal and child cobalamin status is due to shared genetic variants, despite adjusting for several genetic variants affecting cobalamin metabolism and transport. In addition, we did not have information on maternal B-vitamin status during the follow-up phase to compare with that of the children, and so could not assess whether common nutritional factors highlighted this association. Data regarding cobalamin and folate content of breast milk, or child status at younger ages was also missing. However, we assessed the

maternal effects from early pregnancy, and at different time-points throughout the pregnancy, which has never been performed before.

In conclusion, breastfeeding regime appears not to affect child folate nor cobalamin status at 7.5 years of age. Our data suggests that maternal cobalamin status persists as the main determinant of child cobalamin status at 7.5 years. It remains to be determined whether this association is mediated by a common genetic profile.

RESULTS CHAPTER 3

Chapter 3: Maternal one carbon metabolism during pregnancy and offspring growth and adiposity at mid-childhood

Introduction

The impact of environmental exposures on one generation may shape the development of the subsequent generation (458). Developmental plasticity has been found to be critical in early stages of life and can be understood as epigenetic traits that do not modify the genomic sequence (377,378). However, these epigenetic marks, including DNA methylation which controls stable changes in gene expression potential (459), may result in permanent alterations in body physiology and metabolism. Any pathological consequences that they may cause are often observed much later in life. 1C metabolism plays a crucial role in transporting and transferring methyl groups, essential for methylation reactions. In addition, 1C nutrients are widely recognised as pivotal for optimal growth and development in prenatal life and throughout childhood. Strong evidence suggests a protective effect of folate against the risk of NTDs (6,35). In parallel, folic acid supplementation during pregnancy was reported, in a recent meta-analysis of randomised controlled clinical trials, to increase birth weight (mean difference from non-folic acid supplement use: 135.75 g; 95% CI: 47.9, 223.7) (300). In addition, both maternal plasma tHcy and the *MTHFR* 677C>T variant allele have been associated with lower offspring birth weight (38,39). However, available evidence from pregnancy cohort studies examining the associations of pregnancy 1C metabolism and offspring growth and adiposity during childhood is limited. Moreover, previous reports have yielded discrepant findings with either positive, negative or no associations between pregnancy 1C metabolism and child growth or adiposity. In 2008, Yajnik and collaborators reported, for the first time, higher BMI in 6-year old Indian children born to women with high erythrocyte folate compared to lower at 28 GW (389). Krishnaveni et al. did not confirm these results but showed a positive association between

plasma folate and offspring linear growth at 9.5 and 13.5 years (39). A recent report from a Dutch birth cohort reported a negative association between second trimester plasma folate and child BMI at 5-6 years (91). To date no effects on offspring growth or adiposity have been observed for pregnancy cobalamin.

The role of prenatal folate status in the prevention of NTDs in the offspring has been the focal point of most of the epidemiological, clinical and experimental attention to date. Therefore, preconceptional recommendations concerning folic acid supplementation consider solely the prevention of NTDs. To the best of our knowledge, other offspring outcomes including childhood growth and adiposity have been scarcely considered in the context of prenatal folate status. Elevated tHcy in early pregnancy was associated with reduced birth weight, increased IUGR risk and impaired foeto-placental Doppler function in a recent thesis from our group (303). The effects of prenatal 1C metabolism may extend far beyond foetal development, but so far, this is unknown.

Specific objectives

The specific objectives of this chapter are:

- To assess the associations of folate and cobalamin status during pregnancy on mid-childhood linear and ponderal growth and adiposity.

- To investigate the associations between maternal and child genetic variants affecting folate and cobalamin metabolism or transport and mid-childhood linear and ponderal growth and adiposity.

Material and methods

The materials and methods applicable to this chapter have been described in the general Material and methods section.

Statistical analysis

We first examined whether differences in plasma folate and cobalamin biomarkers existed in early pregnancy among the women who participated in the follow-up phase compared with those who did not. Median (P_{25}, P_{75}) or relative frequencies (95% CI) according to offspring participation in the follow-up phase are reported. Differences in continuous or categorical variables were assessed by t-test or χ^2 respectively.

We then evaluated the effects of early, mid and late pregnancy inadequate folate and cobalamin biomarker status on child weight, height and BMI age- and sex- standardised z-scores using multiple linear regression analyses. Subjects were grouped into inadequate plasma folate (≤10 nmol/L) (237), cobalamin (≤221 pmol/L) (442), holoTC (≤40 pmol/L) (460) or MMA (≥0.210 µmol/L) (443) categories. Distribution based cut-offs were used for inadequate RBC folate and plasma tHcy. The lower quartiles of RBC folate (first trimester: ≤651 nmol/L; second trimester: ≤810 nmol/L; third trimester: ≤603 nmol/L) and highest quartiles of plasma tHcy (first trimester: ≥6.0 µmol/L; second trimester: ≥5.4 µmol/L; third trimester: ≥6.1 µmol/L) were used to group subjects as having inadequate RBC folate or tHcy respectively. Offspring growth z-scores were derived from WHO growth charts (422).

The effect of first trimester folate and cobalamin status biomarkers on child growth was assessed using three different models:

- An unadjusted model.

- A model including potential maternal and child confounders (maternal first trimester age, BMI [for child weight and BMI models] or height [for child height models], RBC folate or plasma cobalamin status [for child cobalamin and folate models respectively], socioeconomic status during pregnancy and at follow-up, smoking throughout pregnancy and exclusive breastfeeding duration).
- A model including the previous factors plus two potential mediators (child physical activity score and daily total energy intake). Whether the effects of early pregnancy folate and cobalamin status biomarkers on child growth were modified by breastfeeding (≤1 month vs. >1 month) or sex was analysed by ANCOVA adjusting for the factors listed for the second model above.

Given that maternal folate during pregnancy has been positively associated with offspring BMI (389), we addressed whether elevated plasma folate (≥45 nmol/L) at any time of pregnancy (<12, 24 and at 34 GW) was associated with offspring growth. A previously published lower cut-off point for elevated plasma folate (≥30 nmol/L) (314,315) was also evaluated. Interactions between elevated folate and inadequate cobalamin during pregnancy on offspring growth were assessed by ANCOVA. Given the observed associations between RBC folate status at any time of pregnancy and offspring's height we assessed the effects of different timing of maternal folic acid supplementation on child height using three different multiple linear regression models. We compared offspring age- and sex-standardised height z-scores between folic acid supplement users vs. non-users before conception, during the first trimester and during the second trimester adjusting for potential confounders.

The effects of maternal genetic variants, affecting folate and cobalamin transport or metabolism, on offspring growth were assessed in both additive and dominant models by linear regression analysis. In the additive model, the β-coefficients (95% CI) for the increase in one variant allele for each corresponding genetic polymorphism are reported, and for the dominant model, the reported β-coefficients represent the mean difference in the studied

outcome between variant homozygotes and heterozygotes compared with common homozygotes. These β-coefficients represent the mean difference in weight, height and BMI age- and sex-standardised z-scores from adequate status.

Three different models were used for the additive and dominant models. The first model included the four studied maternal genetic variants (*MTHFR* 677C>T, *MTRR* 66A>G, *MTRR* 524C>T and *TCN2* 776C>G). In order to account for a potential effect of child genotype, child genetic variants were added to the crude model (model 2). Finally, a third model consisted of adjusting model 2 for the potential confounders and mediators previously used, plus first trimester RBC folate and cobalamin status. We also assessed the effects of the four child genetic variants on growth in similar models.

We used linear mixed effects models with height, weight and age- and sex- standardised BMI z-scores as dependent variables to model growth curve trajectories from 1 month until 7.5 years according to the studied maternal genotype to account for within-subject correlations between repeated measures and different numbers of observations at follow-up. The aim of the analysis was to test whether the effects of maternal genotype on offspring growth that we observed at mid-childhood were persistent throughout childhood. Models included fixed effect terms for age, age^2 and age^3 (excluded for the BMI model) in months, as well as exclusive breastfeeding length, birth weight z-score, smoking throughout pregnancy, socioeconomic status at follow-up, maternal BMI (for weight and BMI models) or height (for height models), early pregnancy folate and cobalamin status (inadequate vs. adequate) and offspring corresponding genotype. Random effects included the intercept, age, age^2 and age^3, to account for repeated correlations and allow within-subject trajectories to vary. Random effects for height, weight and BMI were allowed to covary via an unstructured covariance matrix and fitting was accomplished by restricted maximum likelihood estimation. Residual variances were allowed to vary according to age groups (1, 2, 6, 12 months and 1.5, 3, 4, 6 and 7.5 years) for every subject using a diagonal structure. The effects of maternal genotypes (*MTHFR* 677C>T, *TCN2* 776C>G, *MTRR* 66A>G and *MTRR*

524C>T) were individually assessed for each of the three dependent outcomes as fixed factors. Interaction term with age (age, age2 and age3) was added as fixed effects in all models and were consecutively excluded from the models when their associations with the dependent variable were non-significant, from highest to lowest order. In order to evaluate growth curve trajectories we assumed that missing values were missing at random. We assessed the correlations between the number of measurements performed and child socioeconomic status, weight at birth, gestational age at labour, height, weight and BMI at follow-up and exclusive breastfeeding duration. We found no associations between number of measurements and the above listed factors by Pearson linear correlation. We exhaustively checked the models and assumptions to ensure the validity of the models, including the use of log likelihood ratio tests and residual plots.

We also used linear regression analysis to evaluate the effect of studied maternal genotypes (additive model) on height and first trimester weight and BMI, after adjusting for age, smoking throughout the pregnancy and socioeconomic status.

As in the case of the evaluation of the associations between folate and cobalamin biomarkers during pregnancy and offspring growth at mid-childhood, we also evaluated the effects of these biomarkers on offspring adiposity using linear regression models. Exposure variables were categorised as described above, and models included maternal first trimester age, BMI, socioeconomic status during pregnancy and at follow-up, smoking throughout pregnancy, exclusive breastfeeding duration and child height, physical activity score and daily total energy intake. Offspring adiposity outcomes were waist circumference (cm, according to WHO), fat mass (kg), fat mass index (kg/ m^2) and skinfold thickness mean (mm, mean of subscapular, suprailiac, tricipital and bicipital skinfold thicknesses). In addition, the risk of offspring overweight or obesity was evaluated according to maternal folate and cobalamin biomarkers during pregnancy. Overweight and obesity were defined according to the World Obesity Foundation (327). Logistic regression models were used to carry out these analyses, after adjusting for the abovementioned confounding factors.

Cross-sectional analyses were also performed to investigate the associations between folate and cobalamin status biomarkers and mid-childhood growth. Folate and cobalamin status variables were tested as linear and categorical variables. Distribution based cut-offs were used for all biomarkers. The first quartile of plasma (≤12.1 nmol/L) and RBC (≤589 nmol/L) folate and plasma cobalamin (≤568 pmol/L) were used to define inadequate folate or cobalamin status, and the fourth quartile of plasma tHcy (≥6.3 µmol/L) and MMA (≥0.160 µmol/L) to define elevated tHcy and MMA respectively. Multiple linear regression analysis was used to study these effects after controlling for maternal age, smoking throughout pregnancy, first trimester BMI, exclusive breastfeeding duration, socioeconomic status at childhood and child physical activity score and total energy intake.

Results

Maternal folate and cobalamin status in early pregnancy according to RTBC participation

Differences in early pregnancy folate and cobalamin biomarkers between participants and non-participants in the follow-up study are shown in **Table 19**. We found no differences in first trimester metabolic markers of folate and cobalamin status between mothers of children participating in the follow-up phase compared with non-participants. In contrast, mothers of participants had lower energy (mean: 1869; 95% CI: 1773, 1965; p= 0.019), protein (mean: 74.1; 95% CI: 69.8, 78.3; p= 0.009), carbohydrate (mean: 177; 95% CI: 165, 190; p= 0.019) and cobalamin (mean: 4.9; 95% CI: 4.6, 5.3; p= 0.017) intake from food compared with those of non-participants.

Table 19. First trimester maternal nutritional characteristics according to follow-up participation.[1]

	Followed-up (n= 1620	Not followed-up (n= 394)
Plasma folate, nmol/L	27.3 (15.3, 41.4)	28.1 (15.5, 42.0)
Folate ≤10 nmol/L, %	8.8 (5.3, 14.2)	12.7 (9.8, 16.3)
Folate ≥45 nmol/L, %	18.1 (12.9, 24.8)	21.1 (17.3, 25.4)
RBC folate, nmol/L	942 (632, 1478)	1003 (656, 1468)
Folate ≤651 nmol/L, %	26.3 (20.0, 33.6)	24.9 (20.7, 29.6)
Plasma cobalamin, pmol/L	357 (282, 441)	381 (307, 459)
Cobalamin ≤221 pmol/L, %	7.5 (4.3, 12.7)	5.8 (3.9, 8.6)
Plasma holotranscobalamin, pmol/L	70.5 (51.8, 87.6)	72.1 (51.8, 56.1)
HoloTC ≤40 pmol/L, %	8.8 (5.3, 14.2)	8.4 (5.6, 12.4
Plasma tHcy, µmol/L	5.2 (4.5, 5.9)	5.3 (4.5, 6.0)
tHcy ≥6.0 µmol/L, %	23.8 (17.8, 30.9)	25.4 (21.3, 29.9)
Plasma MMA, µmol/L	0.110 (0.100, 0.130)	0.112 (0.100, 0.140)
MMA ≥0.221 µmol/L, %	3.1 (1.3, 7.1)	2.5 (1.4, 4.6)

Abbreviation: holoTC, holotranscobalamin; MMA, methylmalonic acid; RBC, red blood cell; tHcy, total homocysteine.

[1] All data values shown are median (P_{25}, P_{75}) or percentages (95% confidence interval). Differences between participants followed-up and not followed-up were assessed by χ^2 and t-test for categorical and continuous variables respectively.

Longitudinal associations between first trimester folate and cobalamin status and offspring growth

The associations between first trimester folate and cobalamin status biomarkers and weight, height and BMI sex- and age-standardised mid-childhood z-scores are shown in Table 20. None of these vitamins (or any of their functional biomarkers) was associated with mid-childhood weight. However, lower height was observed in offspring born to inadequate compared with adequate RBC folate (mean z score difference from adequate status: -0.30; 95% CI: -0.58, -0.02), plasma cobalamin (mean z score difference from adequate status: -0.69; 95% CI: -1.17, -0.21) and tHcy (mean z score difference from adequate status: -0.33; 95% CI: -0.62, -0.03) statuses. These associations were observed in both crude and fully adjusted models. By adjusting folate and cobalamin models for

cobalamin and folate status respectively, we found that the effects of these metabolites on offspring height were independent from each other. Inadequate compared to adequate first trimester cobalamin status and RBC folate was associated with almost 120% (95% CI: -221.9, -50.2%) and 50% (95% CI: -88.5, -29.9%) lower child height (z score) respectively. First trimester status in these two vitamins explained 8.2% of offspring height variability. Elevated pregnancy plasma MMA was associated with lower child height (mean z score difference from normal status: -0.89; 95% CI: -1.70, -0.10). However, these effects were lost after adjusting for potential confounders (mean z score difference from normal status: -0.64; 95% CI: -1.38, 0.09). Low (≤40 pmol/L) compared to normal first trimester plasma holoTC status was associated with increased offspring BMI at mid-childhood (mean z score difference: 0.66; 95% CI: 0.02, 1.29). No other indicator of maternal folate or cobalamin status was associated with offspring BMI.

We observed no interactions between markers of folate and cobalamin status in early pregnancy and sex or breastfeeding on offspring growth.

Table 20. Associations between first trimester folate and cobalamin status biomarkers and child growth at 7.5 years.

	Weight (z-score)			**Height** (z-score)			**BMI** (z-score)		
	Crude	Model 1	Model 2	Crude	Model 1	Model 2	Crude	Model 1	Model 2
Folate	0.07 [1] (-0.54, 0.69)	-0.04 (-0.67, 0.59)	0.09 (-0.55, 0.72)	-0.10 (-0.59, 0.39)	-0.13 (-0.59, 0.32)	-0.07 (-0.54, 0.39)	0.17 (-0.49, 0.83)	0.07 (-0.61, 0.75)	0.23 (-0.45, 0.92)
RBC folate	-0.37 (-0.76, 0.02)	-0.35 (-0.74, 0.04)	-0.31 (-0.69, 0.08)	**-0.38 (-0.69, -0.07)**	**-0.32 (-0.57, -0.04)**	**-0.30 (-0.58, -0.02)**	-0.19 (-0.62, 0.23)	-0.19 (-0.61, 0.23)	-0.14 (-0.55, 0.28)
Cobalamin	-0.05 (-0.71, 0.61)	-0.21 (-0.87, 0.45)	-0.14 (-0.80, 0.51)	**-0.72 (-1.24, -0.20)**	**-0.72 (-1.20, -0.24)**	**-0.69 (-1.17, -0.21)**	0.48 (-0.22, 1.19)	0.37 (-0.35, 1.08)	0.45 (-0.26, 1.16)
HoloTC	0.32 (-0.29, 0.93)	0.29 (-0.31, 0.89)	0.30 (-0.30, 0.89)	-0.30 (-0.79, 0.19)	-0.23 (-0.68, 0.22)	-0.23 (-0.68, 0.22)	**0.67 (0.01, 1.31)**	**0.65 (0.01, 1.30)**	**0.66 (0.02, 1.29)**
tHcy	-0.14 (-0.54, 0.27)	-0.24 (-0.64, 0.16)	-0.19 (-0.59, 0.21)	-0.31 (-0.63, 0.02)	**-0.35 (-0.64, -0.06)**	**-0.33 (-0.62, -0.03)**	0.02 (-0.42, 0.46)	-0.07 (-0.51, 0.37)	-0.01 (-0.45, 0.42)
MMA	-0.92 (-1.9, 0.07)	-0.77 (-1.77, 0.22)	-0.57 (-1.18, 0.17)	**-0.89 (-1.70, -0.10)**	-0.64 (-1.38, 0.09)	-0.67 (-1.40, 0.07)	-0.54 (-1.37, 0.53)	-0.28 (-1.43, 0.80)	-0.35 (-1.4, 0.70)

Abbreviations: BMI, body mass index; holoTC, holotranscobalamin; MMA, methylmalonic acid; RBC, red blood cell; tHcy, total homocysteine.

[1]Values are β-coefficients (95% CI) which represent the mean difference in age- and sex-standardised weight, height and BMI z-scores from the reference group (adequate status for vitamins or elevated for biomarkers). Adequate status was categorised as: plasma folate (>10 nmol/L), RBC folate (>651 nmol/L), plasma cobalamin (>221 pmol/L), plasma holoTC (>40 pmol/L) and nonelevated biomarkers as plasma tHcy (<6.0 µmol/L) and plasma MMA (<0.210 µmol/L). β-coefficients were derived by multiple linear regression analysis. World Health Organisation growth charts were used to calculate individual standardised z-scores. Sample included 160 mother-child pairs.

Crude model was unadjusted; Model 1 was adjusted for maternal 1st trimester age, BMI (for weight and BMI models) or height (for height models), socioeconomic status during pregnancy and at follow-up, smoking throughout pregnancy and exclusive breastfeeding duration; Model 2 included model 1 plus child physical activity score and total daily energy intake. Adjusted models included plasma cobalamin and RBC folate for folate and cobalamin models respectively.

Longitudinal associations between folate and cobalamin status throughout pregnancy and offspring growth

The effects of second and third trimester folate and cobalamin status indicators on child growth were assessed and are shown in **Figure 15**. Children born to women in the lowest quartile of RBC folate (≤603 nmol/L) in the third trimester only, had higher BMI compared with children born to women with adequate RBC folate status (mean z score difference: 0.55; 95% CI: 0.09, 1.01). These results were consistent with those observed for plasma folate inadequacy (data not shown).

Only inadequate first trimester maternal RBC folate or cobalamin status, affected child height (Figure 15 A and B). However, low holoTC (≤40 pmol/L) at 24-28 GW was associated with higher offspring BMI (mean z score difference: 0.50; 95% CI: 0.04, 0.97) and lower height (mean z score difference: -0.40; 95% CI: -0.72, -0.08) compared with those with adequate holoTC (data not shown). The associations between holoTC and BMI were lost after adjusting for child physical activity score and total daily energy intake.

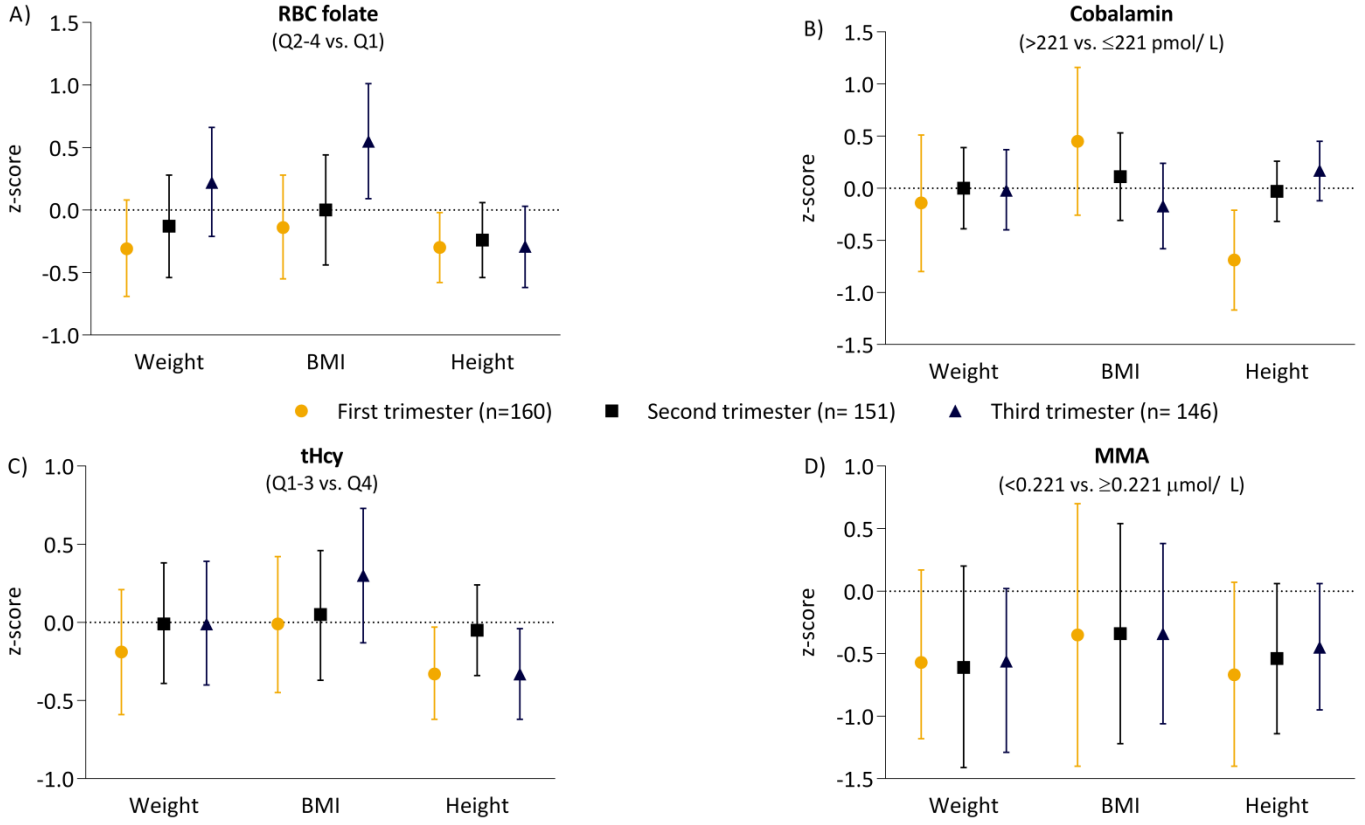
Elevated first and third trimester tHcy was associated with lower height in the offspring at 7.5 years (Figure 15 C; mean z score difference for late pregnancy: -0.33; 95% CI: -0.62, -0.42) compared with those with normal tHcy. There was a borderline association between elevated plasma MMA at any time of pregnancy and height in the offspring, however it was never associated with either weight or BMI (Figure 15 D).

Elevated plasma folate at any time of pregnancy was not associated with mid-childhood weight (mean z score difference in early pregnancy from adequate status: -0.33; 95% CI: -0.78, 0.13; R^2*100: 14.7; n= 160; p for model= 0.012), BMI (mean z score difference in early pregnancy from adequate status: -0.36; 95% CI: -0.85, 0.12; R^2*100: 14.8; n= 160; p for model= 0.011) or height (mean z score difference in early pregnancy from adequate status: -0.09; 95% CI: -0.42, 0.24; R^2*100: 24.1; n= 160; p for model< 0.001) (data not shown). Results were not modified after using a lower cut-off for elevated plasma folate (≥30

nmol/L). In addition, elevated or deficient first trimester plasma folate did not modify the association between marginal cobalamin deficiency and offspring growth at mid-childhood.

Given that the effects of RBC folate on offspring height were only observed for first trimester RBC folate, and that we did not observe any effect of plasma folate, we investigated whether supplementation with folic acid before conception, in early and in mid-pregnancy were associated with child height at 7.5 years. Mid-childhood height (z-score) was over 60% greater in children born to mothers that used folic acid supplements at preconception compared to non-users (mean z score difference: 0.26; 95% CI: 0.01, 0.51; R^2*100: 31.2; n: 160; p for model <0.001) after adjusting for maternal age, height, marginal first trimester cobalamin deficiency, smoking throughout pregnancy, exclusive breastfeeding duration, socioeconomic status during pregnancy and at follow-up, child physical activity score and energy intake. No differences in offspring height were observed between children born to folic acid supplement users and non-users in early (mean z score difference: -0.15; 95% CI: -0.58, 0.28; R^2*100: 30.1; n: 149; p for model <0.001) or mid-pregnancy (mean z score difference: 0.19; 95% CI: -0.08, 0.46; R^2*100: 30.8; n: 151; p for model <0.001).

Figure 15. Associations between folate and cobalamin status biomarkers during pregnancy and offspring growth at 7.5 years.



Associations between RBC folate (A), plasma cobalamin (B), tHcy (C) and MMA (D) throughout pregnancy and offspring age- and sex-standardised weight, BMI and height z-scores (WHO growth charts) at mid-childhood. Symbols (lines) show the mean difference (95% CI) from children born to mothers with adequate status in folate or cobalamin (<221 pmol/L) or with normal tHcy or MMA (<0.210 µmol/L) derived from multiple linear regressions. RBC folate (1st: <651; 2nd: <810; 3rd trimester: <603 nmol/L); tHcy (1st: >6.0; 2nd: >5.4; 3rd trimester: >6.1 µmol/L). Model was adjusted for 1st trimester maternal age, BMI or height, socioeconomic status during pregnancy and at follow-up, smoking throughout pregnancy, exclusive breastfeeding duration, child physical activity score and total energy intake.

Associations between folate and cobalamin related maternal genetic variants and offspring growth

In this study, we tested the associations between maternal genetic variants related to folate or cobalamin transport or metabolism and child growth outcomes. Ideally, to test causality a randomised intervention trial should be used. However, other approaches must be used when such trials are unethical such as in our pregnancy study. The prospective nature of this study was also a strong point in the testing of causal associations. Furthermore, the advantage of genotype is that it is randomised at conception and is unaffected by lifestyle confounders. Therefore genetic variants affecting environmental or lifestyle exposures may be used to overcome the limitation of observational studies, improving the confidence in the causality of the associations, if any actually exists (461). The associations between maternal and child genetic variants in folate and cobalamin transport or metabolism and child growth at mid-childhood are reported in **Table 21**.

We observed no associations between the maternal *MTHFR* 677C>T polymorphism and offspring growth at mid-childhood. The maternal *MTRR* 66A>G genetic variant was not associated with either child weight or BMI, but was positively associated with height in both the additive (β-coefficient: 0.31; 95% CI: 0.07, 0.55) and the dominant (β-coefficient: 0.36; 95% CI: 0.02, 0.07) models after adjusting for child genotype. Associations were lost after adjusting for potential confounders. Maternal *MTRR* 524 CT and TT genotypes compared to CC genotype was associated with lower weight (β-coefficient: -0.45; 95% CI: -0.83, -0.06) and BMI (β-coefficient: -0.46; 95% CI: -0.87, -0.04) in the offspring in the most adjusted model. The most consistent effect was observed for maternal *TCN2* 776C>G. For every additional maternal variant allele, child height (z score) was almost 35% lower (β-coefficient: -0.23; 95% CI: -0.45, -0.01) and children born to women carrying at least one variant allele were shorter than those born to common homozygous mothers (mean difference: -0.38; 95% CI: -0.67, -0.08).

Table 21. Associations between maternal folate and cobalamin related genetic variants child growth at 7.5 years.

	Additive			Dominant		
	Crude	Model 1	Model 2	Crude	Model 1	Model 2
***MTHFR* 677C>T**						
Weight	0.03[1] (-0.22, 0.28)	0.04 (-0.28, 0.36)	0.11 (-0.20, 0.42)	-0.01 (-0.39, 0.36)	0.09 (-0.34, 0.50)	0.10 (-0.32, 0.51)
Height	0.01 (-0.20, 0.21)	-0.04 (-0.29, 0.22)	-0.01 (-0.24, 0.23)	0.08 (-0.23, 0.39)	0.18 (-0.16, 0.52)	0.13 (-0.19, 0.44)
BMI	0.04 (-0.22, 0.30)	0.09 (-0.25, 0.43)	0.17 (-0.17, 0.49)	-0.08 (-0.48, 0.31)	-0.03 (-0.48, 0.42)	-0.00 (-0.44, 0.44)
***MTRR* 66A>G**						
Weight	0.10 (-0.16, 0.35)	0.22 (-0.08, 0.51)	0.10 (-0.19, 0.39)	0.19 (-0.21, 0.59)	0.33 (-0.10, 0.75)	0.10 (-0.32, 0.52)
Height	0.14 (-0.07, 0.19)	**0.31 (0.07, 0.55)**	0.21 (-0.01, 0.43)	0.20 (-0.13, 0.53)	**0.36 (0.02, 0.70)**	0.16 (-0.16, 0.48)
BMI	0.05 (-0.22, 0.31)	0.09 (-0.23, 0.40)	-0.01 (-0.32, 0.30)	0.13 (-0.29, 0.55)	0.19 (-0.26, 0.64)	0.00 (-0.45, 0.45)
***MTRR* 524C>T**						
Weight	-0.16 (-0.43, 0.12)	-0.20 (-0.51, 0.11)	-0.24 (-0.55, 0.06)	-0.31 (-0.67, 0.07)	-0.38 (-0.78, 0.02)	**-0.45 (-0.83, -0.06)**
Height	-0.04 (-0.26, 0.19)	-0.08 (-0.33, 0.18)	-0.07 (-0.29, 0.16)	-0.17 (-0.47, 0.14)	-0.25 (-0.57, 0.08)	-0.20 (-0.50, 0.09)
BMI	-0.18 (-0.47, 0.11)	-0.23 (-0.56, 0.11)	-0.30 (-0.62, 0.03)	-0.29 (-0.68, 0.10)	-0.35 (-0.77, 0.08)	**-0.46 (-0.87, -0.04)**
***TCN2* 776C>G**						
Weight	-0.20 (-0.46, 0.05)	-0.26 (-0.56, 0.04)	-0.27 (-0.56, 0.02)	-0.34 (-0.71, 0.04)	-0.38 (-0.77, 0.01)	**-0.40 (-0.78, -0.01)**
Height	-0.20 (-0.41, 0.01)	**-0.29 (-0.53, -0.04)**	**-0.23 (-0.45, -0.01)**	**-0.38 (-0.69, -0.07)**	**-0.45 (-0.77, -0.14)**	**-0.38 (-0.67, -0.08)**
BMI	-0.12 (-0.39, 0.15)	-0.13 (-0.45, 0.19)	-0.17 (-0.48, 0.13)	-0.18 (-0.48, 0.31)	-0.20 (-0.61, 0.22)	-0.21 (-0.62, 0.20)

Abbreviations: BMI, body mass index; *MTHFR*: *methylenetetrahydrofolate reductase*; *MTRR*: *5-methyltetrahydrofolate-homocysteine methyltransferase reductase*; *TCN2*, *transcobalamin 2*.

[1]Values are β-coefficients (95% CI) representing, in the dominant model, the mean difference in age- and sex-standardised weight, height and BMI z-scores between variant homozygous and heterozygous and common homozygous. β-coefficients were derived by multiple linear regression analysis. World Health Organisation growth charts were used to calculate individual standardised z-scores. Sample included 147 mother-child pairs. Crude model was adjusted for the other maternal genetic variants; model 1 was further adjusted for child polymorphisms. Model 2 included model 1 plus maternal 1st trimester age, BMI (for weight and BMI models) or height (for height model), plasma cobalamin and RBC folate concentrations, socioeconomic status during pregnancy and at follow-up, smoking throughout pregnancy, exclusive breastfeeding duration, child physical activity score and total daily energy intake.

Moreover, the presence of at least one variant allele of the *TCN2* 776C>G genetic variant in the mother was associated with lower offspring weight compared to the common homozygous genotype (mean z score difference: -0.40; 95% CI: -0.78, -0.01), but only in the most adjusted model. However, the associations were borderline significant in the other models. It should be highlighted that these results were found even after adjusting for child genotype.

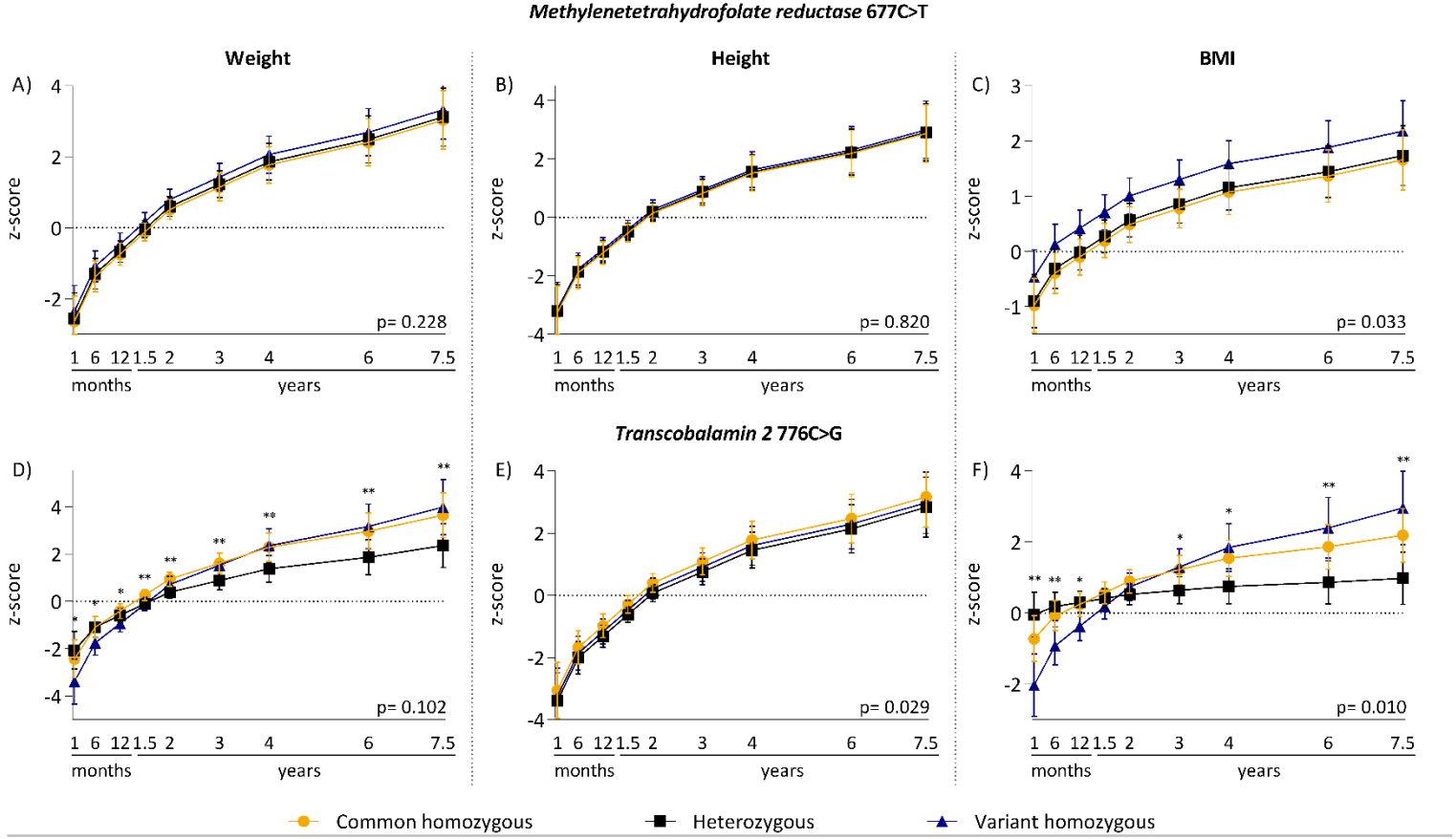
The *MTRR* 66A>G polymorphism in the child was negatively associated with weight (additive model, β-coefficient: -0.30; 95% CI: -0.59, 0.00) and height (additive model, β-coefficient: -0.39; 95% CI: -0.61, -0.16). No associations were found between child *MTHFR* 677C>T, *MTRR* 524C>T and *TCN2* 776C>G genetic variants and mid-childhood growth.

Associations between folate and cobalamin related genetic variants and offspring growth trajectories

We further examined the longitudinal trajectories of offspring age- and sex-standardised weight, height and BMI z-scores from birth until mid-childhood according to folate and cobalamin related genetic variants using mixed effects models. We analysed 970 growth measurements from birth until 7.5 years, with an average of six measurements per child. Offspring height, BMI and weight growth trajectories according to maternal *MTHFR* 677C>T and *TCN2* 776C>G genetic variants are illustrated in **Figure 16**. The maternal *MTHFR* 677C>T polymorphism had no effects on offspring height nor weight but was significantly associated with BMI (Figure 16; A, B and C). The offspring of *MTHFR* 677 TT mothers had persistently higher BMI (mean BMI z-score: 0.86; 95% CI: 0.54, 1.17) from birth until mid-childhood (p= 0.033) compared with CC (mean BMI z-score: 0.34; 95% CI: 0.03, 0.65) or CT (mean BMI z-score: 0.42; 95% CI: 0.12, 0.71) mothers after adjusting for multiple comparisons (p= 0.050 and 0.040 for TT vs. CT and TT vs. CC respectively). The effects were consistent across all age groups.

We observed significant interactions between the maternal *TCN2* 776C>G genetic variant and offspring age on offspring weight (p< 0.01) and BMI (p< 0.01) (Figure 16; D and F). In the first 12 months after birth, offspring born to heterozygous mothers had the highest weight and BMI and offspring born to variant homozygous mothers had the lowest weight and BMI. However, after 12 months, the opposite occurred: children born to heterozygous mothers had the lowest weight and BMI and those born to variant homozygous mothers had the highest BMI and weight. The effects of the maternal *TCN2* 776C>G SNP on BMI became more evident as children grew older, with the biggest difference at 7.5 years. These findings are consistent with an earlier adiposity rebound in children born with low BMI or weight. In agreement with these findings, the offspring of common homozygous mothers were taller (z-scores) from birth and until mid-childhood compared to the offspring

Figure 16. Offspring growth trajectories according to maternal *MTHFR* 677C>T and *TCN2* 776C>G polymorphism.



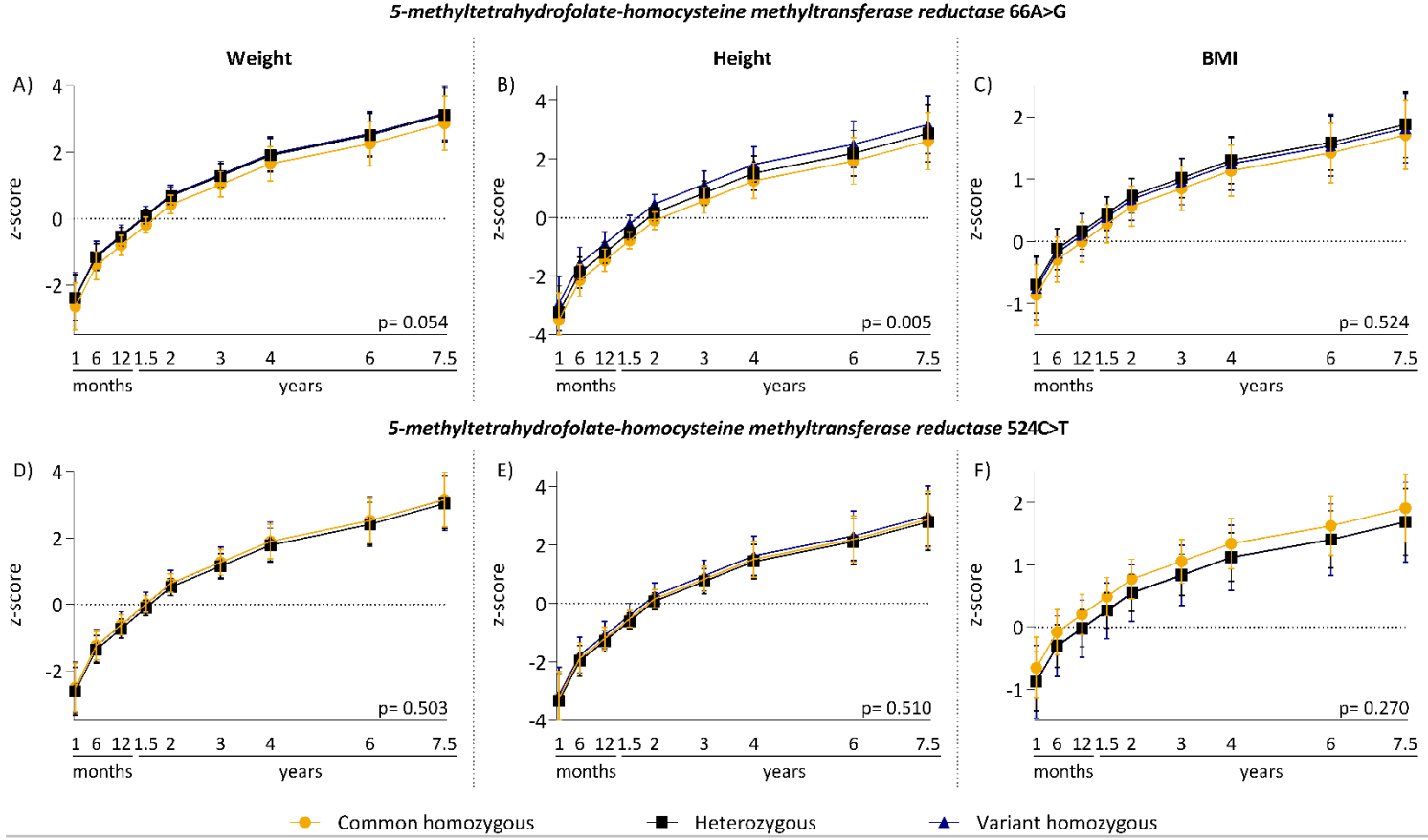
Mean (95% CI) age- and sex-standardised BMI, weight and height z-scores from 1 month through 7.5 years according to maternal *MTHFR* 677C>T (A, B and C) and *TCN2* 776C>G (D, E and F) SNP derived from linear mixed effects models. Fixed effects: age, age^2 and age^3, as well as exclusive breastfeeding length, birth weight z-score, smoking throughout pregnancy, socioeconomic status at follow-up, maternal BMI (for weight and BMI models) or height (for height models), early pregnancy folate and cobalamin status and offspring genotype. Sample size: 1 (n= 94); 6 (n= 112); 12 months (n= 98); 1.5 (n= 102); 2 (n= 94); 3 (n= 56); 4 (n= 74); 6 (n= 70); 7.5 years (n= 160). *p< 0.05; **p< 0.01

of heterozygous mothers. However, the effect size was small (mean difference between common homozygous and heterozygous: 0.33; 95% CI: 0.03, 0.63, p= 0.024 after adjusting for multiple comparisons).

Figure 17 show the mean age- and sex- standardised weight, height and BMI z-score trajectories from birth to mid-childhood according to maternal *MTRR* 66A>G and *MTRR* 524C>T genetic variants by linear mixed effects models. On average, weight (mean, AA: 0.11; 95% CI: -0.14, 0.36; AG: 0.37; 95% CI: 0.15, 0.58; GG: 0.41; 95% CI: 0.15, 0.67) and height (mean, AA: -0.45; 95% CI: -0.72, -0.17; AG: -0.19; 95% CI: -0.43, 0.05; GG: 0.12; 95% CI: -0.18, 0.41) were consistently lower in children born to common compared with variant homozygous mothers for the *MTRR* 66A>G SNP (p= 0.075 and 0.005 for weight and height respectively after adjusting for multiple comparisons). These effects were consistently observed from birth until mid-childhood. However, we observed no effects of maternal *MTRR* 66A>G on offspring BMI trajectory.

The maternal *MTRR* 524C>T genetic variant was not associated with growth trajectories for weight (mean, CC: 0.32; 95% CI: 0.07, 0.57; CT: 0.20; 95% CI: -0.03, 0.43; TT: 0.32; 95% CI: -0.04, 0.67), height (mean, CC: -0.20; 95% CI: -0.48, 0.08; CT: -0.28; 95% CI: -0.54, -0.02; TT: -0.08; 95% CI: -0.49, -0.02) or BMI (mean, CC: 0.63; 95% CI: 0.32, 0.94; CT: 0.41; 95% CI: 0.12, 0.69; TT: 0.40; 95% CI: -0.05, 0.85) in the offspring.

Figure 17. Offspring growth trajectories according to maternal *MTRR* 66A>G and *MTRR* 524C>T polymorphism.



Mean (95% CI) age- and sex-standardised weight, height and BMI z-scores from 1 month until 7.5 years according to maternal *MTRR* 66A>G (A, B and C) and *MTRR* 524C>T (D, E and F) genotypes from linear mixed effects models. Fixed effects: age, age^2 and age^3, as well as exclusive breastfeeding length, birth weight z-score, smoking throughout pregnancy, socioeconomic status at follow-up, maternal BMI (for weight and BMI models) or height (for height models), 1st trimester folate and cobalamin status and offspring genotype. Sample size: 1 (n= 94); 6 (n= 112); 12 months (n= 98); 1.5 (n= 102); 2 (n= 94); 3 (n= 56); 4 (n= 74); 6 (n= 70); 7.5 years (n= 160).

Associations between maternal folate and cobalamin related genetic variants and maternal weight, height and BMI

We assessed whether the effects of maternal genetic variants related to folate and cobalamin transport or metabolism were associated with maternal weight, height and BMI. We found no evidence for any associations between maternal genetic variants and weight or BMI. However, the *MTRR* 66A>G genetic variant was positively associated with maternal height. Per every additional variant allele, height was an average of 0.71 cm (95% CI: 0.05, 1.38) greater.

Effect of birthweight and gestation length on the associations between folate and cobalamin status during pregnancy and offspring growth at mid-childhood

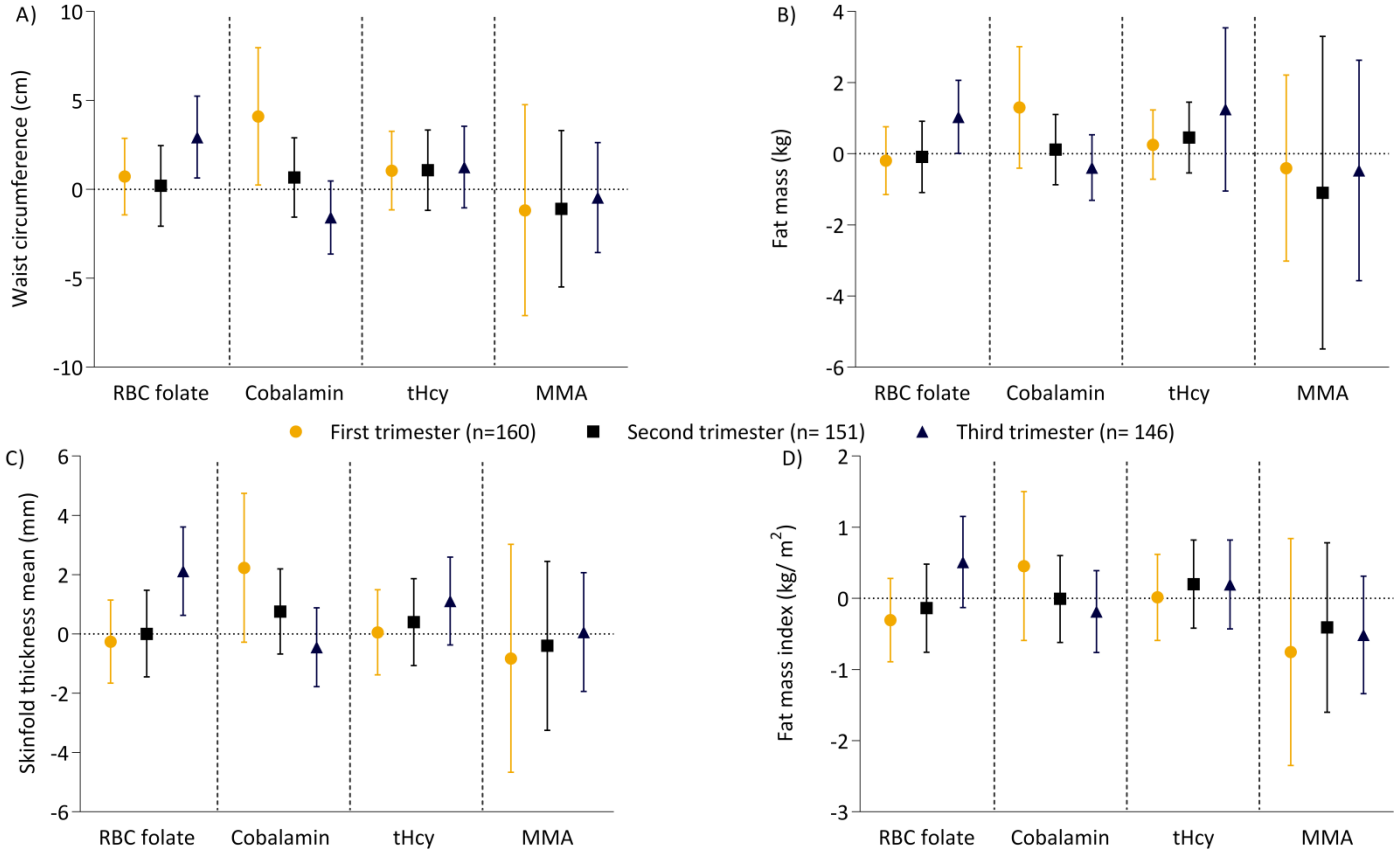
We assessed whether the previously reported effects of maternal folate and cobalamin status during pregnancy on offspring growth were affected by weight at birth and/ or by gestational length. Inclusion of birth weight, gestational age at labour or both factors in multiple linear regression models did not alter the associations between maternal folate and offspring growth at mid-childhood.

Longitudinal associations between folate and cobalamin status during pregnancy and offspring adiposity at mid-childhood

The effects of maternal 1C metabolism on offspring adiposity are shown in **Figure 18**. We found no associations between maternal tHcy and MMA at any time of pregnancy and waist circumference, fat mass, mean skinfold thickness and fat mass index. First trimester cobalamin inadequacy was associated with higher waist circumference, however no effects were observed on any other adiposity measurement. Early and mid-pregnancy RBC folate status was not associated with offspring adiposity. However, inadequate third trimester

RBC folate status (≤603 nmol/L) was associated with higher offspring waist circumference (β-coefficient: 2.93; 95% CI: 0.63, 5.23), fat mass (β-coefficient: 1.03; 95% CI: 0.01, 2.06) and skinfold thickness mean (β-coefficient: 0.71; 95% CI: 0.09, 1.33) compared with adequate RBC folate. No effects were observed on fat mass index (β-coefficient: 0.51; 95% CI: -0.13, 1.15). All analyses were adjusted for first trimester maternal age, BMI, socioeconomic status during pregnancy and at follow-up, smoking throughout pregnancy, exclusive breastfeeding duration, child height (except for fat mass index), physical activity score and energy intake.

Figure 18. Associations between low folate or cobalamin status during pregnancy and offspring adiposity at 7.5 years.



Associations between maternal 1C biomarkers during pregnancy and offspring waist circumference (A), fat mass (B), skinfold thickness mean (C) and fat mass index (D). Symbols (lines) are mean differences (95% CI) observed in the respective low status categories from adequate status [RBC folate (1^{st}: >651; 2^{nd}: >810; 3^{rd} trimester: >603 nmol/L); tHcy (1^{st}: <6.0; 2^{nd}: <5.4; 3^{rd} trimester: <6.1 µmol/L); cobalamin (>221 pmol/L); MMA (<0.210 µmol/L)] by multiple linear regression. Model was adjusted for 1^{st} trimester maternal age, BMI, socioeconomic status and smoking during pregnancy, socioeconomic status at follow-up, exclusive breastfeeding length, child height

We assessed the risk of offspring overweight and obesity according to maternal folate and cobalamin biomarker status by multiple logistic regression analysis after adjusting for the abovementioned factors (**Table 22**). We found no associations between first trimester folate and cobalamin status biomarkers and offspring overweight. After adjusting for potential confounders, we observed that inadequate first trimester plasma holoTC increased the probability of offspring obesity by 7-fold (odds ratio: 6.8; 95% CI: 1.3, 35.7). This association was not maintained in the unadjusted model, and no other prenatal biomarker was associated with offspring obesity.

Table 22. Risk of offspring overweight and obesity according to maternal folate and cobalamin biomarker status at early pregnancy.

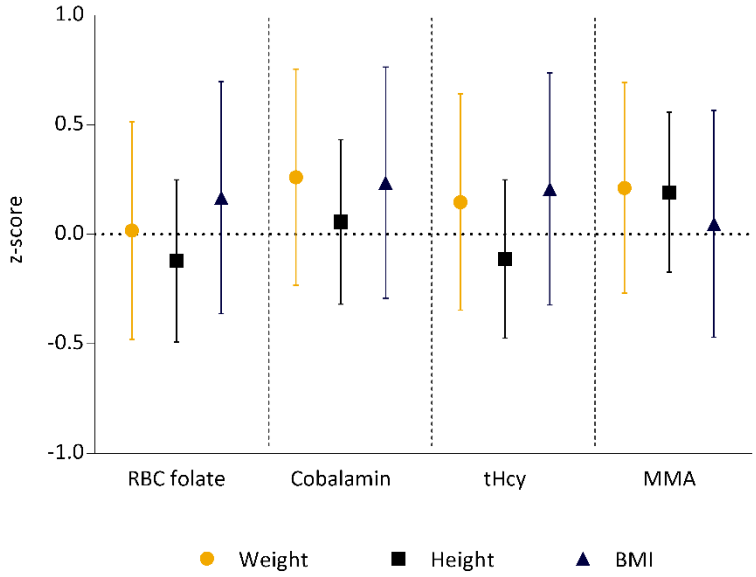
	Overweight (32/ 162)[1]	**Obesity** (13/ 162)
Plasma folate ≤10 nmol/L	4/ 14	2/ 14
Crude	1.69[2] (0.49, 5.77)	2.05 (0.41, 10.32)
Model 1	1.22 (0.29, 5.05)	2.98 (0.48, 18.60)
Model 2	1.70 (0.40, 7.31)	4.12 (0.62, 27.31)
Plasma folate ≥45 nmol/L	2/ 29	1/ 29
Crude	0.25 (0.06, 1.10)	0.35 (0.04, 2.84)
Model 1	0.24 (0.05, 1.15)	0.40 (0.05, 3.54)
Model 2	0.26 (0.05, 1.26)	0.46 (0.05, 4.05)
RBC folate ≤651 nmol/L	6/ 42	2/ 42
Crude	0.59 (0.22, 1.55)	0.49 (0.10, 2.29)
Model 1	0.59 (0.21, 1.65)	0.51 (0.10, 2.53)
Model 2	0.65 (0.23, 1.82)	0.55 (0.11, 2.74)
Plasma cobalamin ≤221 pmol/L	3/ 12	2/ 12
Crude	1.38 (0.35, 5.37)	2.49 (0.48, 12.81)
Model 1	1.32 (0.30, 5.85)	3.10 (0.50, 19.06)
Model 2	1.34 (0.29, 6.26)	3.17 (0.46, 22.06)
Plasma holoTC ≤40 pmol/L	5/ 14	3/ 14
Crude	2.43 (0.75, 7.83)	3.68 (0.88, 15.37)
Model 1	2.91 (0.80, 10.56)	**6.58 (1.32, 32.72)**
Model 2	2.86 (0.76, 10.76)	**6.77 (1.28, 35.74)**
Plasma tHcy ≥6.0 µmol/L	9/ 38	4/ 38
Crude	1.34 (0.56, 3.20)	1.48 (0.43, 5.10)
Model 1	1.02 (0.39, 2.66)	1.40 (0.39, 5.03)
Model 2	1.15 (0.43, 3.03)	1.57 (0.43, 5.73)

Abbreviations: holoTC, holotranscobalamin; RBC, red blood cell; tHcy, total homocysteine.
[1]Child with outcome per total group sample.
[2]Values are odds ratio (95% CI) for offspring overweight and obesity according to the World Obesity Foundation and according to early pregnancy folate and cobalamin marker status. Multiple logistic regression analysis was used. Sample included 160 mother-child pairs.
Crude model was unadjusted. Model 1 was adjusted for maternal 1st trimester age, BMI (for weight and BMI models) or height (height model), socioeconomic status during pregnancy and at follow-up, smoking throughout pregnancy, exclusive breastfeeding duration and folate and cobalamin models were adjusted for cobalamin status and RBC folate status respectively. Model 2 included model 1 plus child physical activity score and total daily energy intake.

Cross-sectional associations between folate and cobalamin status and growth at mid-childhood

We also assessed whether child folate and cobalamin biomarker status influence growth, in order to identify whether the effects observed may have been caused by the influence of maternal folate and cobalamin status on child status. No linear effects were observed between child metabolic markers of folate and cobalamin status and child weight, height and BMI age- and sex-standardised z-scores by multiple linear regression analysis. Inadequate plasma folate (≤12.1 nmol/L), RBC folate (≤589 nmol/L), cobalamin (≤568 pmol/L), tHcy (≥6.3 µmol/L) or MMA (≥0.160 µmol/L) were not associated with any growth measure (**Figure 19**). Models were adjusted for maternal age, smoking throughout pregnancy, first trimester BMI (for weight and BMI models) or height (for height models), exclusive breastfeeding duration, socioeconomic status at childhood and child physical activity score and total energy intake.

Figure 19. Associations between child folate and cobalamin status biomarkers and growth at mid-childhood.



Abbreviations: BMI, body mass index; MMA, methylmalonic acid; RBC, red blood cell; tHcy, total homocysteine.

[1]Associations between RBC folate, plasma cobalamin, tHcy and MMA at mid-childhood and age- and sex-standardised weight, BMI and height z-scores (WHO growth charts). Dots show the mean difference (95% CI) from adequate status with multiple linear regression analysis. Inadequate statuses were defined as plasma folate (≤12.1 nmol/L), RBC folate (≤589 nmol/L), cobalamin (≤568 pmol/L), tHcy (≥6.3 µmol/L) or MMA (≥0.160 µmol/L). Model was adjusted for 1st trimester maternal age, BMI (for weight and BMI models) or height (for height models), socioeconomic status at follow-up, smoking throughout pregnancy, exclusive breastfeeding duration, physical activity score and total energy intake. Sample size n= 107.

Discussion

In this chapter, we investigated whether folate and cobalamin status biomarkers during pregnancy and related maternal genetic variants affect offspring growth and adiposity at mid-childhood. The results show that folate and cobalamin status and related maternal genetic variants affect linear growth and adiposity in the offspring. This study was designed to include biological data from the first trimester of pregnancy, a critical phase for understanding developmental programming yet seldom included in most studies that focus on mid to late pregnancy.

Our first finding showed that children born to women with low first trimester cobalamin, RBC folate or elevated tHcy were shorter than children born to women with adequate status in the vitamins or non-elevated tHcy respectively. Inadequate first or second trimester holoTC (the active form of cobalamin) was associated with higher BMI in the offspring at mid-childhood. Late pregnancy inadequate RBC folate was associated with higher offspring BMI in parallel with other adiposity measures including waist circumference and fat mass. Interestingly, these results were found despite no effects of child folate and cobalamin biomarkers on growth or adiposity. It should be highlighted that inadequate status was defined well above what is considered deficient, supporting the idea that a high proportion of the general population might be exposed to the reported deleterious effects on growth and adiposity. We observed no effects of pregnancy tHcy or MMA on offspring weight. Regarding genotypes affecting folate and cobalamin transport or metabolism, the presence of at least one variant allele of the maternal *TCN2* 776C>G or *MTRR* 524C>T polymorphisms negatively affected growth in the offspring. Those born to heterozygous or variant homozygous compared to common homozygous mothers, for these polymorphisms weighed less and those born to *TCN2* 776 CG or GG compared to CC genotype were also shorter. We observed a consistently higher BMI trajectory from birth until mid-childhood in offspring of *MTHFR* 677C>T homozygous variant compared to heterozygous or common homozygous mothers. The effects of the maternal *TCN2* 776C>G

polymorphism on offspring height were observed as early as 1 month after birth, but its effects on weight and BMI suggested earlier catch-up growth in children born to homozygous variant mothers. Given that the *TCN2* 776 GG genotype has been associated with lower serum and cerebrospinal fluid holoTC in several studies (132,135,462,463), this finding could explain the reported associations between first trimester holoTC and offspring BMI at mid-childhood. Finally, a major susceptibility to developmental programming was observed in offspring exposed to impaired first trimester 1C metabolism compared to those exposed at mid- or late pregnancy. Folic acid supplement intake at preconception was associated with increased height in the offspring at mid-childhood, however first or second trimester folic acid supplementation had no effect on offspring height. Similarly, first trimester, but not second or third trimester, plasma cobalamin inadequacy was associated with lower height in the offspring at 7.5 years.

There is previous evidence of a potential link between maternal folate and cobalamin status during pregnancy and offspring growth in experimental studies in animals and observational studies in humans. One of the most studied mechanisms involves the IGF axis which is crucial in normal somatic growth and development. An increase in methylation at differentially methylated regions of the *IGF-II* gene has been observed in women using folic acid supplements during pregnancy compared to non-users (464,465), but results remain inconsistent (466). It should be noticed that IGF-II and IGF-I are responsible for foetal and postnatal development respectively (467), but available evidence suggesting a role of 1C metabolism on IGF-I is limited. The decrease in IGF-I protein expression observed in embryo skull bone from rats born to folic acid restricted dams (468), in parallel with the finding that IGF-I concentrations are positively correlated with child growth (469) support the results reported in this chapter. In addition, compared with wild type mice, those born to cobalamin deficient dams (genetic murine model) had lower absolute osteoblast per trabecular area and a dramatically decreased bone formation rate (388). These findings were in line with the strong effects on height observed in children born to marginally

cobalamin deficient mothers or mothers carrying the *TCN2* 776C>G variant allele. Again, the effects in mice were mediated by IGF-I histone methylation, which induced a taurine-dependent growth hormone resistance.

The link of 1C metabolism related genetic variants with growth has been scarcely studied. *Mtrr* knockout mice had pups with restricted growth and developmental delay (470). In this case, the observed effects were not mediated by the IGF axis but by decreased histone methylation at the *Igf1* locus. At mid-childhood, we observed similar effects of the maternal *MTRR* 524C>T polymorphism at mid-childhood, and *MTRR* 66A>G polymorphism on weight and height growth trajectories from birth until mid-childhood. We also observed that both the maternal and child *MTRR* 66A>G variant allele compared to the common homozygous genotype was associated with higher maternal and child height respectively. Intriguingly, child but not maternal *MTRR* 66A>G variant allele was associated with higher child IGF-II concentrations (471), results that could potentially explain the divergent effects between the maternal and child *MTRR* 66A>G genetic variant on child linear growth. Why the maternal *MTRR* 66A>G common homozygous genotype negatively influences offspring linear growth while the child genotype has the opposite effect remains unclear. We cannot ascertain whether this is a casual finding, but may manifest the relevance of intrauterine environment on subsequent health.

Studies in 1C nutrient restricted rat dams supports the hypothesis of a link between impaired 1C metabolism and increased offspring adiposity. A reduction in pup myocardium PPAR-α expression was observed after prenatal dietary restriction of vitamin B_{12} and folate which lead to impaired lipid metabolism (379). However, the link between folic acid and adiposity remains unclear in both experimental and human studies. Folic acid supplementation with 5 mg/kg of body weight prevented the effects of protein restriction by decreasing PPAR-α expression (385). In addition to these metabolic effects, increased fat mass percentage is observed in pups born to cobalamin deficient compared to control dams (386). We found a negative association between pregnancy holoTC and offspring

adiposity. To the best of our knowledge, these associations have not been studied before. Interestingly, cobalamin was not associated with offspring BMI or adiposity as reported in other studies, suggesting that intracellular cobalamin deficiency, rather than extracellular cobalamin deficiency, may alter fatty acid metabolism in the offspring. In line with these findings is the observation that children born to *TCN2* 776C>G homozygous variant mothers had a faster rate of BMI growth than children born to homozygous common allele mothers. The *TCN2* 776C>G polymorphism has been associated with lower intracellular cobalamin (129), suggesting a role for holoTC. The role of cobalamin in the β-oxidation pathway involves MMA; however, this is unlikely to explain the transgenerational effects reported in this chapter.

The effects of the maternal *MTHFR* 677C>T genetic variant on offspring body composition have been previously assessed in a cohort of 5783 pregnant women, whose offspring were followed-up at 9 years of age (390). No effect of the maternal nor the child genetic variant on body composition was observed, however, this study did not adjust for folate status, which has been found to be an effect modifier of the association between *MTHFR* 677C>T and tHcy (29). In addition, we observed effects in children from birth until mid-childhood, but no longer at mid-childhood. This suggests that the effects of maternal *MTHFR* 677C>T on offspring adiposity are limited to earlier stages of life.

Experiments altering early pregnancy environmental exposures have shown that the periods of blastocyst development and pre-implantation are highly susceptible to epigenetic changes (472). A potential explanation is that DNA demethylation normally occurs during the cleavage stage and remethylation during the implantation period (between 6 and 12 days after conception) (473,474). Our results support this hypothesis. We found a link between RBC but not plasma folate at <12 GW and offspring height. RBC folate indicates long-term folate status (the previous 120 days or ≈17 weeks) and plasma folate indicates recent folate intake. Hence, first trimester RBC folate indicates folate status during the periconceptional period. In addition, folic acid supplement use at preconception

but not in the first or second trimesters was associated with higher offspring height. Findings reported in studies evaluating the quasi-experimental Dutch famine (475) and results arising from the restriction of methyl donors in female sheep preovulatory oocytes (476) have also shed light on the relevance of exposure timing in developmental programming.

Previous studies have evaluated the influence of 1C metabolism during pregnancy on growth and adiposity beyond foetal development. Yajnik and coworkers reported, in an Indian population (Pune), a positive effect of RBC folate at 28 GW on offspring adiposity at 6 years (389). However, these effects were not replicated later by Krishnaveni et al., who found no associations between maternal folate status and offspring adiposity at either 5, 9.5 or 13.5 years (39). In contrast, in the Netherlands, Krikke found a negative association between mid-pregnancy folate and offspring BMI at 5-6 years (91). In our study, late, but not early pregnancy RBC folate seem to support the latter findings in the Netherlands. In addition, we did not find any effect of elevated plasma folate throughout pregnancy on offspring growth nor adiposity. These discrepancies may arise due to population differences in mean protein intake. As stated earlier, folic acid supplementation in protein-restricted dams had the same effect on pup PPAR-α expression as folic acid deprivation in protein non-restricted dams. In Pune (India), median dietary protein intake was low (41.7 g at 28 GW) (389) compared to the expected intake in the Netherlands and compared to the intake in the studied population from the present study (77 g/day). Folate status in subjects with low protein intake would reduce fatty acid catabolism, thus favouring its accumulation. The opposite effect would be observed in subjects with normal to elevated protein intake: folate status would increase fatty acid oxidation. Given that both underweight and overweight are linked with increased risk of mortality (477), folate status during pregnancy seems to optimally modulate the expression of genes according to the received environmental stimuli. Indeed, in a randomised controlled clinical trial, infant formula with

lower protein content compared to infant formulas with higher protein content reduced BMI and obesity risk, in children with apparently normal folate status (383). These findings could be mediated through the IGF1 axis, as suggested by the authors (478). Nevertheless, this proposed protein-dependent differential impact of folate during pregnancy on offspring BMI remains speculative. It has also been suggested that balanced folate and cobalamin status is necessary for optimal growth (389), but we found no interactions between folate and cobalamin status during pregnancy on offspring growth.

To date, no effects on offspring growth or adiposity have been attributed to cobalamin status during pregnancy. In normal pregnancies, cobalamin declines by approximately 25% from preconception and throughout pregnancy (76). However, this decrease is not accompanied by an alteration in functional biomarkers of cobalamin status, despite increases in tHcy and MMA in late pregnancy (38,76). Therefore, the influence of pregnancy on between-subject variability is greater the later in pregnancy the blood sample is collected.

The study has strengths and limitations. The most important limitation is the relatively small sample size (162 mother-child pairs). However, it consisted of a very well characterised population both genetically and phenotypically from the first trimester throughout pregnancy. Ideally, a random control trial to test the effect of first trimester folate or cobalamin status on growth and adiposity in the offspring would enable us to test whether the association is causal. However, such a trial would be unethical in humans given the established risk of grave neural tube defects in the foetus in the presence of maternal folate or cobalamin deficiency. Our prospective study did enable us to test the effect of exposure to low early pregnancy folate or cobalamin status on growth and adiposity at mid-childhood and to adjust all of the analyses for numerous potential confounders allowing us to assume a high degree of confidence when interpreting the results. Although unmeasured residual confounding cannot be ruled out, our models were adjusted for established confounding factors to date. An alternative interpretation to our results could be that

women not using preconceptional folic acid supplements or those with inadequate cobalamin or folate status at early pregnancy are less health-conscious, negatively affecting offspring achievement of optimal growth compared to more health-conscious women. However, we found that maternal and not child genetic variants affecting intrauterine folate and cobalamin transport or metabolism were associated with offspring growth and growth trajectories. Thus, we observed via these randomly occurring genetic variants that prenatal and not postnatal exposure to impaired 1C metabolism has a role in developmental programming.

Our observation that folic acid supplementation at preconception and inadequate first trimester RBC but not plasma folate affected offspring growth, highlights the relevance of the preconceptional period in developmental programming. In addition, inadequate folate and cobalamin status during pregnancy are associated with impaired offspring growth or adiposity and we found no harmful effects of regular early pregnancy folic acid supplement use, often in excess of 400 µg/day on offspring growth and adiposity. Given the associations between adiposity and height with other cardiometabolic risk factors (335,479,480), the influence of prenatal folate and cobalamin biomarkers on these risk factors should also be investigated.

In summary, our study shows that prenatal exposure to inadequate 1C metabolism, as early as during the preconceptional period, induces growth impairment in the offspring at mid childhood. Europe has failed to implement an effective strategy for the prevention of NTDs with folic acid. Considering the reported results, the benefits of mandatory fortification of cereal grains with folic acid may not be limited to the prevention of NTDs and could potentially enhance postnatal optimal growth attainment.

DISCUSSION

Discussion

Global context

The importance of optimal early development in determining lifelong healthy ageing trajectories is gaining recognition (5). The 1C metabolic network has proven to be highly important in embryogenesis, foetal development and in determining pregnancy outcome. Its recognised role in neural tube development led to the implementation of mandatory fortification of flour with folic acid in the USA and Canada in 1998. An impressive reduction in NTD prevalence (8,9) was observed after its implementation, as well as other health benefits affecting the general population, such as a reduction in plasma tHcy (11). It has been suggested that this metabolic effect was accompanied by a reduction in stroke mortality in the post-fortification era (12). Unlike many other countries, European countries have not implemented mandatory fortification with folic acid to date, while the debate regarding the proven benefits and possible risks is ongoing. The current strategies to prevent NTD occurrence such as allowing voluntary folic acid fortification and the recommendation for the use of folic acid at preconception are widely practised by member states of the European Union but have not been successful in reducing NTD prevalence (298).

The studied mother-child pairs were not exposed to mandatory fortification, but high doses of folic acid supplements (>1 mg/day) were frequently used during the first trimester of pregnancy. The exposure to such doses has been identified as of concern for four priority areas of research by the National Toxicology Program and Office of Dietary Supplements at the USA National Institutes of Health (481). Generally, the concern is with regard to the combined exposure to folic acid from fortified foods and supplements, of population subgroups with underlying metabolic imbalances (such as cobalamin deficiency or obesity), that may render them susceptible to adverse effects of high folate status. We found no

evidence to suggest deleterious effects of elevated folate status in pregnant women with cobalamin inadequacy on either metabolic or haematological indicators of cobalamin deficiency. However, we did observe that women with concomitant elevated folate and low cobalamin status (1.1 % of those studied) had lower MCV than those with adequate cobalamin. This might be interpreted as evidence of masking of cobalamin deficiency, a major concern of folic acid fortification, but the relatively small sample size hindered our ability to identify macrocytosis. It would be interesting to investigate this further in pregnant women from a population such as the USA where combined exposure to fortification and supplement use is commonplace, and globally folate status of pregnant women is likely to be higher. There is evidence that folic acid supplement use in the preconception period is more effective in preventing NTDs in normal weight compared to overweight and obese women (482) and affects RBC and plasma folate compartments differently in women with high BMI (264). We confirmed a BMI-folate intake interaction on RBC folate status and observed that the RBC folate-plasma folate ratio was higher in overweight and obese compared to normal weight women. These observations may be relevant in informing future research and folic acid fortification policies on doses of the vitamin for effective improvement in folate status in overweight or obese women in a society where overweight and obesity are becoming increasing prevalent.

The results from this thesis suggest that prenatal 1C metabolism affects offspring growth far beyond foetal development. To the best of our knowledge, the following findings are novel:

- BMI is negatively associated with plasma folate in pregnant women, but its association with RBC folate depends on total daily folate intake.
- Specific indicators of pregnancy cobalamin status are associated with cobalamin and MMA in the offspring.

- Inadequate folate or cobalamin, elevated tHcy in early pregnancy or no use of folic acid supplements at preconception is associated with lower offspring linear growth.
- Folate and cobalamin transport or metabolism related maternal genetic variants are associated with growth and adiposity in the offspring.

The fact that subtle imbalances, and not clinical deficiency, in folate or cobalamin during pregnancy were linked with growth and adiposity in the offspring at mid-childhood suggests that prenatal exposure to folic acid and probably vitamin B_{12} may potentially affect long-term health and development in the subsequent generation. This study expands the evidence supporting prenatal 1C metabolism programming of growth and adiposity in the offspring and suggests that maternal folic acid supplement use at the levels observed is not harmful to growth or adiposity in the offspring.

Strengths and limitations

Biological, clinical and lifestyle data were collected from the first trimester of pregnancy. Only a limited number of longitudinal pregnancy studies with prospectively collected data throughout pregnancy have been carried out to date. It is exceptionally difficult to obtain first trimester data because it depends on the timely initiation of prenatal care by the mother. Prenatal data available from most prospective mother child cohorts is usually from late pregnancy, and exceptionally from mid-pregnancy.

The prospective nature of the study design enabled us to test hypotheses regarding early prenatal exposures and outcomes in the offspring in a well characterised (both phenotypically and genotypically) mother-child cohort.

Discussion

The exposure-outcome associations of interest in our study are limited in the sense of not meeting some criteria to test causality. It would be unethical to perform a randomised control trial to test the nutrient imbalances of interest on child development and metabolic outcomes. Thus, the only option was to carry out an observational study despite the potential limitations of unmeasured residual confounding factors that this entails. However, the prospective nature of the study with time points representative of each trimester of pregnancy and in which each mother acted as her own control was a strong point of the study design.

The relatively small sample size in the child follow-up group to date may appear to be a potential limitation. The number of children eligible to participate (having reached 7.5 years) and the participation rate of 50% of these at follow-up, limited the sample size. We had anticipated a higher participation rate based on previous experience in another mother-child cohort. The relocation of our hospital from the city centre to the suburbs, as well as the necessity for children to miss school during the morning of the check-up may have affected the participation rate. Sample size is expected to increase because the child recruitment phase is still ongoing. However, we had sufficient statistical power to explore numerous hypotheses linking early prenatal 1C metabolism and exposures with outcomes in the children.

It is possible that the requirement of seeking prenatal care before completing the first trimester of pregnancy and subsequently that the parents voluntarily bring their child for a health check-up may have introduced a selection bias into the study. Most women were Caucasian and had moderate-high socioeconomic status. This limits the extrapolation of conclusions to other populations.

Future perspectives

Further investigation is required to completely test some hypotheses that this thesis set out to address and new hypotheses that have stemmed from the findings of the thesis. The results should be replicated in studies of different populations incorporating Mendelian randomisation to provide information on the causal relevance of the different components in 1C metabolism to understand and complete current knowledge on the *in utero* programming of growth and adiposity.

The safety of elevated folic acid in subjects with cobalamin inadequacy should be carefully monitored in already existing clinical trials and observational studies in order to gain information on whether mandatory fortification with folic acid is safe for the whole population. Ethical issues exist on the inclusion of people with cobalamin deficiency in clinical trials without treating their deficiency. In this sense, animal models could be used to examine the effects of folic acid supplementation in the presence of cobalamin deficiency. The inclusion of subjects with marginally deficient cobalamin status in intervention studies with different doses of folic acid would pose no ethical concerns, and would provide valuable information. There is also the need to establish reference ranges for elevated folate and cobalamin deficiency and marginal deficiency during pregnancy and childhood.

Regarding the long-term effects of prenatal 1C metabolism on offspring growth and adiposity, replication studies in a variety of populations would be of great interest to corroborate the present findings, and to shed light on the differential phenotypic effects that this metabolic network may have. The present cohort has great potential and is of remarkable value given the early recruitment and the longitudinal follow-up during pregnancy, including the collection of six blood samples. However, the sample size should be increased in order to gain sufficient statistical power to test hypotheses affecting some small subgroups in the cohort. Moreover, the effects of 1C metabolism during pregnancy on other offspring health outcomes should be assessed. Whether the observed effects on

growth and adiposity reported in this thesis are accompanied by cognitive impairment, elevated blood pressure or altered metabolic profile remains unknown. Therefore, the studied outcomes should include offspring cognitive development and blood pressure, as well as biomarkers of glucose metabolism, lipid profile, inflammation and endothelial function. To test whether some of the observed effects reported here are mediated through the IGF1 axis, would assess a new hypothesis generated by this and previous research. Further follow-up of this cohort of children at a later stage of development is of interest in order to evaluate the persistence of these effects over time.

The use of Mendelian randomisation bypasses the intrinsic limitations to observational studies. Given the wide range of genetic variants associated with alterations in 1C metabolism, the effects of these variants should also be investigated. Moreover, it is necessary to clarify whether the timing of exposure is essential in the relationship between prenatal exposure and long-term outcomes. In this sense, paternal effects have not been examined in this thesis but would shed light on this issue given their implications in normal placentation and in the initial demethylation of whole genome.

Finally, the findings of this thesis in parallel with further studies (derived or not from this cohort) will provide strong evidence to inform public health strategies such as the fortification with folic acid and vitamin B_{12} to enhance balance in 1C nutrient status and to counteract the deleterious effects of common SNPs.

CONCLUSIONS

Conclusions

Specific objectives

Objective: To describe cobalamin and folate metabolic indicators in the first trimester of pregnancy.

First trimester marginal plasma cobalamin deficiency affected 6.3% of the pregnancies, but functional biomarkers were normal.

First trimester RBC folate status was not optimal for the prevention of NTDs, despite folic acid supplementation. First trimester plasma folate deficiency and elevated plasma folate affected 11.4% and 20.2% of the mothers, respectively.

Objective: To determine the association between genetic, nutritional and other lifestyle factors and first trimester folate and cobalamin status.

First trimester folic acid supplement intake, pregnancy planning and maternal *MTHFR* 677C>T genetic variant accounted for most of the RBC folate variability. Maternal *MTHFR* 677C>T and *MTRR* 66A>G variant homozygous genotypes compared to common homozygous genotypes were associated with higher plasma tHcy.

Conclusions

First trimester maternal BMI was negatively associated with plasma folate, and folate intake modified the association between BMI and RBC folate.

Objective: To investigate cobalamin and folate status interactions and their association with metabolic and haematological markers of low cobalamin status during pregnancy.

Elevated folate status did not exacerbate biochemical or haematological abnormalities related to low cobalamin status in a population unexposed to mandatory fortification with folic acid but with high use of folic acid supplements.

Objective: To describe cobalamin and folate metabolic indicators in children at mid-childhood (7.5 years).

All of the children studied had optimal plasma cobalamin status but elevated plasma MMA was observed in 5.7% of them. Plasma folate deficiency and elevated plasma folate affected 12.3% and 5.7% of children respectively.

Objective: To determine the association between genetic, nutritional and other lifestyle factors and mid-childhood folate and cobalamin status.

Child *MTHFR* 677C>T variant homozygous compared to common homozygous genotype was associated with lower mid-childhood plasma and RBC folate status.

Breastfeeding and exclusive breastfeeding length were not associated with either child folate or cobalamin status at mid-childhood.

Objective: To investigate the association between prenatal folate and cobalamin status and mid-childhood one carbon metabolism status indicators.

Pregnancy cobalamin status was the most important determinant of, and was positively associated with mid-childhood cobalamin status. Pregnancy folate status was not associated with mid-childhood folate status.

Objective: To assess the associations of folate and cobalamin status during pregnancy on mid-childhood linear and ponderal growth and adiposity.

Inadequate first trimester plasma cobalamin, RBC folate or elevated tHcy were associated with lower linear, but not ponderal, mid-childhood growth.

Folic acid supplement use at preconception, but not in the first or second trimester, was associated with higher linear mid-childhood growth.

Inadequate first or second trimester plasma holoTC or inadequate third trimester RBC folate were associated with higher mid-childhood adiposity.

Child biomarkers of folate and cobalamin status were not associated with either linear or ponderal growth or adiposity at mid-childhood.

Conclusions

Objective: To investigate the associations between maternal and child genetic variants affecting folate and cobalamin metabolism or transport and mid-childhood linear and ponderal growth and adiposity.

The maternal *TCN2* variant 776 CG genotype compared to CC genotype was associated with persistently lower linear growth from birth until mid-childhood. Children from GG compared to CG or CC mothers had lower BMI during infancy but a faster BMI rebound from early- to mid-childhood.

The maternal *MTHFR* 677 TT compared to CT or CC genotypes was associated with higher BMI growth from birth until mid-childhood.

Children born to mothers with the *MTRR* 66 AA genotype compared to GG genotype were shorter and weighed less from birth until mid-childhood.

Children with the *MTRR* 66 variant allele compared to AA genotype had lower linear and ponderal growth at mid-childhood.

BIBLIOGRAPHY

Bibliography

1. United Nations, Department of Economic and Social Affairs, Population Division. World Population Prospects: The 2015 Revision, Key Findings and Advance Tables. 2015.

2. Crimmins EM, Beltrán-Sánchez H. Mortality and morbidity trends: is there compression of morbidity? J Gerontol B Psychol Sci Soc Sci. 2011;66:75–86.

3. World Health Organization. Global status report on noncommunicable diseases 2014. Geneva; 2014.

4. Murray CJLJL, Barber RMM, Foreman KJJ, Ozgoren AAA, Abd-Allah F, Abera SFF, Aboyans V, Abraham JPP, Abubakar I, et al. Global, regional, and national disability-adjusted life years (DALYs) for 306 diseases and injuries and healthy life expectancy (HALE) for 188 countries, 1990–2013: quantifying the epidemiological transition. Lancet. 2015;386:2145–91.

5. World Health Organization. World report on ageing and health. Geneva; 2015.

6. MRC Vitamin Study Research Group. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group. Lancet (London, England). 1991;338:131–7.

7. Czeizel AE, Dudás I. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. N Engl J Med. 1992;327:1832–5.

8. National Birth Defects Prevention Network. Birth defects state profile - Oklahoma. 2010.

9. De Wals P, Tairou F, Van Allen MI, Uh S-H, Lowry RB, Sibbald B, Evans JA, Van den Hof MC, Zimmer P, et al. Reduction in neural-tube defects after folic acid fortification in Canada. N Engl J Med. 2007;357:135–42.

10. Bentley TGK, Willett WC, Weinstein MC, Kuntz KM. Population-level changes in folate intake by age, gender, and race/ethnicity after folic acid fortification. Am J Public Health. 2006;96:2040–7.

11. Pfeiffer CM, Osterloh JD, Kennedy-Stephenson J, Picciano MF, Yetley EA, Rader JI, Johnson CL. Trends in circulating concentrations of total homocysteine among US adolescents and adults: findings from the 1991-1994 and 1999-2004 National Health and Nutrition Examination Surveys. Clin Chem. 2008;54:801–13.

12. Yang Q, Botto LD, Erickson JD, Berry RJ, Sambell C, Johansen H, Friedman JM. Improvement in stroke mortality in Canada and the United States, 1990 to 2002. Circulation. 2006;113:1335–43.

13. Finkelstein JD. Methionine metabolism in mammals. J Nutr Biochem. 1990;1:228–37.

14. Blount BC, Mack MM, Wehr CM, MacGregor JT, Hiatt RA, Wang G, Wickramasinghe SN, Everson RB, Ames BN. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. Proc Natl Acad Sci U S A. 1997;94:3290–5.

15. Zatz M, Dudley PA, Kloog Y, Markey SP. Nonpolar lipid methylation. Biosynthesis of fatty acid methyl esters by rat lung membranes using S-adenosylmethionine. J Biol Chem. 1981;256:10028–32.

16. Spector AA, Yorek MA. Membrane lipid composition and cellular function. J Lipid Res. 1985;26:1015–35.

17. Noga AA, Vance DE. A gender-specific role for phosphatidylethanolamine N-methyltransferase-derived phosphatidylcholine in the regulation of plasma high density and very low density lipoproteins in mice. J Biol Chem. 2003;278:21851–9.

18. Blusztajn JK, Wurtman RJ. Choline and cholinergic neurons. Science. 1983;221:614–20.

19. Vance DE, Ridgway ND. The methylation of phosphatidylethanolamine. Prog Lipid Res. 1988;27:61–79.

20. Katada S, Imhof A, Sassone-Corsi P. Connecting threads: epigenetics and metabolism. Cell. 2012;148:24–8.

21. Gluckman PD. Epigenetics and metabolism in 2011: Epigenetics, the life-course and metabolic disease. Nat Rev Endocrinol. 2012;8:74–6.

22. Teperino R, Schoonjans K, Auwerx J. Histone methyl transferases and demethylases; can they link metabolism and transcription? Cell Metab. 2010;12:321–7.

23. Feinberg AP. Phenotypic plasticity and the epigenetics of human disease. Nature. 2007;447:433–40.

24. Zeisel SH, Zola T, daCosta KA, Pomfret EA. Effect of choline deficiency on S-adenosylmethionine and methionine concentrations in rat liver. Biochem J. 1989;259:725–9.

25. Ogier de Baulny H, Gérard M, Saudubray JM, Zittoun J. Remethylation defects: guidelines for clinical diagnosis and treatment. Eur J Pediatr. 1998;157 Suppl :S77–83.

26. Reynolds EH. The neurology of folic acid deficiency. Handb Clin Neurol. 2014;120:927–43.

27. Selhub J, Jacques PF, Wilson PW, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. JAMA. 1993;270:2693–8.

28. Nygård O, Vollset SE, Refsum H, Stensvold I, Tverdal A, Nordrehaug JE, Ueland M, Kvåle G. Total plasma homocysteine and cardiovascular risk profile. The Hordaland Homocysteine Study. JAMA. 1995;274:1526–33.

29. Crider KS, Zhu J-H, Hao L, Yang Q-H, Yang TP, Gindler J, Maneval DR, Quinlivan EP, Li Z, et al.

MTHFR 677C->T genotype is associated with folate and homocysteine concentrations in a large, population-based, double-blind trial of folic acid supplementation. Am J Clin Nutr. 2011;93:1365–72.

30. Boushey CJ, Beresford SA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. JAMA. 1995;274:1049–57.

31. Selhub J, Jacques PF, Rosenberg IH, Rogers G, Bowman BA, Gunter EW, Wright JD, Johnson CL. Serum total homocysteine concentrations in the third National Health and Nutrition Examination Survey (1991-1994): population reference ranges and contribution of vitamin status to high serum concentrations. Ann Intern Med. 1999;131:331–9.

32. Landgren F, Israelsson B, Lindgren A, Hultberg B, Andersson A, Brattström L. Plasma homocysteine in acute myocardial infarction: homocysteine-lowering effect of folic acid. J Intern Med. 1995;237:381–8.

33. Lindenbaum J, Healton EB, Savage DG, Brust JC, Garrett TJ, Podell ER, Marcell PD, Stabler SP, Allen RH. Neuropsychiatric disorders caused by cobalamin deficiency in the absence of anemia or macrocytosis. N Engl J Med. 1988;318:1720–8.

34. Ubbink JB, Vermaak WJ, van der Merwe A, Becker PJ. Vitamin B-12, vitamin B-6, and folate nutritional status in men with hyperhomocysteinemia. Am J Clin Nutr. 1993;57:47–53.

35. Berry RJ, Li Z, Erickson JD, Li S, Moore CA, Wang H, Mulinare J, Zhao P, Wong LY, et al. Prevention of neural-tube defects with folic acid in China. China-U.S. Collaborative Project for Neural Tube Defect Prevention. N Engl J Med. 1999;341:1485–90.

36. van Beynum IM, Kapusta L, Bakker MK, den Heijer M, Blom HJ, de Walle HEK. Protective effect of periconceptional folic acid supplements on the risk of congenital heart defects: a registry-based case-control study in the northern Netherlands. Eur Heart J. 2010;31:464–71.

37. Refsum H, Nurk E, Smith AD, Ueland PM, Gjesdal CG, Bjelland I, Tverdal A, Tell GS, Nygård O, Vollset SE. The Hordaland Homocysteine Study: a community-based study of homocysteine, its determinants, and associations with disease. J Nutr. 2006;136:1731S – 1740S.

38. Murphy MM, Scott JM, Arija V, Molloy AM, Fernandez-Ballart JD. Maternal homocysteine before conception and throughout pregnancy predicts fetal homocysteine and birth weight. Clin Chem. 2004;50:1406–12.

39. Yajnik CS, Chandak GR, Joglekar C, Katre P, Bhat DS, Singh SN, Janipalli CS, Refsum H, Krishnaveni G, et al. Maternal homocysteine in pregnancy and offspring birthweight: epidemiological associations and Mendelian randomization analysis. Int J Epidemiol. 2014;43:1487–97.

40. Huo Y, Li J, Qin X, Huang Y, Wang X, Gottesman RF, Tang G, Wang B, Chen D, et al. Efficacy of folic acid therapy in primary prevention of stroke among adults with hypertension in China: the CSPPT randomized clinical trial. JAMA. 2015;313:1325–35.

41. Han L, Wu Q, Wang C, Hao Y, Zhao J, Zhang L, Fan R, Liu Y, Li R, et al. Homocysteine, Ischemic Stroke, and Coronary Heart Disease in Hypertensive Patients. Stroke. 2015;46:1777–86.

42. Cheng T-YD, Makar KW, Neuhouser ML, Miller JW, Song X, Brown EC, Beresford SAA, Zheng Y, Poole EM, et al. Folate-mediated one-carbon metabolism genes and interactions with nutritional factors on colorectal cancer risk: Women's Health Initiative Observational Study. Cancer. 2015;121:3684–91.

43. de Batlle J, Ferrari P, Chajes V, Park JY, Slimani N, McKenzie F, Overvad K, Roswall N, Tjønneland A, et al. Dietary folate intake and breast cancer risk: European prospective investigation into cancer and nutrition. J Natl Cancer Inst. 2015;107:367.

44. Molloy AM, Kirke PN, Troendle JF, Burke H, Sutton M, Brody LC, Scott JM, Mills JL. Maternal vitamin B12 status and risk of neural tube defects in a population with high neural tube defect prevalence and no folic Acid fortification. Pediatrics. 2009;123:917–23.

45. Kirke PN, Mills JL, Molloy AM, Brody LC, O'Leary VB, Daly L, Murray S, Conley M, Mayne PD, et al. Impact of the MTHFR C677T polymorphism on risk of neural tube defects: case-control study. BMJ. 2004;328:1535–6.

46. Martens JH, Barg H, Warren MJ, Jahn D. Microbial production of vitamin B12. Appl Microbiol Biotechnol. 2002;58:275–85.

47. Farquharson J, Adams JF. The forms of vitamin B12 in foods. Br J Nutr. 1976;36:127–36.

48. Tucker KL, Rich S, Rosenberg I, Jacques P, Dallal G, Wilson PW, Selhub J. Plasma vitamin B-12 concentrations relate to intake source in the Framingham Offspring study. Am J Clin Nutr. 2000;71:514–22.

49. Vogiatzoglou A, Smith AD, Nurk E, Berstad P, Drevon CA, Ueland PM, Vollset SE, Tell GS, Refsum H. Dietary sources of vitamin B-12 and their association with plasma vitamin B-12 concentrations in the general population: the Hordaland Homocysteine Study. Am J Clin Nutr. 2009;89:1078–87.

50. Durá-Travé T, Gallinas-Victoriano F. Milk and dairy products intake in child-juvenile population in Navarre, Spain. Nutr Hosp. 2014;30:794–9.

51. Allen LH. Bioavailability of vitamin B12. Int J Vitam Nutr Res Int Zeitschrift für Vitamin- und Ernährungsforschung J Int Vitaminol Nutr. 2010;80:330–5.

52. Watanabe F. Vitamin B12 Sources and Bioavailability. Exp Biol Med. 2007;232:1266–74.

53. Berlin H, Berlin R, Brante G. Oral treatment of pernicious anemia with high doses of vitamin B12 without intrinsic factor. Acta Med Scand. 1968;184:247–58.

54. Doets EL, In 't Veld PH, Szczecińska A, Dhonukshe-Rutten RAM, Cavelaars AEJM, van 't Veer P, Brzozowska A, de Groot LCPGM. Systematic review on daily vitamin B12 losses and bioavailability for deriving recommendations on vitamin B12 intake with the factorial

approach. Ann Nutr Metab. 2013;62:311–22.

55. Doscherholmen A, McMahon J, Economon P. Vitamin B12 absorption from fish. Proc Soc Exp Biol Med. 1981;167:480–4.

56. Doscherholmen A, McMahon J, Ripley D. Vitamin B12 absorption from eggs. Proc Soc Exp Biol Med. 1975;149:987–90.

57. Doscherholmen A, McMahon J, Ripley D. Vitamin B12 assimilation from chicken meat. Am J Clin Nutr. 1978;31:825–30.

58. Heyssel RM, Bozian RC, Darby WJ, Bell MC. Vitamin B12 turnover in man. The assimilation of vitamin B12 from natural foodstuff by man and estimates of minimal daily dietary requirements. Am J Clin Nutr. 1966;18:176–84.

59. Institute of Medicine. Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline. Washington, D.C.: National Academies Press; 2000.

60. Federación Española de Sociedades de Nutrición A y D. Ingestas Dietéticas de Referencia (IDR) para la Población Española, 2010. Act dietética. 2010;14:196–7.

61. Ray JG, Wyatt PR, Thompson MD, Vermeulen MJ, Meier C, Wong P-Y, Farrell SA, Cole DEC. Vitamin B12 and the risk of neural tube defects in a folic-acid-fortified population. Epidemiology. 2007;18:362–6.

62. Brown J, Robertson J, Gallagher N. Humoral regulation of vitamin B12 absorption by pregnant mouse small intestine. Gastroenterology. 1977;72:881–8.

63. HELLEGERS A, OKUDA K, NESBITT RE, SMITH DW, CHOW BF. Vitamin B12 absorption in pregnancy and in the newborn. Am J Clin Nutr. 5:327–31.

64. Greibe E, Andreasen BH, Lildballe DL, Morkbak AL, Hvas A-M, Nexo E. Uptake of cobalamin and markers of cobalamin status: a longitudinal study of healthy pregnant women. Clin Chem Lab Med. 2011;49:1877–82.

65. Bae S, West AA, Yan J, Jiang X, Perry CA, Malysheva O, Stabler SP, Allen RH, Caudill MA. Vitamin B-12 Status Differs among Pregnant, Lactating, and Control Women with Equivalent Nutrient Intakes. J Nutr. 2015;145:1507–14.

66. Schneede J, Dagnelie PC, van Staveren WA, Vollset SE, Refsum H, Ueland PM. Methylmalonic acid and homocysteine in plasma as indicators of functional cobalamin deficiency in infants on macrobiotic diets. Pediatr Res. 1994;36:194–201.

67. Lildballe DL, Hardlei TF, Allen LH, Nexo E. High concentrations of haptocorrin interfere with routine measurement of cobalamins in human serum and milk. A problem and its solution. Clin Chem Lab Med. 2009;47:182–7.

68. Greibe E, Fedosov S, Sorensen BS, Højrup P, Poulsen SS, Nexo E. A single rainbow trout cobalamin-binding protein stands in for three human binders. J Biol Chem. 2012;287:33917–25.

69. Hall CA. The carriers of native vitamin B12 in normal human serum. Clin Sci Mol Med. 1977;53:453–7.

70. Quadros E V, Jacobsen DW. The dynamics of cobalamin utilization in L-1210 mouse leukemia cells: a model of cellular cobalamin metabolism. Biochim Biophys Acta. 1995;1244:395–403.

71. Youngdahl-Turner P, Mellman IS, Allen RH, Rosenberg LE. Protein mediated vitamin uptake. Adsorptive endocytosis of the transcobalamin II-cobalamin complex by cultured human fibroblasts. Exp Cell Res. 1979;118:127–34.

72. Quadros E V, Sai P, Rothenberg SP. Characterization of the human placental membrane receptor for transcobalamin II-cobalamin. Arch Biochem Biophys. 1994;308:192–9.

73. Lai S-C, Nakayama Y, Sequeira JM, Wlodarczyk BJ, Cabrera RM, Finnell RH, Bottiglieri T, Quadros E V. The transcobalamin receptor knockout mouse: a model for vitamin B12 deficiency in the central nervous system. FASEB J. 2013;27:2468–75.

74. Weissbach H, Taylor RT. Roles of vitamin B 12 and folic acid in methionine synthesis. Vitam Horm. 1970;28:415–40.

75. Savage DG, Lindenbaum J, Stabler SP, Allen RH. Sensitivity of serum methylmalonic acid and total homocysteine determinations for diagnosing cobalamin and folate deficiencies. Am J Med. 1994;96:239–46.

76. Murphy MM, Molloy AM, Ueland PM, Fernandez-Ballart JD, Schneede J, Arija V, Scott JM. Longitudinal study of the effect of pregnancy on maternal and fetal cobalamin status in healthy women and their offspring. J Nutr. 2007;137:1863–7.

77. Morkbak AL, Hvas A-M, Milman N, Nexo E. Holotranscobalamin remains unchanged during pregnancy. Longitudinal changes of cobalamins and their binding proteins during pregnancy and postpartum. Haematologica. 2007;92:1711–2.

78. Duggan C, Srinivasan K, Thomas T, Samuel T, Rajendran R, Muthayya S, Finkelstein JL, Lukose A, Fawzi W, et al. Vitamin B-12 supplementation during pregnancy and early lactation increases maternal, breast milk, and infant measures of vitamin B-12 status. J Nutr. 2014;144:758–64.

79. Minet JC, Bissé E, Aebischer CP, Beil A, Wieland H, Lütschg J. Assessment of vitamin B-12, folate, and vitamin B-6 status and relation to sulfur amino acid metabolism in neonates. Am J Clin Nutr. 2000;72:751–7.

80. Bjørke Monsen AL, Ueland PM, Vollset SE, Guttormsen AB, Markestad T, Solheim E, Refsum H. Determinants of cobalamin status in newborns. Pediatrics. 2001;108:624–30.

81. Monsen A-LB, Refsum H, Markestad T, Ueland PM. Cobalamin status and its biochemical markers methylmalonic acid and homocysteine in different age groups from 4 days to 19 years. Clin Chem. 2003;49:2067–75.

82. Thompson GN, Walter JH, Bresson JL, Ford GC, Lyonnet SL, Chalmers RA, Saudubray JM, Leonard J V, Halliday D. Sources of propionate in inborn errors of propionate metabolism. Metabolism. 1990;39:1133–7.

83. Bjørke-Monsen A-L, Torsvik I, Saetran H, Markestad T, Ueland PM. Common metabolic profile in infants indicating impaired cobalamin status responds to cobalamin supplementation. Pediatrics. 2008;122:83–91.

84. Allen LH. How common is vitamin B-12 deficiency? Am J Clin Nutr. 2009;89:693S – 6S.

85. Stabler SP, Allen RH. Vitamin B12 deficiency as a worldwide problem. Annu Rev Nutr. 2004;24:299–326.

86. Rosenthal J, Lopez-Pazos E, Dowling NF, Pfeiffer CM, Mulinare J, Vellozzi C, Zhang M, Lavoie DJ, Molina R, et al. Folate and Vitamin B12 Deficiency Among Non-pregnant Women of Childbearing-Age in Guatemala 2009-2010: Prevalence and Identification of Vulnerable Populations. Matern Child Health J. 2015;19:2272–85.

87. Samuel TM, Duggan C, Thomas T, Bosch R, Rajendran R, Virtanen SM, Srinivasan K, Kurpad A V. Vitamin B(12) intake and status in early pregnancy among urban South Indian women. Ann Nutr Metab. 2013;62:113–22.

88. Muthayya S, Kurpad A V, Duggan CP, Bosch RJ, Dwarkanath P, Mhaskar A, Mhaskar R, Thomas A, Vaz M, et al. Low maternal vitamin B12 status is associated with intrauterine growth retardation in urban South Indians. Eur J Clin Nutr. 2006;60:791–801.

89. Carmel R, Sinow RM, Karnaze DS. Atypical cobalamin deficiency. Subtle biochemical evidence of deficiency is commonly demonstrable in patients without megaloblastic anemia and is often associated with protein-bound cobalamin malabsorption. J Lab Clin Med. 1987;109:454–63.

90. Carmel R, Sinow RM, Siegel ME, Samloff IM. Food cobalamin malabsorption occurs frequently in patients with unexplained low serum cobalamin levels. Arch Intern Med. 1988;148:1715–9.

91. Krikke GG, Grooten IJ, Vrijkotte T, van Eijsden M, Roseboom TJ, Painter RC. Vitamin B12 and folate status in early pregnancy and cardiometabolic risk factors in the offspring at age 5-6 years: findings from the ABCD multi-ethnic birth cohort. BJOG. 2016;123:384–92.

92. Bjørke-Monsen AL, Roth C, Magnus P, Midttun Ø, Nilsen RM, Reichborn-Kjennerud T, Stoltenberg C, Susser E, Vollset SE, Ueland PM. Maternal B vitamin status in pregnancy week 18 according to reported use of folic acid supplements. Mol Nutr Food Res. 2013;57:645–52.

93. Knight BA, Shields BM, Brook A, Hill A, Bhat DS, Hattersley AT, Yajnik CS. Lower Circulating

B12 Is Associated with Higher Obesity and Insulin Resistance during Pregnancy in a Non-Diabetic White British Population. PLoS One. 2015;10:e0135268.

94. Rogers LM, Boy E, Miller JW, Green R, Sabel JC, Allen LH. High prevalence of cobalamin deficiency in Guatemalan schoolchildren: associations with low plasma holotranscobalamin II and elevated serum methylmalonic acid and plasma homocysteine concentrations. Am J Clin Nutr. 2003;77:433–40.

95. Casterline JE, Allen LH, Ruel MT. Vitamin B-12 deficiency is very prevalent in lactating Guatemalan women and their infants at three months postpartum. J Nutr. 1997;127:1966–72.

96. Guerra-Shinohara EM, Morita OE, Peres S, Pagliusi RA, Sampaio Neto LF, D'Almeida V, Irazusta SP, Allen RH, Stabler SP. Low ratio of S-adenosylmethionine to S-adenosylhomocysteine is associated with vitamin deficiency in Brazilian pregnant women and newborns. Am J Clin Nutr. 2004;80:1312–21.

97. Hay G, Johnston C, Whitelaw A, Trygg K, Refsum H. Folate and cobalamin status in relation to breastfeeding and weaning in healthy infants. Am J Clin Nutr. 2008;88:105–14.

98. Adaikalakoteswari A, Vatish M, Lawson A, Wood C, Sivakumar K, McTernan PG, Webster C, Anderson N, Yajnik CS, et al. Low maternal vitamin B12 status is associated with lower cord blood HDL cholesterol in white Caucasians living in the UK. Nutrients. 2015;7:2401–14.

99. Visentin CE, Masih SP, Plumptre L, Schroder TH, Sohn K-J, Ly A, Lausman AY, Berger H, Croxford R, et al. Low Serum Vitamin B-12 Concentrations Are Prevalent in a Cohort of Pregnant Canadian Women. J Nutr. 2016;

100. Bergen NE, Jaddoe VW V, Timmermans S, Hofman A, Lindemans J, Russcher H, Raat H, Steegers-Theunissen RPM, Steegers EAP. Homocysteine and folate concentrations in early pregnancy and the risk of adverse pregnancy outcomes: the Generation R Study. BJOG. 2012;119:739–51.

101. Eneroth H, El Arifeen S, Persson L-A, Lönnerdal B, Hossain MB, Stephensen CB, Ekström E-C. Maternal multiple micronutrient supplementation has limited impact on micronutrient status of Bangladeshi infants compared with standard iron and folic acid supplementation. J Nutr. 2010;140:618–24.

102. Siddiqua TJ, Ahmad SM, Ahsan KB, Rashid M, Roy A, Rahman SM, Shahab-Ferdows S, Hampel D, Ahmed T, et al. Vitamin B12 supplementation during pregnancy and postpartum improves B12 status of both mothers and infants but vaccine response in mothers only: a randomized clinical trial in Bangladesh. Eur J Nutr. 2016;55:281–93.

103. Christian P, Jiang T, Khatry SK, LeClerq SC, Shrestha SR, West KP. Antenatal supplementation with micronutrients and biochemical indicators of status and subclinical infection in rural Nepal. Am J Clin Nutr. 2006;83:788–94.

104. Jones KM, Ramirez-Zea M, Zuleta C, Allen LH. Prevalent vitamin B-12 deficiency in twelve-

month-old Guatemalan infants is predicted by maternal B-12 deficiency and infant diet. J Nutr. 2007;137:1307–13.

105. Hay G, Clausen T, Whitelaw A, Trygg K, Johnston C, Henriksen T, Refsum H. Maternal folate and cobalamin status predicts vitamin status in newborns and 6-month-old infants. J Nutr. 2010;140:557–64.

106. Bhate V, Deshpande S, Bhat D, Joshi N, Ladkat R, Watve S, Fall C, de Jager CA, Refsum H, Yajnik C. Vitamin B12 status of pregnant Indian women and cognitive function in their 9-year-old children. Food Nutr Bull. 2008;29:249–54.

107. Hure AJ, Collins CE, Smith R. A longitudinal study of maternal folate and vitamin B12 status in pregnancy and postpartum, with the same infant markers at 6 months of age. Matern Child Health J. 2012;16:792–801.

108. Koletzko B, Mrotzek M, Bremer HJ. Fatty acid composition of mature human milk in Germany. Am J Clin Nutr. 1988;47:954–9.

109. Fewtrell MS, Morgan JB, Duggan C, Gunnlaugsson G, Hibberd PL, Lucas A, Kleinman RE. Optimal duration of exclusive breastfeeding: what is the evidence to support current recommendations? Am J Clin Nutr. 2007;85:635S – 638S.

110. NF B, MG L-A, C G. Nutrient adequacy of exclusive breast feeeding for the term infant during the first six months of life. Geneva; 2002.

111. Fréry N, Huel G, Leroy M, Moreau T, Savard R, Blot P, Lellouch J. Vitamin B12 among parturients and their newborns and its relationship with birthweight. Eur J Obstet Gynecol Reprod Biol. 1992;45:155–63.

112. Whitehead VM. Acquired and inherited disorders of cobalamin and folate in children. Br J Haematol. 2006;134:125–36.

113. González-Gross M, Benser J, Breidenassel C, Albers U, Huybrechts I, Valtueña J, Spinneker A, Segoviano M, Widhalm K, et al. Gender and age influence blood folate, vitamin B12, vitamin B6, and homocysteine levels in European adolescents: the Helena Study. Nutr Res. 2012;32:817–26.

114. Wright JD, Bialostosky K, Gunter EW, Carroll MD, Najjar MF, Bowman BA, Johnson CL. Blood folate and vitamin B12: United States, 1988-94. Vital Health Stat 11. 1998;1–78.

115. McNulty H, Eaton-Evans J, Cran G, Woulahan G, Boreham C, Savage JM, Fletcher R, Strain JJ. Nutrient intakes and impact of fortified breakfast cereals in schoolchildren. Arch Dis Child. 1996;75:474–81.

116. Gibson S. Micronutrient intakes, micronutrient status and lipid profiles among young people consuming different amounts of breakfast cereals: further analysis of data from the National Diet and Nutrition Survey of Young People aged 4 to 18 years. Public Health Nutr. 2003;6:815–20.

117. van den Boom A, Serra-Majem L, Ribas L, Ngo J, Pérez-Rodrigo C, Aranceta J, Fletcher R. The contribution of ready-to-eat cereals to daily nutrient intake and breakfast quality in a Mediterranean setting. J Am Coll Nutr. 2006;25:135–43.

118. Ortega RM, Mena MC, Faci M, Santana JF, Serra-Majem L. Vitamin status in different groups of the Spanish population: a meta-analysis of national studies performed between 1990 and 1999. Public Health Nutr. 2001;4:1325–9.

119. Pfeiffer CM, Hughes JP, Lacher DA, Bailey RL, Berry RJ, Zhang M, Yetley EA, Rader JI, Sempos CT, Johnson CL. Estimation of trends in serum and RBC folate in the U.S. population from pre- to postfortification using assay-adjusted data from the NHANES 1988-2010. J Nutr. 2012;142:886–93.

120. Kerr MA, Livingstone B, Bates CJ, Bradbury I, Scott JM, Ward M, Pentieva K, Mansoor MA, McNulty H. Folate, related B vitamins, and homocysteine in childhood and adolescence: potential implications for disease risk in later life. Pediatrics. 2009;123:627–35.

121. De Laet C, Wautrecht JC, Brasseur D, Dramaix M, Boeynaems JM, Decuyper J, Kahn A. Plasma homocysteine concentration in a Belgian school-age population. Am J Clin Nutr. 1999;69:968–72.

122. Papandreou D, Mavromichalis I, Makedou A, Rousso I, Arvanitidou M. Reference range of total serum homocysteine level and dietary indexes in healthy Greek schoolchildren aged 6-15 years. Br J Nutr. 2006;96:719–24.

123. Li N, Seetharam S, Lindemans J, Alpers DH, Arwert F, Seetharam B. Isolation and sequence analysis of variant forms of human transcobalamin II. Biochim Biophys Acta. 1993;1172:21–30.

124. Li N, Sood GK, Seetharam S, Seetharam B. Polymorphism of human transcobalamin II: substitution of proline and/or glutamine residues by arginine. Biochim Biophys Acta. 1994;1219:515–20.

125. Guéant J-L, Chabi NW, Guéant-Rodriguez R-M, Mutchinick OM, Debard R, Payet C, Lu X, Villaume C, Bronowicki J-P, et al. Environmental influence on the worldwide prevalence of a 776C->G variant in the transcobalamin gene (TCN2). J Med Genet. 2007;44:363–7.

126. Bowen RAR, Wong BYL, Cole DEC. Population-based differences in frequency of the transcobalamin II Pro259Arg polymorphism. Clin Biochem. 2004;37:128–33.

127. Namour F, Guy M, Aimone-Gastin I, de Nonancourt M, Mrabet N, Guéant JL. Isoelectrofocusing phenotype and relative concentration of transcobalamin II isoproteins related to the codon 259 Arg/Pro polymorphism. Biochem Biophys Res Commun. 1998;251:769–74.

128. Namour F, Olivier J, Abdelmouttaleb I, Adjalla C, Debard R, Salvat C, Guéant J. Transcobalamin codon 259 polymorphism in HT-29 and Caco-2 cells and in Caucasians: relation to transcobalamin and homocysteine concentration in blood. Blood. 2001;97:1092–

8.

129. Afman LA, Van Der Put NM, Thomas CM, Trijbels JM, Blom HJ. Reduced vitamin B12 binding by transcobalamin II increases the risk of neural tube defects. QJM. 2001;94:159–66.

130. von Castel-Dunwoody KM, Kauwell GPA, Shelnutt KP, Vaughn JD, Griffin ER, Maneval DR, Theriaque DW, Bailey LB. Transcobalamin 776C->G polymorphism negatively affects vitamin B-12 metabolism. Am J Clin Nutr. 2005;81:1436–41.

131. Castro R, Barroso M, Rocha M, Esse R, Ramos R, Ravasco P, Rivera I, de Almeida IT. The TCN2 776CNG polymorphism correlates with vitamin B(12) cellular delivery in healthy adult populations. Clin Biochem. 2010;43:645–9.

132. Miller JW, Ramos MI, Garrod MG, Flynn MA, Green R. Transcobalamin II 775G>C polymorphism and indices of vitamin B12 status in healthy older adults. Blood. American Society of Hematology; 2002;100:718–20.

133. Stanisławska-Sachadyn A, Woodside J V, Sayers CM, Yarnell JW, Young IS, Evans AE, Mitchell LE, Whitehead AS. The transcobalamin (TCN2) 776C>G polymorphism affects homocysteine concentrations among subjects with low vitamin B(12) status. Eur J Clin Nutr. 2010;64:1338–43.

134. Garrod MG, Allen LH, Haan MN, Green R, Miller JW. Transcobalamin C776G genotype modifies the association between vitamin B12 and homocysteine in older Hispanics. Eur J Clin Nutr. 2010;64:503–9.

135. Lievers KJA, Afman LA, Kluijtmans LAJ, Boers GHJ, Verhoef P, den Heijer M, Trijbels FJM, Blom HJ. Polymorphisms in the transcobalamin gene: association with plasma homocysteine in healthy individuals and vascular disease patients. Clin Chem. 2002;48:1383–9.

136. Aléssio ACM, Höehr NF, Siqueira LH, Bydlowski SP, Annichino-Bizzacchi JM. Polymorphism C776G in the transcobalamin II gene and homocysteine, folate and vitamin B12 concentrations. Association with MTHFR C677T and A1298C and MTRR A66G polymorphisms in healthy children. Thromb Res. 2007;119:571–7.

137. Olteanu H, Banerjee R. Human methionine synthase reductase, a soluble P-450 reductase-like dual flavoprotein, is sufficient for NADPH-dependent methionine synthase activation. J Biol Chem. 2001;276:35558–63.

138. Leclerc D, Wilson A, Dumas R, Gafuik C, Song D, Watkins D, Heng HH, Rommens JM, Scherer SW, et al. Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria. Proc Natl Acad Sci U S A. 1998;95:3059–64.

139. Yamada K, Gravel RA, Toraya T, Matthews RG. Human methionine synthase reductase is a molecular chaperone for human methionine synthase. Proc Natl Acad Sci U S A. 2006;103:9476–81.

140. Olteanu H, Munson T, Banerjee R. Differences in the efficiency of reductive activation of

methionine synthase and exogenous electron acceptors between the common polymorphic variants of human methionine synthase reductase. Biochemistry. 2002;41:13378–85.

141. Sharp L, Little J. Polymorphisms in genes involved in folate metabolism and colorectal neoplasia: a HuGE review. Am J Epidemiol. 2004;159:423–43.

142. Yang Q-H, Botto LD, Gallagher M, Friedman JM, Sanders CL, Koontz D, Nikolova S, Erickson JD, Steinberg K. Prevalence and effects of gene-gene and gene-nutrient interactions on serum folate and serum total homocysteine concentrations in the United States: findings from the third National Health and Nutrition Examination Survey DNA Bank. Am J Clin Nutr. 2008;88:232–46.

143. Fredriksen A, Meyer K, Ueland PM, Vollset SE, Grotmol T, Schneede J. Large-scale population-based metabolic phenotyping of thirteen genetic polymorphisms related to one-carbon metabolism. Hum Mutat. 2007;28:856–65.

144. Bueno O, Molloy AM, Fernandez-Ballart JD, García-Minguillán CJ, Ceruelo S, Ríos L, Ueland PM, Meyer K, Murphy MM. Common Polymorphisms That Affect Folate Transport or Metabolism Modify the Effect of the MTHFR 677C > T Polymorphism on Folate Status. J Nutr. 2015;

145. Vinukonda G, Shaik Mohammad N, Md Nurul Jain J, Prasad Chintakindi K, Rama Devi Akella R. Genetic and environmental influences on total plasma homocysteine and coronary artery disease (CAD) risk among South Indians. Clin Chim Acta. 2009;405:127–31.

146. Laraqui A, Allami A, Carrié A, Raisonnier A, Coiffard A-S, Benkouka F, Bendriss A, Benjouad A, Bennouar N, et al. Relation between plasma homocysteine, gene polymorphisms of homocysteine metabolism-related enzymes, and angiographically proven coronary artery disease. Eur J Intern Med. 2007;18:474–83.

147. Guéant-Rodriguez R-M, Juilliére Y, Candito M, Adjalla CE, Gibelin P, Herbeth B, Van Obberghen E, Gueánt J-L. Association of MTRRA66G polymorphism (but not of MTHFR C677T and A1298C, MTRA2756G, TCN C776G) with homocysteine and coronary artery disease in the French population. Thromb Haemost. 2005;94:510–5.

148. Gaughan DJ, Kluijtmans LA, Barbaux S, McMaster D, Young IS, Yarnell JW, Evans A, Whitehead AS. The methionine synthase reductase (MTRR) A66G polymorphism is a novel genetic determinant of plasma homocysteine concentrations. Atherosclerosis. 2001;157:451–6.

149. Nagele P, Meissner K, Francis A, Födinger M, Saccone NL. Genetic and environmental determinants of plasma total homocysteine levels: impact of population-wide folate fortification. Pharmacogenet Genomics. 2011;21:426–31.

150. Summers CM, Mitchell LE, Stanislawska-Sachadyn A, Baido SF, Blair IA, Von Feldt JM, Whitehead AS. Genetic and lifestyle variables associated with homocysteine concentrations and the distribution of folate derivatives in healthy premenopausal women. Birth Defects Res A Clin Mol Teratol. 2010;88:679–88.

151. van Driel LMJW, Eijkemans MJC, de Jonge R, de Vries JHM, van Meurs JBJ, Steegers EAP, Steegers-Theunissen RPM. Body mass index is an important determinant of methylation biomarkers in women of reproductive ages. J Nutr. 2009;139:2315–21.

152. Barbosa PR, Stabler SP, Machado ALK, Braga RC, Hirata RDC, Hirata MH, Sampaio-Neto LF, Allen RH, Guerra-Shinohara EM. Association between decreased vitamin levels and MTHFR, MTR and MTRR gene polymorphisms as determinants for elevated total homocysteine concentrations in pregnant women. Eur J Clin Nutr. 2008;62:1010–21.

153. Brouns R, Ursem N, Lindemans J, Hop W, Pluijm S, Steegers E, Steegers-Theunissen R. Polymorphisms in genes related to folate and cobalamin metabolism and the associations with complex birth defects. Prenat Diagn. 2008;28:485–93.

154. Feix A, Winkelmayer WC, Eberle C, Sunder-Plassmann G, Födinger M. Methionine synthase reductase MTRR 66A > G has no effect on total homocysteine, folate, and Vitamin B12 concentrations in renal transplant patients. Atherosclerosis. 2004;174:43–8.

155. Kluijtmans LAJ, Young IS, Boreham CA, Murray L, McMaster D, McNulty H, Strain JJ, McPartlin J, Scott JM, Whitehead AS. Genetic and nutritional factors contributing to hyperhomocysteinemia in young adults. Blood. 2003;101:2483–8.

156. García-Minguillán CJ, Fernandez-Ballart JD, Ceruelo S, Ríos L, Bueno O, Berrocal-Zaragoza MI, Molloy AM, Ueland PM, Meyer K, Murphy MM. Riboflavin status modifies the effects of methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) polymorphisms on homocysteine. Genes Nutr. 2014;9:435.

157. Gomes S, Lopes C, Pinto E. Folate and folic acid in the periconceptional period: recommendations from official health organizations in thirty-six countries worldwide and WHO. Public Health Nutr. 2015;1–14.

158. Ronnenberg AG, Goldman MB, Chen D, Aitken IW, Willett WC, Selhub J, Xu X. Preconception homocysteine and B vitamin status and birth outcomes in Chinese women. Am J Clin Nutr. 2002;76:1385–91.

159. Ronnenberg AG, Venners SA, Xu X, Chen C, Wang L, Guang W, Huang A, Wang X. Preconception B-vitamin and homocysteine status, conception, and early pregnancy loss. Am J Epidemiol. 2007;166:304–12.

160. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet. 2012;380:2095–128.

161. Swamy GK, Ostbye T, Skjaerven R. Association of preterm birth with long-term survival, reproduction, and next-generation preterm birth. JAMA. 2008;299:1429–36.

162. Ancel P-Y, Goffinet F, Kuhn P, Langer B, Matis J, Hernandorena X, Chabanier P, Joly-Pedespan L, Lecomte B, et al. Survival and Morbidity of Preterm Children Born at 22 Through 34 Weeks'

Gestation in France in 2011. JAMA Pediatr. 2015;169:230.

163. Hübner U, Alwan A, Jouma M, Tabbaa M, Schorr H, Herrmann W. Low serum vitamin B12 is associated with recurrent pregnancy loss in Syrian women. Clin Chem Lab Med. 2008;46:1265–9.

164. Haggarty P, McCallum H, McBain H, Andrews K, Duthie S, McNeill G, Templeton A, Haites N, Campbell D, Bhattacharya S. Effect of B vitamins and genetics on success of in-vitro fertilisation: prospective cohort study. Lancet (London, England). 2006;367:1513–9.

165. Takimoto H, Mito N, Umegaki K, Ishiwaki A, Kusama K, Abe S, Yamawaki M, Fukuoka H, Ohta C, Yoshiike N. Relationship between dietary folate intakes, maternal plasma total homocysteine and B-vitamins during pregnancy and fetal growth in Japan. Eur J Nutr. 2007;46:300–6.

166. Dwarkanath P, Barzilay JR, Thomas T, Thomas A, Bhat S, Kurpad A V. High folate and low vitamin B-12 intakes during pregnancy are associated with small-for-gestational age infants in South Indian women: a prospective observational cohort study. Am J Clin Nutr. 2013;98:1450–8.

167. Ma A-G, Chen X-C, Wang Y, Xu R-X, Zheng M-C, Li J-S. The multiple vitamin status of Chinese pregnant women with anemia and nonanemia in the last trimester. J Nutr Sci Vitaminol (Tokyo). 2004;50:87–92.

168. de Azevedo Paiva A, Rondó PHC, Guerra-Shinohara EM, Silva CS. The influence of iron, vitamin B(12), and folate levels on soluble transferrin receptor concentration in pregnant women. Clin Chim Acta. 2003;334:197–203.

169. Gibson RS, Abebe Y, Stabler S, Allen RH, Westcott JE, Stoecker BJ, Krebs NF, Hambidge KM. Zinc, gravida, infection, and iron, but not vitamin B-12 or folate status, predict hemoglobin during pregnancy in Southern Ethiopia. J Nutr. 2008;138:581–6.

170. Shields RC, Caric V, Hair M, Jones O, Wark L, McColl MD, Ramsay JE. Pregnancy-specific reference ranges for haematological variables in a Scottish population. J Obstet Gynaecol. 2011;31:286–9.

171. Cherian A, Seena S, Bullock RK, Antony AC. Incidence of neural tube defects in the least-developed area of India: a population-based study. Lancet (London, England). 2005;366:930–1.

172. Yajnik CS, Deshpande SS, Lubree HG, Naik SS, Bhat DS, Uradey BS, Deshpande JA, Rege SS, Refsum H, Yudkin JS. Vitamin B12 deficiency and hyperhomocysteinemia in rural and urban Indians. J Assoc Physicians India. 2006;54:775–82.

173. Refsum H, Yajnik CS, Gadkari M, Schneede J, Vollset SE, Orning L, Guttormsen AB, Joglekar A, Sayyad MG, et al. Hyperhomocysteinemia and elevated methylmalonic acid indicate a high prevalence of cobalamin deficiency in Asian Indians. Am J Clin Nutr. 2001;74:233–41.

174. World Health Organization. Optimal serum and red blood cell folate concentrations in women of reproductive age for prevention of neural tube defects. World Health Organization; 2015;

175. Swanson DA, Pangilinan F, Mills JL, Kirke PN, Conley M, Weiler A, Frey T, Parle-McDermott A, O'Leary VB, et al. Evaluation of transcobalamin II polymorphisms as neural tube defect risk factors in an Irish population. Birth Defects Res A Clin Mol Teratol. 2005;73:239–44.

176. Guéant-Rodriguez RM, Rendeli C, Namour B, Venuti L, Romano A, Anello G, Bosco P, Debard R, Gérard P, et al. Transcobalamin and methionine synthase reductase mutated polymorphisms aggravate the risk of neural tube defects in humans. Neurosci Lett. 2003;344:189–92.

177. Biselli JM, Brumati D, Frigeri VF, Zampieri BL, Goloni-Bertollo EM, Pavarino-Bertelli EC. A80G polymorphism of reduced folate carrier 1 (RFC1) and C776G polymorphism of transcobalamin 2 (TC2) genes in Down's syndrome etiology. São Paulo Med J = Rev Paul Med. 2008;126:329–32.

178. Zampieri BL, Biselli JM, Goloni-Bertollo EM, Vannucchi H, Carvalho VM, Cordeiro JA, Pavarino EC. Maternal risk for Down syndrome is modulated by genes involved in folate metabolism. Dis Markers. 2012;32:73–81.

179. Zetterberg H, Regland B, Palmér M, Rymo L, Zafiropoulos A, Arvanitis DA, Spandidos DA, Blennow K. The transcobalamin codon 259 polymorphism influences the risk of human spontaneous abortion. Hum Reprod. 2002;17:3033–6.

180. Zetterberg H, Zafiropoulos A, Spandidos DA, Rymo L, Blennow K. Gene-gene interaction between fetal MTHFR 677C>T and transcobalamin 776C>G polymorphisms in human spontaneous abortion. Hum Reprod. 2003;18:1948–50.

181. Wilson A, Platt R, Wu Q, Leclerc D, Christensen B, Yang H, Gravel RA, Rozen R. A common variant in methionine synthase reductase combined with low cobalamin (vitamin B12) increases risk for spina bifida. Mol Genet Metab. 1999;67:317–23.

182. van der Linden IJM, den Heijer M, Afman LA, Gellekink H, Vermeulen SHHM, Kluijtmans LAJ, Blom HJ. The methionine synthase reductase 66A>G polymorphism is a maternal risk factor for spina bifida. J Mol Med (Berl). 2006;84:1047–54.

183. O'Leary VB, Mills JL, Pangilinan F, Kirke PN, Cox C, Conley M, Weiler A, Peng K, Shane B, et al. Analysis of methionine synthase reductase polymorphisms for neural tube defects risk association. Mol Genet Metab. 2005;85:220–7.

184. Santos-Rebouças CB, Corrêa JC, Bonomo A, Fintelman-Rodrigues N, Moura KC V, Rodrigues CSC, Santos JM, Pimentel MMG. The impact of folate pathway polymorphisms combined to nutritional deficiency as a maternal predisposition factor for Down syndrome. Dis Markers. 2008;25:149–57.

185. Brandalize APC, Bandinelli E, Borba JB, Félix TM, Roisenberg I, Schüler-Faccini L.

Polymorphisms in genes MTHFR, MTR and MTRR are not risk factors for cleft lip/palate in South Brazil. Brazilian J Med Biol Res = Rev Bras Pesqui médicas e biológicas / Soc Bras Biofísica . [et al]. 2007;40:787–91.

186. Shaw GM, Lu W, Zhu H, Yang W, Briggs FBS, Carmichael SL, Barcellos LF, Lammer EJ, Finnell RH. 118 SNPs of folate-related genes and risks of spina bifida and conotruncal heart defects. BMC Med Genet. 2009;10:49.

187. Candito M, Rivet R, Herbeth B, Boisson C, Rudigoz R-C, Luton D, Journel H, Oury J-F, Roux F, et al. Nutritional and genetic determinants of vitamin B and homocysteine metabolisms in neural tube defects: a multicenter case-control study. Am J Med Genet A. 2008;146A:1128–33.

188. Verkleij-Hagoort AC, van Driel LMJW, Lindemans J, Isaacs A, Steegers EAP, Helbing WA, Uitterlinden AG, Steegers-Theunissen RPM. Genetic and lifestyle factors related to the periconception vitamin B12 status and congenital heart defects: a Dutch case-control study. Mol Genet Metab. 2008;94:112–9.

189. Cai B, Zhang T, Zhong R, Zou L, Zhu B, Chen W, Shen N, Ke J, Lou J, et al. Genetic variant in MTRR, but not MTR, is associated with risk of congenital heart disease: an integrated meta-analysis. PLoS One. 2014;9:e89609.

190. Amorim MR, Lima MAC. MTRR 66A>G polymorphism as maternal risk factor for Down syndrome: a meta-analysis. Genet Test Mol Biomarkers. 2013;17:69–73.

191. Engel SM, Olshan AF, Siega-Riz AM, Savitz DA, Chanock SJ. Polymorphisms in folate metabolizing genes and risk for spontaneous preterm and small-for-gestational age birth. Am J Obstet Gynecol. 2006;195:1231.e1–11.

192. Bokor S, Meirhaeghe A, Ruiz JR, Zaccaria M, Widhalm K, Gonzalez-Gross M, Amouyel P, Moreno LA, Molnàr D, Dallongeville J. Common polymorphisms in six genes of the methyl group metabolism pathway and obesity in European adolescents. Int J Pediatr Obes. 2011;6:e336–44.

193. Matherly LH, Czajkowski CA, Muench SP, Psiakis JT. Role for cytosolic folate-binding proteins in the compartmentation of endogenous tetrahydrofolates and the 5-formyl tetrahydrofolate-mediated enhancement of 5-fluoro-2'-deoxyuridine antitumor activity in vitro. Cancer Res. 1990;50:3262–9.

194. Scott JM, Weir DG. The methyl folate trap. A physiological response in man to prevent methyl group deficiency in kwashiorkor (methionine deficiency) and an explanation for folic-acid induced exacerbation of subacute combined degeneration in pernicious anaemia. Lancet. 1981;2:337–40.

195. Field MS, Kamynina E, Agunloye OC, Liebenthal RP, Lamarre SG, Brosnan ME, Brosnan JT, Stover PJ. Nuclear enrichment of folate cofactors and methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) protect de novo thymidylate biosynthesis during folate deficiency. J Biol Chem. 2014;289:29642–50.

196. Konings EJ, Roomans HH, Dorant E, Goldbohm RA, Saris WH, van den Brandt PA. Folate intake of the Dutch population according to newly established liquid chromatography data for foods. Am J Clin Nutr. 2001;73:765–76.

197. Nguyen MT, Indrawati, Hendrickx M. Model studies on the stability of folic acid and 5-methyltetrahydrofolic acid degradation during thermal treatment in combination with high hydrostatic pressure. J Agric Food Chem. 2003;51:3352–7.

198. Food and Drug Administration. Food standards: amendment of standards of identity for enriched grain products to require addition of folic acid. Federal Register. 1996.

199. Canada Gazette. Canada Gazette Part II. Food and drugs regulation SOR/96-527. Canada Gaz. 1996;130.

200. Yang Q, Cogswell ME, Hamner HC, Carriquiry A, Bailey LB, Pfeiffer CM, Berry RJ. Folic acid source, usual intake, and folate and vitamin B-12 status in US adults: National Health and Nutrition Examination Survey (NHANES) 2003-2006. Am J Clin Nutr. 2010;91:64–72.

201. Melse-Boonstra A, de Bree A, Verhoef P, Bjørke-Monsen AL, Verschuren WMM. Dietary monoglutamate and polyglutamate folate are associated with plasma folate concentrations in Dutch men and women aged 20-65 years. J Nutr. 2002;132:1307–12.

202. Subar AF, Block G, James LD. Folate intake and food sources in the US population. Am J Clin Nutr. 1989;50:508–16.

203. Brouwer IA, van Dusseldorp M, West CE, Meyboom S, Thomas CM, Duran M, van het Hof KH, Eskes TK, Hautvast JG, Steegers-Theunissen RP. Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. J Nutr. 1999;129:1135–9.

204. Hannon-Fletcher MP, Armstrong NC, Scott JM, Pentieva K, Bradbury I, Ward M, Strain JJ, Dunn AA, Molloy AM, et al. Determining bioavailability of food folates in a controlled intervention study. Am J Clin Nutr. 2004;80:911–8.

205. Yang TL, Hung J, Caudill MA, Urrutia TF, Alamilla A, Perry CA, Li R, Hata H, Cogger EA. A long-term controlled folate feeding study in young women supports the validity of the 1.7 multiplier in the dietary folate equivalency equation. J Nutr. 2005;135:1139–45.

206. Winkels RM, Brouwer IA, Siebelink E, Katan MB, Verhoef P. Bioavailability of food folates is 80% of that of folic acid. Am J Clin Nutr. 2007;85:465–73.

207. Sauberlich HE, Kretsch MJ, Skala JH, Johnson HL, Taylor PC. Folate requirement and metabolism in nonpregnant women. Am J Clin Nutr. 1987;46:1016–28.

208. McNulty H, Pentieva K. Folate bioavailability. In: Bailey LB, editor. Folate in Health and disease. CRC Press Taylor and Francis Group; 2010. p. 25–48.

209. Pfeiffer CM, Rogers LM, Bailey LB, Gregory JF. Absorption of folate from fortified cereal-grain

products and of supplemental folate consumed with or without food determined by using a dual-label stable-isotope protocol. Am J Clin Nutr. 1997;66:1388–97.

210. Bailey LB. Dietary reference intakes for folate: the debut of dietary folate equivalents. Nutr Rev. 1998;56:294–9.

211. Selhub J, Rosenberg IH. Folate transport in isolated brush border membrane vesicles from rat intestine. J Biol Chem. 1981;256:4489–93.

212. Qiu A, Jansen M, Sakaris A, Min SH, Chattopadhyay S, Tsai E, Sandoval C, Zhao R, Akabas MH, Goldman ID. Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. Cell. 2006;127:917–28.

213. Zhao R, Matherly LH, Goldman ID. Membrane transporters and folate homeostasis: intestinal absorption and transport into systemic compartments and tissues. Expert Rev Mol Med. 2009;11:e4.

214. Bailey SW, Ayling JE. The extremely slow and variable activity of dihydrofolate reductase in human liver and its implications for high folic acid intake. Proc Natl Acad Sci U S A. 2009;106:15424–9.

215. Boilson A, Staines A, Kelleher CC, Daly L, Shirley I, Shrivastava A, Bailey SW, Alverson PB, Ayling JE, et al. Unmetabolized folic acid prevalence is widespread in the older Irish population despite the lack of a mandatory fortification program. Am J Clin Nutr. 2012;96:613–21.

216. Obeid R, Kirsch SH, Kasoha M, Eckert R, Herrmann W. Concentrations of unmetabolized folic acid and primary folate forms in plasma after folic acid treatment in older adults. Metabolism. 2011;60:673–80.

217. Pfeiffer CM, Sternberg MR, Fazili Z, Lacher DA, Zhang M, Johnson CL, Hamner HC, Bailey RL, Rader JI, et al. Folate status and concentrations of serum folate forms in the US population: National Health and Nutrition Examination Survey 2011-2. Br J Nutr. 2015;113:1965–77.

218. Pentieva K, Selhub J, Paul L, Molloy AM, McNulty B, Ward M, Marshall B, Dornan J, Reilly R, et al. Evidence from a Randomized Trial That Exposure to Supplemental Folic Acid at Recommended Levels during Pregnancy Does Not Lead to Increased Unmetabolized Folic Acid Concentrations in Maternal or Cord Blood. J Nutr. 2016;146:494–500.

219. Stark M, Rothem L, Jansen G, Scheffer GL, Goldman ID, Assaraf YG. Antifolate resistance associated with loss of MRP1 expression and function in Chinese hamster ovary cells with markedly impaired export of folate and cholate. Mol Pharmacol. 2003;64:220–7.

220. Whetstine JR, Flatley RM, Matherly LH. The human reduced folate carrier gene is ubiquitously and differentially expressed in normal human tissues: identification of seven non-coding exons and characterization of a novel promoter. Biochem J. 2002;367:629–40.

221. Ratnam M, Marquardt H, Duhring JL, Freisheim JH. Homologous membrane folate binding

proteins in human placenta: cloning and sequence of a cDNA. Biochemistry. 1989;28:8249–54.

222. Maddox DM, Manlapat A, Roon P, Prasad P, Ganapathy V, Smith SB. Reduced-folate carrier (RFC) is expressed in placenta and yolk sac, as well as in cells of the developing forebrain, hindbrain, neural tube, craniofacial region, eye, limb buds and heart. BMC Dev Biol. 2003;3:6.

223. Weitman SD, Weinberg AG, Coney LR, Zurawski VR, Jennings DS, Kamen BA. Cellular localization of the folate receptor: potential role in drug toxicity and folate homeostasis. Cancer Res. 1992;52:6708–11.

224. Shane B, Garrow T, Brenner A, Chen L, Choi YJ, Hsu JC, Stover P. Folylpoly-gamma-glutamate synthetase. Adv Exp Med Biol. 1993;338:629–34.

225. Lowe KE, Osborne CB, Lin BF, Kim JS, Hsu JC, Shane B. Regulation of folate and one-carbon metabolism in mammalian cells. II. Effect of folylpoly-gamma-glutamate synthetase substrate specificity and level on folate metabolism and folylpoly-gamma-glutamate specificity of metabolic cycles of one-carbon metaboli. J Biol Chem. 1993;268:21665–73.

226. Schirch V, Strong WB. Interaction of folylpolyglutamates with enzymes in one-carbon metabolism. Arch Biochem Biophys. 1989;269:371–80.

227. HERBERT V, ZALUSKY R. Interrelations of vitamin B12 and folic acid metabolism: folic acid clearance studies. J Clin Invest. 1962;41:1263–76.

228. Cichowicz DJ, Shane B. Mammalian folylpoly-gamma-glutamate synthetase. 2. Substrate specificity and kinetic properties. Biochemistry. 1987;26:513–21.

229. Cook JD, Cichowicz DJ, George S, Lawler A, Shane B. Mammalian folylpoly-gamma-glutamate synthetase. 4. In vitro and in vivo metabolism of folates and analogues and regulation of folate homeostasis. Biochemistry. 1987;26:530–9.

230. Fort DW, Lark RH, Smith AK, Marling-Cason M, Weitman SD, Shane B, Kamen BA. Accumulation of 5-methyltetrahydrofolic acid and folylpolyglutamate synthetase expression by mitogen stimulated human lymphocytes. Br J Haematol. 1993;84:595–601.

231. Green JM, Ballou DP, Matthews RG. Examination of the role of methylenetetrahydrofolate reductase in incorporation of methyltetrahydrofolate into cellular metabolism. FASEB J. 1988;2:42–7.

232. Friso S, Choi S-W, Girelli D, Mason JB, Dolnikowski GG, Bagley PJ, Olivieri O, Jacques PF, Rosenberg IH, et al. A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. Proc Natl Acad Sci. 2002;99:5606–11.

233. McLean E, de Benoist B, Allen LH. Review of the magnitude of folate and vitamin B12 deficiencies worldwide. Food Nutr Bull. 2008;29:S38–51.

234. Chango A, Potier De Courcy G, Boisson F, Guilland JC, Barbé F, Perrin MO, Christidès JP, Rabhi K, Pfister M, et al. 5,10-methylenetetrahydrofolate reductase common mutations, folate status and plasma homocysteine in healthy French adults of the Supplementation en Vitamines et Mineraux Antioxydants (SU.VI.MAX) cohort. Br J Nutr. 2000;84:891–6.

235. Herbert V. Making sense of laboratory tests of folate status: folate requirements to sustain normality. Am J Hematol. 1987;26:199–207.

236. Selhub J, Jacques PF, Dallal G, Choumenkovitch S, Rogers G. The use of blood concentrations of vitamins and their respective functional indicators to define folate and vitamin B12 status. Food Nutr Bull. 2008;29:S67–73.

237. de Benoist B. Conclusions of a WHO Technical Consultation on folate and vitamin B12 deficiencies. Food Nutr Bull. 2008;29:S238–44.

238. Daly LE, Kirke PN, Molloy A, Weir DG, Scott JM. Folate levels and neural tube defects. Implications for prevention. JAMA. 1995;274:1698–702.

239. Crider KS, Devine O, Hao L, Dowling NF, Li S, Molloy AM, Li Z, Zhu J, Berry RJ. Population red blood cell folate concentrations for prevention of neural tube defects: Bayesian model. BMJ. 2014;349:g4554.

240. Tinker SC, Hamner HC, Qi YP, Crider KS. U.S. women of childbearing age who are at possible increased risk of a neural tube defect-affected pregnancy due to suboptimal red blood cell folate concentrations, National Health and Nutrition Examination Survey 2007 to 2012. Birth Defects Res A Clin Mol Teratol. 2015;103:517–26.

241. Tamura T, Picciano MF. Folate and human reproduction. Am J Clin Nutr. 2006;83:993–1016.

242. Velzing-Aarts F V, Holm PI, Fokkema MR, van der Dijs FP, Ueland PM, Muskiet FA. Plasma choline and betaine and their relation to plasma homocysteine in normal pregnancy. Am J Clin Nutr. 2005;81:1383–9.

243. Ubeda N, Reyes L, González-Medina A, Alonso-Aperte E, Varela-Moreiras G. Physiologic changes in homocysteine metabolism in pregnancy: a longitudinal study in Spain. Nutrition. 2011;27:925–30.

244. Fernàndez-Roig S, Cavallé-Busquets P, Fernandez-Ballart JD, Ballesteros M, Berrocal-Zaragoza MI, Salat-Batlle J, Ueland PM, Murphy MM. Low folate status enhances pregnancy changes in plasma betaine and dimethylglycine concentrations and the association between betaine and homocysteine. Am J Clin Nutr. 2013;97:1252–9.

245. Kim KN, Kim YJ, Chang N. Effects of the interaction between the C677T 5,10-methylenetetrahydrofolate reductase polymorphism and serum B vitamins on homocysteine levels in pregnant women. Eur J Clin Nutr. 2004;58:10–6.

246. Chanarin I, Rothman D, Ward A, Perry J. Folate status and requirement in pregnancy. Br Med J. 1968;2:390–4.

247. Ek J, Magnus EM. Plasma and red blood cell folate during normal pregnancies. Acta Obstet Gynecol Scand. 1981;60:247–51.

248. Cikot RJ, Steegers-Theunissen RP, Thomas CM, de Boo TM, Merkus HM, Steegers EA. Longitudinal vitamin and homocysteine levels in normal pregnancy. Br J Nutr. 2001;85:49–58.

249. McNulty B, McNulty H, Marshall B, Ward M, Molloy AM, Scott JM, Dornan J, Pentieva K. Impact of continuing folic acid after the first trimester of pregnancy: findings of a randomized trial of Folic Acid Supplementation in the Second and Third Trimesters. Am J Clin Nutr. 2013;98:92–8.

250. Murphy MM, Scott JM, McPartlin JM, Fernandez-Ballart JD. The pregnancy-related decrease in fasting plasma homocysteine is not explained by folic acid supplementation, hemodilution, or a decrease in albumin in a longitudinal study. Am J Clin Nutr. 2002;76:614–9.

251. Allen LH, Rosado JL, Casterline JE, López P, Muñoz E, Garcia OP, Martinez H. Lack of hemoglobin response to iron supplementation in anemic mexican preschoolers with multiple micronutrient deficiencies. Am J Clin Nutr. 2000;71:1485–94.

252. Siekmann JH, Allen LH, Bwibo NO, Demment MW, Murphy SP, Neumann CG. Kenyan school children have multiple micronutrient deficiencies, but increased plasma vitamin B-12 is the only detectable micronutrient response to meat or milk supplementation. J Nutr. 2003;133:3972S – 3980S.

253. Allen LH, Rosado JL, Casterline JE, Martinez H, Lopez P, Muñoz E, Black AK. Vitamin B-12 deficiency and malabsorption are highly prevalent in rural Mexican communities. Am J Clin Nutr. 1995;62:1013–9.

254. Centers for Disease Control and Prevention. Knowledge and use of folic acid among women of reproductive age--Michigan, 1998. MMWR Morb Mortal Wkly Rep. 2001;50:185–9.

255. Flour Fortification Initiative. Flour Fortification Initiative home page [Internet]. 2015 [cited 2011 May 20]. Available from: http://www.ffinetwork.org/about/stay_informed/releases/2014Review.html

256. Jacques PF, Selhub J, Bostom AG, Wilson PW, Rosenberg IH. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. N Engl J Med. 1999;340:1449–54.

257. Ray JG. Folic acid food fortification in Canada. Nutr Rev. 2004;62:S35–9.

258. Hertrampf E, Cortés F. Folic acid fortification of wheat flour: Chile. Nutr Rev. 2004;62:S44–8; discussion S49.

259. Hoey L, McNulty H, Askin N, Dunne A, Ward M, Pentieva K, Strain J, Molloy AM, Flynn CA, Scott JM. Effect of a voluntary food fortification policy on folate, related B vitamin status, and homocysteine in healthy adults. Am J Clin Nutr. 2007;86:1405–13.

260. Belcastro V, Striano P. Antiepileptic drugs, hyperhomocysteinemia and B-vitamins supplementation in patients with epilepsy. Epilepsy Res. 2012;102:1–7.

261. Linnebank M, Moskau S, Semmler A, Widman G, Stoffel-Wagner B, Weller M, Elger CE. Antiepileptic drugs interact with folate and vitamin B12 serum levels. Ann Neurol. 2011;69:352–9.

262. Stabler SP. Clinical folate deficiency. In: Bailey LB, editor. Folate in Health and disease. Second. CRC Press Taylor and Francis Group; 2010.

263. Dierkes J, Luley C, Westphal S. Effect of lipid-lowering and anti-hypertensive drugs on plasma homocysteine levels. Vasc Health Risk Manag. 2007;3:99–108.

264. Tinker SC, Hamner HC, Berry RJ, Bailey LB, Pfeiffer CM. Does obesity modify the association of supplemental folic acid with folate status among nonpregnant women of childbearing age in the United States? Birth Defects Res A Clin Mol Teratol. 2012;94:749–55.

265. Huemer M, Vonblon K, Födinger M, Krumpholz R, Hubmann M, Ulmer H, Simma B. Total homocysteine, folate, and cobalamin, and their relation to genetic polymorphisms, lifestyle and body mass index in healthy children and adolescents. Pediatr Res. 2006;60:764–9.

266. Pfeiffer CM, Sternberg MR, Schleicher RL, Rybak ME. Dietary supplement use and smoking are important correlates of biomarkers of water-soluble vitamin status after adjusting for sociodemographic and lifestyle variables in a representative sample of U.S. adults. J Nutr. 2013;143:957S – 65S.

267. Bostom AG, Lathrop L. Hyperhomocysteinemia in end-stage renal disease: prevalence, etiology, and potential relationship to arteriosclerotic outcomes. Kidney Int. 1997;52:10–20.

268. Nygård O, Refsum H, Ueland PM, Vollset SE. Major lifestyle determinants of plasma total homocysteine distribution: the Hordaland Homocysteine Study. Am J Clin Nutr. 1998;67:263–70.

269. Nakazato M, Maeda T, Takamura N, Wada M, Yamasaki H, Johnston KE, Tamura T. Relation of body mass index to blood folate and total homocysteine concentrations in Japanese adults. Eur J Nutr. 2011;50:581–5.

270. Kimmons JE, Blanck HM, Tohill BC, Zhang J, Khan LK. Associations between body mass index and the prevalence of low micronutrient levels among US adults. MedGenMed. 2006;8:59.

271. Mojtabai R. Body mass index and serum folate in childbearing age women. Eur J Epidemiol. 2004;19:1029–36.

272. da Silva VR, Hausman DB, Kauwell GPA, Sokolow A, Tackett RL, Rathbun SL, Bailey LB. Obesity affects short-term folate pharmacokinetics in women of childbearing age. Int J Obes (Lond). 2013;37:1608–10.

273. Bird JK, Ronnenberg AG, Choi S-W, Du F, Mason JB, Liu Z. Obesity is associated with increased

red blood cell folate despite lower dietary intakes and serum concentrations. J Nutr. 2015;145:79–86.

274. Gunanti IR, Marks GC, Al-Mamun A, Long KZ. Low serum vitamin B-12 and folate concentrations and low thiamin and riboflavin intakes are inversely associated with greater adiposity in Mexican American children. J Nutr. 2014;144:2027–33.

275. Kant AK. Interaction of body mass index and attempt to lose weight in a national sample of US adults: association with reported food and nutrient intake, and biomarkers. Eur J Clin Nutr. 2003;57:249–59.

276. Cho SJ, Lee HA, Park BH, Ha EH, Kim YJ, Park EA, Park H. Combined effect of folate and adiposity on homocysteine in children at three years of age. Nutr Res Pract. 2016;10:74–80.

277. O'Connor DL, Green T, Picciano MF. Maternal folate status and lactation. J Mammary Gland Biol Neoplasia. 1997;2:279–89.

278. Colman N, Hettiarachchy N, Herbert V. Detection of a milk factor that facilitates folate uptake by intestinal cells. Science. 1981;211:1427–9.

279. Delvin EE, Rozen R, Merouani A, Genest J, Lambert M. Influence of methylenetetrahydrofolate reductase genotype, age, vitamin B-12, and folate status on plasma homocysteine in children. Am J Clin Nutr. 2000;72:1469–73.

280. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet. 1995;10:111–3.

281. Wilcken B, Bamforth F, Li Z, Zhu H, Ritvanen A, Renlund M, Stoll C, Alembik Y, Dott B, et al. Geographical and ethnic variation of the 677C>T allele of 5,10 methylenetetrahydrofolate reductase (MTHFR): findings from over 7000 newborns from 16 areas world wide. J Med Genet. 2003;40:619–25.

282. Tsang BL, Devine OJ, Cordero AM, Marchetta CM, Mulinare J, Mersereau P, Guo J, Qi YP, Berry RJ, et al. Assessing the association between the methylenetetrahydrofolate reductase (MTHFR) 677C>T polymorphism and blood folate concentrations: a systematic review and meta-analysis of trials and observational studies. Am J Clin Nutr. 2015;101:1286–94.

283. van Beynum IM, den Heijer M, Thomas CMG, Afman L, Oppenraay-van Emmerzaal D, Blom HJ. Total homocysteine and its predictors in Dutch children. Am J Clin Nutr. 2005;81:1110–6.

284. Papoutsakis C, Yiannakouris N, Manios Y, Papaconstantinou E, Magkos F, Schulpis KH, Zampelas A, Matalas AL. Plasma homocysteine concentrations in Greek children are influenced by an interaction between the methylenetetrahydrofolate reductase C677T genotype and folate status. J Nutr. 2005;135:383–8.

285. Papoutsakis C, Yiannakouris N, Manios Y, Papaconstantinou E, Magkos F, Schulpis KH, Zampelas A, Matalas AL. The effect of MTHFR(C677T) genotype on plasma homocysteine

concentrations in healthy children is influenced by gender. Eur J Clin Nutr. 2006;60:155–62.

286. Kato I, Dnistrian AM, Schwartz M, Toniolo P, Koenig K, Shore RE, Akhmedkhanov A, Zeleniuch-Jacquotte A, Riboli E. Serum folate, homocysteine and colorectal cancer risk in women: a nested case-control study. Br J Cancer. 1999;79:1917–22.

287. Collin SM, Metcalfe C, Refsum H, Lewis SJ, Zuccolo L, Smith GD, Chen L, Harris R, Davis M, et al. Circulating folate, vitamin B12, homocysteine, vitamin B12 transport proteins, and risk of prostate cancer: a case-control study, systematic review, and meta-analysis. Cancer Epidemiol Biomarkers Prev. 2010;19:1632–42.

288. Kennedy DA, Stern SJ, Moretti M, Matok I, Sarkar M, Nickel C, Koren G. Folate intake and the risk of colorectal cancer: a systematic review and meta-analysis. Cancer Epidemiol. 2011;35:2–10.

289. Xu X, Chen J. One-carbon metabolism and breast cancer: an epidemiological perspective. J Genet Genomics. 2009;36:203–14.

290. Tower RL, Spector LG. The epidemiology of childhood leukemia with a focus on birth weight and diet. Crit Rev Clin Lab Sci. 2007;44:203–42.

291. Mason JB, Dickstein A, Jacques PF, Haggarty P, Selhub J, Dallal G, Rosenberg IH. A temporal association between folic acid fortification and an increase in colorectal cancer rates may be illuminating important biological principles: a hypothesis. Cancer Epidemiol Biomarkers Prev. 2007;16:1325–9.

292. Vollset SE, Clarke R, Lewington S, Ebbing M, Halsey J, Lonn E, Armitage J, Manson JE, Hankey GJ, et al. Effects of folic acid supplementation on overall and site-specific cancer incidence during the randomised trials: meta-analyses of data on 50,000 individuals. Lancet (London, England). 2013;381:1029–36.

293. Bailey LB, Stover PJ, McNulty H, Fenech MF, Gregory JF, Mills JL, Pfeiffer CM, Fazili Z, Zhang M, et al. Biomarkers of Nutrition for Development-Folate Review. J Nutr. 2015;145:1636S – 80S.

294. Smith AD, Smith SM, de Jager CA, Whitbread P, Johnston C, Agacinski G, Oulhaj A, Bradley KM, Jacoby R, Refsum H. Homocysteine-lowering by B vitamins slows the rate of accelerated brain atrophy in mild cognitive impairment: a randomized controlled trial. PLoS One. 2010;5:e12244.

295. Jager CA, Oulhaj A, Jacoby R, Refsum H, Smith AD. Cognitive and clinical outcomes of homocysteine-lowering B-vitamin treatment in mild cognitive impairment: a randomized controlled trial. Int J Geriatr Psychiatry. 2012;27:592–600.

296. Malouf R, Grimley Evans J. Folic acid with or without vitamin B12 for the prevention and treatment of healthy elderly and demented people. Cochrane database Syst Rev. 2008;CD004514.

297. Clarke R, Bennett D, Parish S, Lewington S, Skeaff M, Eussen SJPM, Lewerin C, Stott DJ, Armitage J, et al. Effects of homocysteine lowering with B vitamins on cognitive aging: meta-analysis of 11 trials with cognitive data on 22,000 individuals. Am J Clin Nutr. 2014;100:657–66.

298. Khoshnood B, Loane M, Walle H de, Arriola L, Addor M-C, Barisic I, Beres J, Bianchi F, Dias C, et al. Long term trends in prevalence of neural tube defects in Europe: population based study. BMJ. 2015;351:h5949.

299. De-Regil LM, Peña-Rosas JP, Fernández-Gaxiola AC, Rayco-Solon P. Effects and safety of periconceptional oral folate supplementation for preventing birth defects. Cochrane database Syst Rev. 2015;12:CD007950.

300. Lassi ZS, Salam RA, Haider BA, Bhutta ZA. Folic acid supplementation during pregnancy for maternal health and pregnancy outcomes. Cochrane database Syst Rev. 2013;3:CD006896.

301. Dodds L, Fell DB, Dooley KC, Armson BA, Allen AC, Nassar BA, Perkins S, Joseph KS. Effect of homocysteine concentration in early pregnancy on gestational hypertensive disorders and other pregnancy outcomes. Clin Chem. 2008;54:326–34.

302. Lee HA, Park EA, Cho SJ, Kim HS, Kim YJ, Lee H, Gwak HS, Kim KN, Chang N, et al. Mendelian randomization analysis of the effect of maternal homocysteine during pregnancy, as represented by maternal MTHFR C677T genotype, on birth weight. J Epidemiol. 2013;23:371–5.

303. Cavallé-Busquets P. Contribució de l'estat nutricional matern i de la funció placentària sobre el desenvolupament del retard de creixement intrauterí. Universitat Rovira i Virgili; 2013.

304. Botto LD, Yang Q. 5,10-Methylenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review. Am J Epidemiol. 2000;151:862–77.

305. Yin M, Dong L, Zheng J, Zhang H, Liu J, Xu Z. Meta analysis of the association between MTHFR C677T polymorphism and the risk of congenital heart defects. Ann Hum Genet. 2012;76:9–16.

306. Wang X, Wu H, Qiu X. Methylenetetrahydrofolate reductase (MTHFR) gene C677T polymorphism and risk of preeclampsia: an updated meta-analysis based on 51 studies. Arch Med Res. 2013;44:159–68.

307. Chen J, Chen L, Zhu L-H, Zhang S-T, Wu Y-L. Association of methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism with preterm delivery and placental abruption: a systematic review and meta-analysis. Acta Obstet Gynecol Scand. 2016;95:157–65.

308. Ganji V, Kafai MR. Hemoglobin and hematocrit values are higher and prevalence of anemia is lower in the post-folic acid fortification period than in the pre-folic acid fortification period in US adults. Am J Clin Nutr. 2009;89:363–71.

309. Odewole OA, Williamson RS, Zakai NA, Berry RJ, Judd SE, Qi YP, Adedinsewo DA, Oakley GP.

Near-elimination of folate-deficiency anemia by mandatory folic acid fortification in older US adults: Reasons for Geographic and Racial Differences in Stroke study 2003-2007. Am J Clin Nutr. 2013;98:1042–7.

310. Miller JW, Garrod MG, Allen LH, Haan MN, Green R. Metabolic evidence of vitamin B-12 deficiency, including high homocysteine and methylmalonic acid and low holotranscobalamin, is more pronounced in older adults with elevated plasma folate. Am J Clin Nutr. 2009;90:1586–92.

311. Selhub J, Morris MS, Jacques PF. In vitamin B12 deficiency, higher serum folate is associated with increased total homocysteine and methylmalonic acid concentrations. Proc Natl Acad Sci U S A. 2007;104:19995–20000.

312. Morris MS, Jacques PF, Rosenberg IH, Selhub J. Folate and vitamin B-12 status in relation to anemia, macrocytosis, and cognitive impairment in older Americans in the age of folic acid fortification. Am J Clin Nutr. 2007;85:193–200.

313. Morris MS, Jacques PF, Rosenberg IH, Selhub J. Circulating unmetabolized folic acid and 5-methyltetrahydrofolate in relation to anemia, macrocytosis, and cognitive test performance in American seniors. Am J Clin Nutr. 2010;91:1733–44.

314. Clarke R, Sherliker P, Hin H, Molloy AM, Nexo E, Ueland PM, Emmens K, Scott JM, Evans JG. Folate and vitamin B12 status in relation to cognitive impairment and anaemia in the setting of voluntary fortification in the UK. Br J Nutr. 2008;100:1054–9.

315. Mills JL, Carter TC, Scott JM, Troendle JF, Gibney ER, Shane B, Kirke PN, Ueland PM, Brody LC, Molloy AM. Do high blood folate concentrations exacerbate metabolic abnormalities in people with low vitamin B-12 status? Am J Clin Nutr. 2011;94:495–500.

316. Moore EM, Ames D, Mander AG, Carne RP, Brodaty H, Woodward MC, Boundy K, Ellis KA, Bush AI, et al. Among vitamin B12 deficient older people, high folate levels are associated with worse cognitive function: combined data from three cohorts. J Alzheimers Dis. 2014;39:661–8.

317. Berry RJ, Carter HK, Yang Q. Cognitive impairment in older Americans in the age of folic acid fortification. Am J Clin Nutr. 2007;86:265–7; author reply 267–9.

318. Carmel R. Does high folic acid intake affect unrecognized cobalamin deficiency, and how will we know it if we see it? Am J Clin Nutr. 2009;90:1449–50.

319. Quinlivan EP. In vitamin B12 deficiency, higher serum folate is associated with increased homocysteine and methylmalonic acid concentrations. Proc Natl Acad Sci U S A. 2008;105:E7; author reply E8.

320. Samaniego-Vaesken M de L, Alonso-Aperte E, Varela-Moreiras G. Contribution of folic acid-fortified foods to fertile women's folate Recommended Nutrient Intake through breakfast simulation models. Public Health Nutr. 2015;18:1960–8.

321. Smith AD, Kim Y-I, Refsum H. Is folic acid good for everyone? Am J Clin Nutr. 2008;87:517–33.

322. UK Standing Advisory Committee on Nutrition. Folate and Disease Prevention Report. London; 2006.

323. Morris JK, Rankin J, Draper ES, Kurinczuk JJ, Springett A, Tucker D, Wellesley D, Wreyford B, Wald NJ. Prevention of neural tube defects in the UK: a missed opportunity. Arch Dis Child. 2015;

324. Khan A, Choudhury N, Uddin S, Hossain L, Baur LA. Longitudinal trends in global obesity research and collaboration: a review using bibliometric metadata. Obes Rev. 2016;

325. Power C, Lake JK, Cole TJ. Measurement and long-term health risks of child and adolescent fatness. Int J Obes Relat Metab Disord. 1997;21:507–26.

326. Berrington de Gonzalez A, Hartge P, Cerhan JR, Flint AJ, Hannan L, MacInnis RJ, Moore SC, Tobias GS, Anton-Culver H, et al. Body-mass index and mortality among 1.46 million white adults. N Engl J Med. 2010;363:2211–9.

327. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. BMJ. 2000;320:1240–3.

328. Rolland-Cachera MF, Sempé M, Guilloud-Bataille M, Patois E, Péquignot-Guggenbuhl F, Fautrad V. Adiposity indices in children. Am J Clin Nutr. 1982;36:178–84.

329. Cole TJ, Freeman J V, Preece MA. Body mass index reference curves for the UK, 1990. Arch Dis Child. 1995;73:25–9.

330. World Obesity Foundation. Overweight/ Obesity by age category [Internet]. 2015 [cited 2016 Feb 25]. Available from: http://www.worldobesity.org/resources/overweight-obesity-age-category/?map=age-children

331. van Stralen MM, te Velde SJ, van Nassau F, Brug J, Grammatikaki E, Maes L, De Bourdeaudhuij I, Verbestel V, Galcheva S, et al. Weight status of European preschool children and associations with family demographics and energy balance-related behaviours: a pooled analysis of six European studies. Obes Rev. 2012;13 Suppl 1:29–41.

332. Wijnhoven TMA, van Raaij JMA, Spinelli A, Starc G, Hassapidou M, Spiroski I, Rutter H, Martos É, Rito AI, et al. WHO European Childhood Obesity Surveillance Initiative: body mass index and level of overweight among 6-9-year-old children from school year 2007/2008 to school year 2009/2010. BMC Public Health. 2014;14:806.

333. Skinner AC, Skelton JA. Prevalence and trends in obesity and severe obesity among children in the United States, 1999-2012. JAMA Pediatr. 2014;168:561–6.

334. Shashaj B, Bedogni G, Graziani MP, Tozzi AE, DiCorpo ML, Morano D, Tacconi L, Veronelli P, Contoli B, Manco M. Origin of cardiovascular risk in overweight preschool children: a cohort study of cardiometabolic risk factors at the onset of obesity. JAMA Pediatr. 2014;168:917–

24.

335. Corvalán C, Uauy R, Kain J, Martorell R. Obesity indicators and cardiometabolic status in 4-y-old children. Am J Clin Nutr. 2010;91:166–74.

336. Skinner AC, Perrin EM, Moss LA, Skelton JA. Cardiometabolic Risks and Severity of Obesity in Children and Young Adults. N Engl J Med. 2015;373:1307–17.

337. Juonala M, Magnussen CG, Berenson GS, Venn A, Burns TL, Sabin MA, Srinivasan SR, Daniels SR, Davis PH, et al. Childhood adiposity, adult adiposity, and cardiovascular risk factors. N Engl J Med. 2011;365:1876–85.

338. Franks PW, Hanson RL, Knowler WC, Sievers ML, Bennett PH, Looker HC. Childhood obesity, other cardiovascular risk factors, and premature death. N Engl J Med. 2010;362:485–93.

339. Baker JL, Olsen LW, Sørensen TIA. Childhood body-mass index and the risk of coronary heart disease in adulthood. N Engl J Med. 2007;357:2329–37.

340. Must A, Jacques PF, Dallal GE, Bajema CJ, Dietz WH. Long-term morbidity and mortality of overweight adolescents. A follow-up of the Harvard Growth Study of 1922 to 1935. N Engl J Med. 1992;327:1350–5.

341. Olshansky SJ, Passaro DJ, Hershow RC, Layden J, Carnes BA, Brody J, Hayflick L, Butler RN, Allison DB, Ludwig DS. A potential decline in life expectancy in the United States in the 21st century. N Engl J Med. 2005;352:1138–45.

342. World Health Assembly. Resolution 65.6.Comprehensive implementation plan on maternal, infant and young child nutrition. Geneva; 2014.

343. European Commission. EU Action Plan on Childhood Obesity 2014-2020. 2014.

344. Foster BA, Farragher J, Parker P, Sosa ET. Treatment Interventions for Early Childhood Obesity: A Systematic Review. Acad Pediatr. 2015;15:353–61.

345. Hung L-S, Tidwell DK, Hall ME, Lee ML, Briley CA, Hunt BP. A meta-analysis of school-based obesity prevention programs demonstrates limited efficacy of decreasing childhood obesity. Nutr Res. 2015;35:229–40.

346. Wang Y, Cai L, Wu Y, Wilson RF, Weston C, Fawole O, Bleich SN, Cheskin LJ, Showell NN, et al. What childhood obesity prevention programmes work? A systematic review and meta-analysis. Obes Rev. 2015;16:547–65.

347. Peirson L, Fitzpatrick-Lewis D, Morrison K, Warren R, Usman Ali M, Raina P. Treatment of overweight and obesity in children and youth: a systematic review and meta-analysis. C open. 3:E35–46.

348. Cesa CC, Sbruzzi G, Ribeiro RA, Barbiero SM, de Oliveira Petkowicz R, Eibel B, Machado NB, Marques R das V, Tortato G, et al. Physical activity and cardiovascular risk factors in children:

meta-analysis of randomized clinical trials. Prev Med (Baltim). 2014;69:54–62.

349. Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, Lindgren CM, Perry JRB, Elliott KS, Lango H, et al. A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science. 2007;316:889–94.

350. Felix JF, Bradfield JP, Monnereau C, van der Valk RJP, Stergiakouli E, Chesi A, Gaillard R, Feenstra B, Thiering E, et al. Genome-wide association analysis identifies three new susceptibility loci for childhood body mass index. Hum Mol Genet. 2016;25:389–403.

351. Reilly JJ, Armstrong J, Dorosty AR, Emmett PM, Ness A, Rogers I, Steer C, Sherriff A. Early life risk factors for obesity in childhood: cohort study. BMJ. 2005;330:1357.

352. Howe LD, Tilling K, Galobardes B, Smith GD, Ness AR, Lawlor DA. Socioeconomic disparities in trajectories of adiposity across childhood. Int J Pediatr Obes. 2011;6:e144–53.

353. Owen CG, Nightingale CM, Rudnicka AR, Sattar N, Cook DG, Ekelund U, Whincup PH. Physical activity, obesity and cardiometabolic risk factors in 9- to 10-year-old UK children of white European, South Asian and black African-Caribbean origin: the Child Heart And health Study in England (CHASE). Diabetologia. 2010;53:1620–30.

354. Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet (London, England). 1986;1:1077–81.

355. Leon DA, Lithell HO, Vâgerö D, Koupilová I, Mohsen R, Berglund L, Lithell UB, McKeigue PM. Reduced fetal growth rate and increased risk of death from ischaemic heart disease: cohort study of 15 000 Swedish men and women born 1915-29. BMJ. 1998;317:241–5.

356. Eriksson JG, Forsén T, Tuomilehto J, Osmond C, Barker DJP. Early adiposity rebound in childhood and risk of Type 2 diabetes in adult life. Diabetologia. 2003;46:190–4.

357. Rich-Edwards JW, Stampfer MJ, Manson JE, Rosner B, Hankinson SE, Colditz GA, Willett WC, Hennekens CH. Birth weight and risk of cardiovascular disease in a cohort of women followed up since 1976. BMJ. 1997;315:396–400.

358. Hales CN, Barker DJ, Clark PM, Cox LJ, Fall C, Osmond C, Winter PD. Fetal and infant growth and impaired glucose tolerance at age 64. BMJ. 1991;303:1019–22.

359. Frankel S, Elwood P, Sweetnam P, Yarnell J, Smith GD. Birthweight, body-mass index in middle age, and incident coronary heart disease. Lancet (London, England). 1996;348:1478–80.

360. Risnes KR, Vatten LJ, Baker JL, Jameson K, Sovio U, Kajantie E, Osler M, Morley R, Jokela M, et al. Birthweight and mortality in adulthood: a systematic review and meta-analysis. Int J Epidemiol. 2011;40:647–61.

361. Yu ZB, Han SP, Zhu GZ, Zhu C, Wang XJ, Cao XG, Guo XR. Birth weight and subsequent risk of obesity: a systematic review and meta-analysis. Obes Rev. 2011;12:525–42.

362. Parkinson JRC, Hyde MJ, Gale C, Santhakumaran S, Modi N. Preterm birth and the metabolic syndrome in adult life: a systematic review and meta-analysis. - PubMed - NCBI [Internet]. Pediatrics. 2013 [cited 2016 Feb 25]. p. e1240–63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/?term=Preterm+Birth+and+the+Metabolic+Syndrome+in+Adult+Life%3A+A+Systematic+Review+and+Meta-analysis

363. Sharp GC, Lawlor DA, Richmond RC, Fraser A, Simpkin A, Suderman M, Shihab HA, Lyttleton O, McArdle W, et al. Maternal pre-pregnancy BMI and gestational weight gain, offspring DNA methylation and later offspring adiposity: findings from the Avon Longitudinal Study of Parents and Children. Int J Epidemiol. 2015;44:1288–304.

364. Derraik JGB, Ayyavoo A, Hofman PL, Biggs JB, Cutfield WS. Increasing maternal prepregnancy body mass index is associated with reduced insulin sensitivity and increased blood pressure in their children. Clin Endocrinol (Oxf). 2015;83:352–6.

365. Zhu Y, Olsen SF, Mendola P, Yeung EH, Vaag A, Bowers K, Liu A, Bao W, Li S, et al. Growth and obesity through the first 7 y of life in association with levels of maternal glycemia during pregnancy: a prospective cohort study. Am J Clin Nutr. 2016;

366. Mamun AA, Mannan M, Doi SAR. Gestational weight gain in relation to offspring obesity over the life course: a systematic review and bias-adjusted meta-analysis. Obes Rev. 2014;15:338–47.

367. Crowther CA, Hiller JE, Moss JR, McPhee AJ, Jeffries WS, Robinson JS. Effect of treatment of gestational diabetes mellitus on pregnancy outcomes. N Engl J Med. 2005;352:2477–86.

368. Landon MB, Spong CY, Thom E, Carpenter MW, Ramin SM, Casey B, Wapner RJ, Varner MW, Rouse DJ, et al. A multicenter, randomized trial of treatment for mild gestational diabetes. N Engl J Med. 2009;361:1339–48.

369. Landon MB, Rice MM, Varner MW, Casey BM, Reddy UM, Wapner RJ, Rouse DJ, Biggio JR, Thorp JM, et al. Mild gestational diabetes mellitus and long-term child health. Diabetes Care. 2015;38:445–52.

370. Adair LS, Fall CHD, Osmond C, Stein AD, Martorell R, Ramirez-Zea M, Sachdev HS, Dahly DL, Bas I, et al. Associations of linear growth and relative weight gain during early life with adult health and human capital in countries of low and middle income: findings from five birth cohort studies. Lancet (London, England). 2013;382:525–34.

371. Gadgil M, Joshi K, Pandit A, Otiv S, Joshi R, Brenna JT, Patwardhan B. Imbalance of folic acid and vitamin B12 is associated with birth outcome: an Indian pregnant women study. Eur J Clin Nutr. 2014;68:726–9.

372. Demerath EW, Reed D, Choh AC, Soloway L, Lee M, Czerwinski SA, Chumlea WC, Siervogel RM, Towne B. Rapid postnatal weight gain and visceral adiposity in adulthood: the Fels Longitudinal Study. Obesity (Silver Spring). 2009;17:2060–6.

373. Araújo de França G V, Lucia Rolfe E De, Horta BL, Gigante DP, Yudkin JS, Ong KK, Victora CG.

Associations of birth weight, linear growth and relative weight gain throughout life with abdominal fat depots in adulthood: the 1982 Pelotas (Brazil) birth cohort study. Int J Obes (Lond). 2016;40:14–21.

374. Araújo de França G V, Restrepo-Méndez MC, Loret de Mola C, Victora CG. Size at birth and abdominal adiposity in adults: a systematic review and meta-analysis. Obes Rev. 2014;15:77–91.

375. Sipola-Leppänen M, Vääräsmäki M, Tikanmäki M, Matinolli H-M, Miettola S, Hovi P, Wehkalampi K, Ruokonen A, Sundvall J, et al. Cardiometabolic risk factors in young adults who were born preterm. Am J Epidemiol. 2015;181:861–73.

376. West-Eberhard MJ. Phenotypic Plasticity and the Origins of Diversity. Annu Rev Ecol Syst. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA; 1989;20:249–78.

377. Holliday R, Grigg GW. DNA methylation and mutation. Mutat Res. 1993;285:61–7.

378. Felsenfeld G, Groudine M. Controlling the double helix. Nature. 2003;421:448–53.

379. Garcia MM, Guéant-Rodriguez R-M, Pooya S, Brachet P, Alberto J-M, Jeannesson E, Maskali F, Gueguen N, Marie P-Y, et al. Methyl donor deficiency induces cardiomyopathy through altered methylation/acetylation of PGC-1α by PRMT1 and SIRT1. J Pathol. 2011;225:324–35.

380. Pooya S, Blaise S, Moreno Garcia M, Giudicelli J, Alberto J-M, Guéant-Rodriguez R-M, Jeannesson E, Gueguen N, Bressenot A, et al. Methyl donor deficiency impairs fatty acid oxidation through PGC-1α hypomethylation and decreased ER-α, ERR-α, and HNF-4α in the rat liver. J Hepatol. 2012;57:344–51.

381. Kersten S, Desvergne B, Wahli W. Roles of PPARs in health and disease. Nature. 2000;405:421–4.

382. Meher AP, Joshi AA, Joshi SR. Maternal micronutrients, omega-3 fatty acids, and placental PPARγ expression. Appl Physiol Nutr Metab = Physiol Appl Nutr métabolisme. 2014;39:793–800.

383. Weber M, Grote V, Closa-Monasterolo R, Escribano J, Langhendries J-P, Dain E, Giovannini M, Verduci E, Gruszfeld D, et al. Lower protein content in infant formula reduces BMI and obesity risk at school age: follow-up of a randomized trial. Am J Clin Nutr. 2014;99:1041–51.

384. Gruszfeld D, Weber M, Gradowska K, Socha P, Grote V, Xhonneux A, Dain E, Verduci E, Riva E, et al. Association of early protein intake and pre-peritoneal fat at five years of age: Follow-up of a randomized clinical trial. Nutr Metab Cardiovasc Dis. 2016;

385. Lillycrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC. Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. J Nutr. 2005;135:1382–6.

386. Kumar KA, Lalitha A, Pavithra D, Padmavathi IJN, Ganeshan M, Rao KR, Venu L, Balakrishna N, Shanker NH, et al. Maternal dietary folate and/or vitamin B12 restrictions alter body composition (adiposity) and lipid metabolism in Wistar rat offspring. J Nutr Biochem. 2013;24:25–31.

387. Wang Z, Dou X, Yao T, Song Z. Homocysteine inhibits adipogenesis in 3T3-L1 preadipocytes. Exp Biol Med (Maywood). 2011;236:1379–88.

388. Roman-Garcia P, Quiros-Gonzalez I, Mottram L, Lieben L, Sharan K, Wangwiwatsin A, Tubio J, Lewis K, Wilkinson D, et al. Vitamin B_{12}-dependent taurine synthesis regulates growth and bone mass. J Clin Invest. 2014;124:2988–3002.

389. Yajnik CS, Deshpande SS, Jackson AA, Refsum H, Rao S, Fisher DJ, Bhat DS, Naik SS, Coyaji KJ, et al. Vitamin B12 and folate concentrations during pregnancy and insulin resistance in the offspring: the Pune Maternal Nutrition Study. Diabetologia. 2008;51:29–38.

390. Lewis SJ, Leary S, Davey Smith G, Ness A. Body composition at age 9 years, maternal folate intake during pregnancy and methyltetrahydrofolate reductase (MTHFR) C677T genotype. Br J Nutr. 2009;102:493–6.

391. Stewart CP, Christian P, Schulze KJ, Leclerq SC, West KP, Khatry SK. Antenatal micronutrient supplementation reduces metabolic syndrome in 6- to 8-year-old children in rural Nepal. J Nutr. 2009;139:1575–81.

392. Stewart CP, Christian P, LeClerq SC, West KP, Khatry SK. Antenatal supplementation with folic acid + iron + zinc improves linear growth and reduces peripheral adiposity in school-age children in rural Nepal. Am J Clin Nutr. 2009;90:132–40.

393. Stewart CP, Christian P, Schulze KJ, Arguello M, LeClerq SC, Khatry SK, West KP. Low maternal vitamin B-12 status is associated with offspring insulin resistance regardless of antenatal micronutrient supplementation in rural Nepal. J Nutr. 2011;141:1912–7.

394. Tobias JH, Steer CD, Emmett PM, Tonkin RJ, Cooper C, Ness AR. Bone mass in childhood is related to maternal diet in pregnancy. Osteoporos Int. 2005;16:1731–41.

395. Ganpule A, Yajnik CS, Fall CHD, Rao S, Fisher DJ, Kanade A, Cooper C, Naik S, Joshi N, et al. Bone mass in Indian children--relationships to maternal nutritional status and diet during pregnancy: the Pune Maternal Nutrition Study. J Clin Endocrinol Metab. 2006;91:2994–3001.

396. Steer CD, Emmett PM, Lewis SJ, Smith GD, Tobias JH. Methylenetetrahydrofolate Reductase (MTHFR) C677T Polymorphism Is Associated With Spinal BMD in 9-Year-Old Children. J Bone Miner Res. 2009;24:117–24.

397. Steer CD, Tobias JH. Insights into the programming of bone development from the Avon Longitudinal Study of Parents and Children (ALSPAC). Am J Clin Nutr. 2011;94:1861S – 1864S.

398. Generalitat de Catalunya. Departament de Salut. Protocol de seguiment de l'embaràs a Catalunya. [Catalonia pregnancy monitoring protocol]. Pública DG, editor. 2005.

399. Spanish Society of Gynaecology and Obstetrics (SEGO) and Spanish National, Health System. Recomendaciones sobre suplementación con ácido fólico para la prevención de defectos del tubo neural. [Folic acid supplementation recommendations for the prevention of neural tube defects.]. Inf Ter del Sist Nac Salud. 2001;25:66–7.

400. Rodríguez IT, Ballart JF, Pastor GC, Jordà EB, Val VA. [Validation of a short questionnaire on frequency of dietary intake: reproducibility and validity]. Nutr Hosp. 2008;23:242–52.

401. Grupo de Trabajo de la Sociedad Española de Epidemiología. La medición de la clase social en Ciencias de la Salud. [Measurement of social class in health sciences.]. SG, editor. Barcelona, Spain: Grupo de Trabajo de la Sociedad Española de Epidemiología; 1995.

402. Catalunya D de SG de. Protocol de seguiment de l'embaràs a Catalunya. Barcelona: Direcció General de Salut Pública; 2005.

403. Harris ML. Classification, Diagnostic Criteria, and Screening for Diabetes. Diabetes in America. 1979. p. 15–36.

404. World Health Organization. International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) [Internet]. 2016 [cited 2016 Jan 29]. Available from: http://apps.who.int/classifications/icd10/browse/2016/en

405. Obstetricia SEG y. Protocolo crecimiento intrauterino restringido [Protocol for intrauterine growth restriction]. 2009;

406. Organization WH. WHO Child Growth Standards. WHO Anthro (version 3.2.2, January 2011) and macros [Internet]. [cited 2016 Feb 6]. Available from: http://www.who.int/childgrowth/software/en/

407. Molloy AM, Scott JM. Microbiological assay for serum, plasma, and red cell folate using cryopreserved, microtiter plate method. Methods Enzymol. 1997;281:43–53.

408. Bevital A/S [Internet]. [cited 2016 Feb 5]. Available from: http://bevital.no/

409. Kelleher BP, Broin SD. Microbiological assay for vitamin B12 performed in 96-well microtitre plates. J Clin Pathol. 1991;44:592–5.

410. Ueland PM, Midttun O, Windelberg A, Svardal A, Skålevik R, Hustad S. Quantitative profiling of folate and one-carbon metabolism in large-scale epidemiological studies by mass spectrometry. Clin Chem Lab Med. 2007;45:1737–45.

411. Biochemical verification of tobacco use and cessation. Nicotine Tob Res. 2002;4:149–59.

412. Perrone RD, Madias NE, Levey AS. Serum creatinine as an index of renal function: new insights into old concepts. Clin Chem. 1992;38:1933–53.

413. Meyer K, Fredriksen A, Ueland PM. High-level multiplex genotyping of polymorphisms involved in folate or homocysteine metabolism by matrix-assisted laser

desorption/ionization mass spectrometry. Clin Chem. 2004;50:391–402.

414. Jean-Claude F, Ireland-Ripert J, Toque C, Feinberg M, Ireland J, Toque C, Feinberg M. Répertoire général des aliments : Tables de composition. XXVII. INRA, editor. Paris; 1995. 897 p.

415. Kowalski K, Corcker P, Donen R. The physical activity questionnaire for older children (PAQ-C) and adolescents (PAQ-A) manual. Canada: College of Kinesiology, University of Saskatchewan; 2004. 1-38 p.

416. Martínez-Gómez D, Gómez-Martínez S, Wärnberg J, Welk GJ, Marcos A, Veiga OL. Convergent validity of a questionnaire for assessing physical activity in Spanish adolescents with overweight. Med Clin (Barc). 2011;136:13–5.

417. Moore JB, Hanes JC, Barbeau P, Gutin B, Treviño RP, Yin Z. Validation of the Physical Activity Questionnaire for Older Children in children of different races. Pediatr Exerc Sci. 2007;19:6–19.

418. Thompson AM, Baxter-Jones ADG, Mirwald RL, Bailey DA. Comparison of physical activity in male and female children: does maturation matter? Med Sci Sports Exerc. 2003;35:1684–90.

419. de Onis M, Onyango AW, Van den Broeck J, Chumlea WC, Martorell R. Measurement and standardization protocols for anthropometry used in the construction of a new international growth reference. Food Nutr Bull. 2004;25:S27–36.

420. WHO. WHO | STEPwise approach to surveillance (STEPS). Geneva. World Health Organization (WHO); 2008;

421. Davies PS. Body composition assessment. Arch Dis Child. 1993;69:337–8.

422. de Onis M, Onyango AW, Borghi E, Siyam A, Nishida C, Siekmann J. Development of a WHO growth reference for school-aged children and adolescents. Bull World Health Organ. 2007;85:660–7.

423. Wells JCK, Williams JE, Chomtho S, Darch T, Grijalva-Eternod C, Kennedy K, Haroun D, Wilson C, Cole TJ, Fewtrell MS. Body-composition reference data for simple and reference techniques and a 4-component model: a new UK reference child. Am J Clin Nutr. 2012;96:1316–26.

424. VanItallie TB, Yang MU, Heymsfield SB, Funk RC, Boileau RA. Height-normalized indices of the body's fat-free mass and fat mass: potentially useful indicators of nutritional status. Am J Clin Nutr. 1990;52:953–9.

425. Ray JG, Meier C, Vermeulen MJ, Boss S, Wyatt PR, Cole DEC. Association of neural tube defects and folic acid food fortification in Canada. Lancet (London, England). 360:2047–8.

426. Williams J, Mai CT, Mulinare J, Isenburg J, Flood TJ, Ethen M, Frohnert B, Kirby RS. Updated estimates of neural tube defects prevented by mandatory folic Acid fortification - United

States, 1995-2011. MMWR Morb Mortal Wkly Rep. 2015;64:1–5.

427. Morris MS, Selhub J, Jacques PF. Vitamin B-12 and folate status in relation to decline in scores on the mini-mental state examination in the framingham heart study. J Am Geriatr Soc. 2012;60:1457–64.

428. Doets EL, Ueland PM, Tell GS, Vollset SE, Nygård OK, Van't Veer P, de Groot LCPGM, Nurk E, Refsum H, et al. Interactions between plasma concentrations of folate and markers of vitamin B(12) status with cognitive performance in elderly people not exposed to folic acid fortification: the Hordaland Health Study. Br J Nutr. 2014;111:1085–95.

429. Chen L-W, Lim AL, Colega M, Tint M-T, Aris IM, Tan CS, Chong Y-S, Gluckman PD, Godfrey KM, et al. Maternal folate status, but not that of vitamins B-12 or B-6, is associated with gestational age and preterm birth risk in a multiethnic Asian population. J Nutr. 2015;145:113–20.

430. Furness DLF, Yasin N, Dekker GA, Thompson SD, Roberts CT. Maternal red blood cell folate concentration at 10-12 weeks gestation and pregnancy outcome. J Matern Fetal Neonatal Med. 2012;25:1423–7.

431. Cotter AM, Molloy AM, Scott JM, Daly SF. Elevated plasma homocysteine in early pregnancy: a risk factor for the development of severe preeclampsia. Am J Obstet Gynecol. 2001;185:781–5.

432. Cotter AM, Molloy AM, Scott JM, Daly SF. Elevated plasma homocysteine in early pregnancy: a risk factor for the development of nonsevere preeclampsia. Am J Obstet Gynecol. 2003;189:391–4; discussion 394–6.

433. Krishnaveni G V, Hill JC, Veena SR, Bhat DS, Wills AK, Karat CLS, Yajnik CS, Fall CHD. Low plasma vitamin B12 in pregnancy is associated with gestational "diabesity" and later diabetes. Diabetologia. 2009;52:2350–8.

434. Stern SJ, Matok I, Kapur B, Koren G. A comparison of folic acid pharmacokinetics in obese and nonobese women of childbearing age. Ther Drug Monit. 2011;33:336–40.

435. Shaw GM, Velie EM, Schaffer D. Risk of neural tube defect-affected pregnancies among obese women. JAMA. 1996;275:1093–6.

436. Rasmussen SA, Chu SY, Kim SY, Schmid CH, Lau J. Maternal obesity and risk of neural tube defects: a metaanalysis. Am J Obstet Gynecol. 2008;198:611–9.

437. World Health Organization. Obesity and overweight Fact sheet n°311 [Internet]. 2015. Available from: http://www.who.int/mediacentre/factsheets/fs311/en/

438. Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of childhood and adult obesity in the United States, 2011-2012. JAMA. 2014;311:806–14.

439. von Ruesten A, Steffen A, Floegel A, van der A DL, Masala G, Tjønneland A, Halkjaer J, Palli D,

Wareham NJ, et al. Trend in obesity prevalence in European adult cohort populations during follow-up since 1996 and their predictions to 2015. PLoS One. 2011;6:e27455.

440. Gaunt TR, Rodríguez S, Day IN. Cubic exact solutions for the estimation of pairwise haplotype frequencies: implications for linkage disequilibrium analyses and a web tool "CubeX". BMC Bioinformatics. 2007;8:428.

441. Refsum H, Smith AD, Ueland PM, Nexo E, Clarke R, McPartlin J, Johnston C, Engbaek F, Schneede J, et al. Facts and recommendations about total homocysteine determinations: an expert opinion. Clin Chem. 2004;50:3–32.

442. Stabler SP, Allen RH, Savage DG, Lindenbaum J. Clinical spectrum and diagnosis of cobalamin deficiency. Blood. 1990;76:871–81.

443. Pfeiffer CM, Caudill SP, Gunter EW, Osterloh J, Sampson EJ. Biochemical indicators of B vitamin status in the US population after folic acid fortification: results from the National Health and Nutrition Examination Survey 1999-2000. Am J Clin Nutr. 2005;82:442–50.

444. Partearroyo T, Úbeda N, Montero A, Achón M, Varela-Moreiras G. Vitamin B(12) and folic acid imbalance modifies NK cytotoxicity, lymphocytes B and lymphoprolipheration in aged rats. Nutrients. 2013;5:4836–48.

445. Knudsen VK, Orozova-Bekkevold I, Rasmussen LB, Mikkelsen TB, Michaelsen KF, Olsen SF. Low compliance with recommendations on folic acid use in relation to pregnancy: is there a need for fortification? Public Health Nutr. 2004;7:843–50.

446. Nilsen RM, Vollset SE, Gjessing HK, Magnus P, Meltzer HM, Haugen M, Ueland PM. Patterns and predictors of folic acid supplement use among pregnant women: the Norwegian Mother and Child Cohort Study. Am J Clin Nutr. 2006;84:1134–41.

447. Tort J, Lelong N, Prunet C, Khoshnood B, Blondel B. Maternal and health care determinants of preconceptional use of folic acid supplementation in France: results from the 2010 National Perinatal Survey. BJOG. 2013;120:1661–7.

448. Fayyaz F, Wang F, Jacobs RL, O'Connor DL, Bell RC, Field CJ. Folate, vitamin B12, and vitamin B6 status of a group of high socioeconomic status women in the Alberta Pregnancy Outcomes and Nutrition (APrON) cohort. Appl Physiol Nutr Metab = Physiol Appl Nutr métabolisme. 2014;39:1402–8.

449. Ray JG, Singh G, Burrows RF. Evidence for suboptimal use of periconceptional folic acid supplements globally. BJOG. 2004;111:399–408.

450. Navarrete-Muñoz EM, Valera-Gran D, García de la Hera M, Gimenez-Monzo D, Morales E, Julvez J, Riaño I, Tardón A, Ibarluzea J, et al. Use of high doses of folic acid supplements in pregnant women in Spain: an INMA cohort study. BMJ Open. 2015;5:e009202.

451. Finkelstein JD, Kyle W, Harris BJ. Methionine metabolism in mammals. Regulation of homocysteine methyltransferases in rat tissue. Arch Biochem Biophys. 1971;146:84–92.

452. Yetley EA, Coates PM, Johnson CL. Overview of a roundtable on NHANES monitoring of biomarkers of folate and vitamin B-12 status: measurement procedure issues. Am J Clin Nutr. 2011;94:297S – 302S.

453. Qi YP, Do AN, Hamner HC, Pfeiffer CM, Berry RJ. The prevalence of low serum vitamin B-12 status in the absence of anemia or macrocytosis did not increase among older U.S. adults after mandatory folic acid fortification. J Nutr. 2014;144:170–6.

454. Hustad S, Eussen S, Midttun Ø, Ulvik A, van de Kant PM, Mørkrid L, Gislefoss R, Ueland PM. Kinetic modeling of storage effects on biomarkers related to B vitamin status and one-carbon metabolism. Clin Chem. 2012;58:402–10.

455. Deegan KL, Jones KM, Zuleta C, Ramirez-Zea M, Lildballe DL, Nexo E, Allen LH. Breast milk vitamin B-12 concentrations in Guatemalan women are correlated with maternal but not infant vitamin B-12 status at 12 months postpartum. J Nutr. 2012;142:112–6.

456. Papandreou D, Mavromichalis I, Makedou A, Rousso I, Arvanitidou M. Total serum homocysteine, folate and vitamin B12 in a Greek school age population. Clin Nutr. 2006;25:797–802.

457. Molloy AM, Pangilinan F, Mills JL, Shane B, O'Neill MB, McGaughey DM, Velkova A, Abaan HO, Ueland PM, et al. A Common Polymorphism in HIBCH Influences Methylmalonic Acid Concentrations in Blood Independently of Cobalamin. Am J Hum Genet. 2016;

458. Hardikar AA, Satoor SN, Karandikar MS, Joglekar M V, Puranik AS, Wong W, Kumar S, Limaye A, Bhat DS, et al. Multigenerational Undernutrition Increases Susceptibility to Obesity and Diabetes that Is Not Reversed after Dietary Recuperation. Cell Metab. 2015;22:312–9.

459. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet. 2003;33 Suppl:245–54.

460. Bamonti F, Moscato GA, Novembrino C, Gregori D, Novi C, De Giuseppe R, Galli C, Uva V, Lonati S, Maiavacca R. Determination of serum holotranscobalamin concentrations with the AxSYM active B(12) assay: cut-off point evaluation in the clinical laboratory. Clin Chem Lab Med. 2010;48:249–53.

461. Smith GD, Ebrahim S. "Mendelian randomization": can genetic epidemiology contribute to understanding environmental determinants of disease? Int J Epidemiol. 2003;32:1–22.

462. Zetterberg H, Nexö E, Regland B, Minthon L, Boson R, Palmér M, Rymo L, Blennow K. The transcobalamin (TC) codon 259 genetic polymorphism influences holo-TC concentration in cerebrospinal fluid from patients with Alzheimer disease. Clin Chem. 2003;49:1195–8.

463. Wans S, Schüttler K, Jakubiczka S, Müller A, Luley C, Dierkes J. Analysis of the transcobalamin II 776C>G (259P>R) single nucleotide polymorphism by denaturing HPLC in healthy elderly: associations with cobalamin, homocysteine and holo-transcobalamin II. Clin Chem Lab Med. 2003;41:1532–6.

464. Haggarty P, Hoad G, Campbell DM, Horgan GW, Piyathilake C, McNeill G. Folate in pregnancy and imprinted gene and repeat element methylation in the offspring. Am J Clin Nutr. 2013;97:94–9.

465. Steegers-Theunissen RP, Obermann-Borst SA, Kremer D, Lindemans J, Siebel C, Steegers EA, Slagboom PE, Heijmans BT. Periconceptional maternal folic acid use of 400 microg per day is related to increased methylation of the IGF2 gene in the very young child. PLoS One. 2009;4:e7845.

466. Loke YJ, Galati JC, Morley R, Joo EJ-H, Novakovic B, Li X, Weinrich B, Carson N, Ollikainen M, et al. Association of maternal and nutrient supply line factors with DNA methylation at the imprinted IGF2/H19 locus in multiple tissues of newborn twins. Epigenetics. 2013;8:1069–79.

467. Baker J, Liu JP, Robertson EJ, Efstratiadis A. Role of insulin-like growth factors in embryonic and postnatal growth. Cell. 1993;75:73–82.

468. Santoso MIE, Rohman MS. Decreased TGF-beta1 and IGF-1 protein expression in rat embryo skull bone in folic acid-restricted diet. J Nutr Biochem. 2006;17:51–6.

469. Ouni M, Gunes Y, Belot M-P, Castell A-L, Fradin D, Bougnères P. The IGF1 P2 promoter is an epigenetic QTL for circulating IGF1 and human growth. Clin Epigenetics. 2015;7:22.

470. Padmanabhan N, Jia D, Geary-Joo C, Wu X, Ferguson-Smith AC, Fung E, Bieda MC, Snyder FF, Gravel RA, et al. Mutation in folate metabolism causes epigenetic instability and transgenerational effects on development. Cell. 2013;155:81–93.

471. McKay JA, Groom A, Potter C, Coneyworth LJ, Ford D, Mathers JC, Relton CL. Genetic and non-genetic influences during pregnancy on infant global and site specific DNA methylation: role for folate gene variants and vitamin B12. PLoS One. 2012;7:e33290.

472. Morgan HD, Jin XL, Li A, Whitelaw E, O'Neill C. The culture of zygotes to the blastocyst stage changes the postnatal expression of an epigentically labile allele, agouti viable yellow, in mice. Biol Reprod. 2008;79:618–23.

473. Guo H, Zhu P, Yan L, Li R, Hu B, Lian Y, Yan J, Ren X, Lin S, et al. The DNA methylation landscape of human early embryos. Nature. 2014;511:606–10.

474. Smith ZD, Chan MM, Humm KC, Karnik R, Mekhoubad S, Regev A, Eggan K, Meissner A. DNA methylation dynamics of the human preimplantation embryo. Nature. 2014;511:611–5.

475. Tobi EW, Slieker RC, Stein AD, Suchiman HED, Slagboom PE, van Zwet EW, Heijmans BT, Lumey LH. Early gestation as the critical time-window for changes in the prenatal environment to affect the adult human blood methylome. Int J Epidemiol. 2015;44:1211–23.

476. Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, Bispham J, Thurston A, Huntley JF, Rees WD, et al. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. Proc Natl Acad Sci U S A. 2007;104:19351–6.

477. Seidell JC, Verschuren WM, van Leer EM, Kromhout D. Overweight, underweight, and mortality. A prospective study of 48,287 men and women. Arch Intern Med. 1996;156:958–63.

478. Socha P, Grote V, Gruszfeld D, Janas R, Demmelmair H, Closa-Monasterolo R, Subías JE, Scaglioni S, Verduci E, et al. Milk protein intake, the metabolic-endocrine response, and growth in infancy: data from a randomized clinical trial. Am J Clin Nutr. 2011;94:1776S – 1784S.

479. Ali O, Cerjak D, Kent JW, James R, Blangero J, Zhang Y. Obesity, central adiposity and cardiometabolic risk factors in children and adolescents: a family-based study. Pediatr Obes. 2014;9:e58–62.

480. Furer A, Afek A, Beer Z, Derazne E, Tzur D, Pinhas-Hamiel O, Reichman B, Twig G. Height at Late Adolescence and Incident Diabetes among Young Men. PLoS One. 2015;10:e0136464.

481. National Toxicology Program. Identifying research needs for assessing safe of use of high intakes of folic acid. 2015.

482. Wang M, Wang Z-P, Gao L-J, Gong R, Sun X-H, Zhao Z-T. Maternal body mass index and the association between folic acid supplements and neural tube defects. Acta Paediatr. 2013;102:908–13.

483. Stover PJ. Folate biochemical pathways and their regulation. In: Bailey LB, editor. Folates in Health and Disease. Second edi. CRC Press, Taylor and Francis Group; 2010. p. 49–74.

484. Chanarin I. The megaloblastic anaemias. Blackwell Scientific Publications, editor. London; 1969.

485. Molloy AM. Folate-vitamin B12 interrelationships: Links to disease risk. In: Bailey LB, editor. Folate in Health and disease. CRC Press Taylor and Francis Group; 2010. p. 381–409.

486. R G, Miller JW. Vitamin B12. In: J Z, R B, D M, J S, editors. Handbook of vitamins. Fourth edi. Boca Raton: CRC Press Taylor and Francis Group; 2007. p. 413–58.

487. Alemdaroglu NC, Dietz U, Wolffram S, Spahn-Langguth H, Langguth P. Influence of green and black tea on folic acid pharmacokinetics in healthy volunteers: potential risk of diminished folic acid bioavailability. Biopharm Drug Dispos. 2008;29:335–48.

488. World Obesity Federation [Internet]. 2015 [cited 2016 Feb 28]. Available from: http://www.worldobesity.org/resources/obesity-data-repository/

SCIENTIFIC AND ACADEMIC CONTRIBUTIONS AND OTHER MERITS

Scientific and academic contributions and other merits

Articles

Solé-Navais P, Cavallé-Busquets P, Fernandez-Ballart JD, Murphy MM. *Early pregnancy B vitamin status, one carbon metabolism, pregnancy outcome and child development.* Biochimie 2015; S0300-9084. DOI: 10.1016/j.biochi.2015.12.003. PMID: 26700149.

International conference contributions

Conference: World Forum for Nutrition Research Conference. Reus (Spain), 2013.

Authors: Bueno O, Cavallé-Busquets P, Fernandez-Ballart JD, Ballesteros M, Berrocal-Zaragoza MI, Salat-Batlle J, Fernàndez-Roig S, Garcia-Minguillan CJ, Solé Navais P, Murphy MM.

Title: Long-term pregnancy plasma and red cell folate response to first trimester folic acid supplementation.

Format: Poster.

Conference: 9th Homocysteine and One-Carbon Metabolism. Dublin (Ireland), 2013.

Authors: García-Minguillán CJ; Fernandez-Ballart JD; Cavallé-Busquets P; Ballesteros M; Bueno O; Berrocal-Zaragoza MI; Solé P; Meyer K; Ueland PM; Murphy MM.

Title: The effects of the *MTRR* 66A>G and *MTRR* 524C>T polymorphisms on homocysteine during pregnancy vary depending on riboflavin and cobalamin status.

Format: Oral communication.

Conference: IUNS 20th International Congress of Nutrition. Granada (Spain), 2013.

Authors: Bueno O, Cavallé-Busquets P, Fernandez-Ballart JD, Ballesteros M, Berrocal-Zaragoza MI, Salat-Batlle J, Fernàndez-Roig S, Garcia-Minguillan CJ, Solé Navais P, Murphy MM.

Title: First trimester folic acid supplementation enhances folate status throughout pregnancy and reduces the effect of the *MTHFR* 677C>T polymorphism.

Format: Oral communication.

Conference: IUNS 20th International Congress of Nutrition. Granada (Spain), 2013.

Authors: Garcia-Minguillan CJ, Cavallé-Busquets P, Fernandez-Ballart JD, Ballesteros M, Berrocal-Zaragoza MI, Bueno O, Solé Navais P, Murphy MM.

Title: Riboflavin status is inversely associated with homocysteine and determines the effect of the *MTHFR* 677C>T polymorphism on homocysteine during pregnancy.

Format: Oral communication.

Conference: FASEB: Folic Acid, Vitamin B_{12}, and One Carbon Metabolism. Steamboat Springs (United States), 2014.

Authors: Murphy MM; Fernandez-Ballart JD; Solé-Navais P; Ballesteros M; Bueno O; Ueland PM; Meyer K; Cavallé-Busquets P.

Title: Elevated paternal homocysteine or *MTHFR* 677TT genotype and risk of impaired placental function, gestational hypertension or intrauterine growth retardation.

Format: Oral communication.

Conference: FASEB: Folic Acid, Vitamin B_{12}, and One Carbon Metabolism. Steamboat Springs (United States), 2014.

Authors: Solé-Navais P; Cavallé-Busquets P; Bueno O; Fernandez-Ballart JD; Ballesteros M; Colomina-Muela JM; Ueland PM; Salat-Batlle J; Murphy MM.

Title: First Trimester high folate status combined with low cobalamin status and MMA and tHcy during pregnancy in the Reus Tarragona Birth Cohort.

Format: Poster.

Conference: 10th International conference One Carbon Metabolism and Homocysteine. Nancy (France), 2015.

Authors: Colomina JM; Cavallé-Busquets P; Fernandez-Roig S; Solé-Navais P; Fernandez-Ballart JD; Ballesteros M; Ornosa G; Ueland PM; Meyer K; Murphy MM.

Title: Effects of the *BHMT* c.716G>A polymorphism on the betaine dimethylglycine pathway during pregnancy.

Format: Oral communication.

Conference: 10th International conference One Carbon Metabolism and Homocysteine. Nancy (France), 2015.

Authors: Solé-Navais P; Ornosa G; Fernandez-Ballart JD; Cavallé-Busquets P; Ballesteros M; Colomina JM; Ueland PM; Murphy MM.

Title: Early pregnancy cobalamin status on anthropometry and adiposity in the offspring at 7.5 years (Reus-Tarragona Birth Cohort).

Format: Poster.

Conference: 10th International conference One Carbon Metabolism and Homocysteine. Nancy (France), 2015.

Authors: Murphy MM; Solé-Navais P; Fernandez-Ballart JD; Ballesteros M; Cavallé-Busquets P.

Title: Early pregnancy B vitamin status, one carbon metabolism and pregnancy outcome.

Format: Oral communication.

Book chapters (In Press)

Authors: Obeid R, Solé-Navais P, Murphy MM. **Chapter title:** Cobalamin during pregnancy and lactation. **Book title:** Vitamin B_{12}: advances and insights (Obeid R ed.). **Publisher:** CRC Press/ Taylor & Francis Group.

Authors: Solé-Navais P, Obeid R, Murphy MM. **Chapter title:** Cobalamin – folate interactions. **Book title:** Vitamin B_{12}: advances and insights (Obeid R ed.). **Publisher:** CRC Press/ Taylor & Francis Group.

Authors: Obeid R, Solé-Navais P, Murphy MM. **Chapter title:** Cobalamin after birth and during early life. **Book title:** Vitamin B_{12}: advances and insights (Obeid R ed.). **Publisher:** CRC Press/ Taylor & Francis Group.

Teaching and academic activities

Medical school (approximately 60 hours/ annum since 2012)

- Research and Documentation Bases (70%)
- General Epidemiology (30%)

Myofascial Pain Specialist Course (2 hours, 2016)

- Introduction to SPSS statistical package (SPSS Inc, Chicago, IL, USA)

Experimental Animal Care and Handling Course (4 hours, 2016)

- Biostatistics and sample power calculation

Awards

Poster prize: Best Poster Award for "*Early pregnancy cobalamin status on anthropometry and adiposity in the offspring at 7.5 years (Reus-Tarragona Birth Cohort)*". 10th International Conference one carbon metabolism, vitamins B and homocysteine, Nancy (France), 2015.

Other contributions

- Active role in the fieldwork of the pregnancy and follow-up phases of the Reus-Tarragona Birth Cohort.
- Active role setting-up and designing the follow-up phase at 7.5 years at both University Hospitals (Reus and Tarragona).

Involvement in scientific projects

Title: Links between *in utero* one carbon metabolism and development and health in the offspring at 7.5-8 years. **Principal Investigator:** Michelle Murphy. **Duration**: 3 years. **REF**: PI13/02500. **Funding source**: Instituto de Salud Carlos III/ Fondo FEDER.

APPENDICES

Appendices

12 setmanes

Estudi NUTCIR 1

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

QÜESTIONARI DE FREQÜÈNCIA DE CONSUM ALIMENTARI 1

INSTRUCCIONS PER OMPLIR-LO

Procuri contestar tranquil·lament aquest qüestionari. Prengui el temps que consideri necessari.

Aquest qüestionari li pregunta la freqüència amb la que vostè consumia de forma **habitual** determinats aliments abans d'estat embarassada.

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

- Si el consumeix **tots els dies de la setmana**, posi un 7 en la columna **A LA SETMANA.**
- Si el consumeix **alguna vegada a la setmana,** posi les vegades: 1-2-3-4-5 o 6 en la columna **A LA SETMANA.**

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

Exemple: Una dona esmorza habitualment un got de llet (7 vegades) amb magdalenes(7 vegades), i per sopar a vegades pren llet (4vegades) i a vegades pren iogurt (3 vegades) de postres. A més, pren peix algunes vegades a la setmana per dinar (2 vegades) i altres vegades per sopar (4 vegades). El llegums en consumeix alguna vegada al mes (aproximadament 4 vegades) . Si no menja mai un aliment deixeu en blanc, sense contestar res.

Aquest consum l'apuntaria de la següent manera:

LLISTAT D'ALIMENTS	QUANTES VEGADES MENJA...?	
	A LA SETMANA	AL MES
Llet	*11*	
Iogurt	*3*	
Coc ràpid, magdalenes..	*7*	
...		
Peix	*6*	
...		
Llegum		*4*
Formatge de règim		

QÜESTIONARI DE FREQÜÈNCIA DE CONSUM ALIMENTARI

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

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

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

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

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

	A LA SETMANA	AL MES
Begudes ensucrades ("coca-cola", "Fanta"...)		
Begudes baixes en calories (coca-cola light...)		
Vi, sangria		
Cervesa		
Cervesa sense alcohol		
Begudes destil·lades: (Whisky, ginebra, conyac,...)		

Indiqui amb una X la resposta que vostè vulgui assenyalar:

1.- A taula, s'afegeix sal a les menjades?

Mai__ / Alguna vegada__ / Freqüentment __/ Gairebé sempre __

2.- Com definiríeu la vostra gana? Molta __ Força __ Normal __ Poca __ Gens__

3.- Quin tipus de llet prens habitualment?: Sencera __ Semidescremada __ Descremada __

4.- Quin tipus de iogurt prens habitualment?

a) Natural ___ b) Natural descremat ___
c) De sabors ___ d) De sabors descremat ___
e) Amb trossets de fruita ___ f) Amb trossets de fruites descremat ___

5.- Quin tipus de pa prens habitualment?: Blanc __ Integral __

6.- Et poses tomàquet i oli en els entrepans?: Sempre__ Habitualment__ Alguna vegada__ Gairebé mai_

20 setmanas

Nom: Data:

ENQUESTA 1 SOBRE HÀBITS I ESTIL DE VIDA
(referida a la primera meitat de l'embaràs)

ANOTI LES RESPOSTES EN ELS ESPAIS CORRESPONENTS A CADA PREGUNTA.
Aquestes dades serviran a la Universitat Rovira i Virgili per fer un estudi comparatiu entre diferents poblacions. En els resultats mai apareixerà el seu nom.

ÚS DE SUPLEMENTS DE VITAMINES / MINERALS

Per diferents motius, els suplements de vitamines i minerals recomanats no es prenen sempre: per oblit, per sentiment de que no són necessaris, per no trobar-se bé, perquè donen molèsties, etc. Si us plau, contesti sincerament aquestes preguntes per ajudar-nos a valorar la realitat de l'ús dels suplements.

- Ha pres per iniciativa pròpia o receptat per un metge algun tipus de suplement vitamínic / mineral?

Mai n'he pres ☐ **Si n'he pres ☐**

En el cas que sí, escrigui el nom del preparat i indiqui les vegades a la setmana que ho ha pres marcant el quadrat. Marqui el quadrat corresponent als mesos que ho ha pres.

Exemple, una dona que ha pres cada dia FOLIDOCE durant els primers 3 mesos, escriuria...

Nom del preparat	¿Quantes vegades a la setmana?	Mesos de l'embaràs 1	2	3	4	5
ÀCID FÒLIC	■ Cada dia	■	■	■	☐	☐
	☐ La majoria dels dies (4-6 vegades)	☐	☐	☐	☐	☐
Quin? : FOLIDOCE	☐ Alguns dies (1-3 vegades)	☐	☐	☐	☐	☐

Nom del preparat	Quantes vegades a la setmana?	Mesos de l'embaràs 1	2	3	4	5
ÀCID FÒLIC	☐ Cada dia	☐	☐	☐	☐	☐
	☐ La majoria dels dies (4-6 vegades)	☐	☐	☐	☐	☐
Quin?:_____	☐ Alguns dies (1-3 vegades)	☐	☐	☐	☐	☐
FERRO	☐ Cada dia	☐	☐	☐	☐	☐
	☐ La majoria dels dies (4-6 vegades)	☐	☐	☐	☐	☐
Quin?:_____	☐ Alguns dies (1-3 vegades)	☐	☐	☐	☐	☐
MULTI-VITAMINES	☐ Cada dia	☐	☐	☐	☐	☐
	☐ La majoria dels dies (4-6 vegades)	☐	☐	☐	☐	☐
Quin?:_____	☐ Alguns dies (1-3 vegades)	☐	☐	☐	☐	☐

- Va prendre àcid fòlic en els 3 mesos abans de quedar-se embarassada? **Sí ☐** **No ☐**
- Va prendre ferro en els 3 mesos abans de quedar-se embarassada? **Sí ☐** **No ☐**

ESMORZAR (durant l'embaràs)

	Sí	No
Té el costum d'esmorzar?	☐	☐
Esmorza cereals inflats habitualment (p.ex. tipus Kelloggs / Nestlé etc) ?	☐	☐
Pren cafè amb cafeïna?	☐	☐
Pren cafè descafeïnat ?	☐	☐

Nom: Data:

TABAC

- És fumadora passiva (exposada al fum de tabac habitualment a casa o a la feina)? **Sí** ☐ **No** ☐
- És fumadora activa? **Sí** ☐ **No** ☐

Només per fumadores en els últims 5 anys

	0 cigs/dia	1-5 cigs/dia	6-10 cigs/dia	> 10 cigs/dia
Actualment fumo	☐	☐	☐	☐
Fumava durant els 12 mesos abans de l'embaràs	☐	☐	☐	☐

	No	Abans dels 3 mesos	Entre els 3 i els 6 mesos	Després dels 6 mesos
He deixat de fumar durant l'embaràs	☐	☐	☐	☐

ALCOHOL

	Mai / ocasionalment	< 3 copes / setmana	Cada dia com aperitiu i/o amb els àpats	> 7 copes / setmana
Actualment bec alcohol	☐	☐	☐	☐
En els 12 mesos abans de l'embaràs	☐	☐	☐	☐

	No	Abans dels 3 mesos	Entre els 3 i els 6 mesos	Després dels 6 mesos
He deixat de beure alcohol durant l'embaràs	☐	☐	☐	☐

PES, TALLA, EDAT, ORIGEN I PARTICIPACIÓ EN ESTUDIS

	Abans de l'embaràs	Última vegada que es va pesar abans de realitzar l'entrevista (data; SG)
Pes:	. Kg	. Kg (/ / ; SG)

Talla: . m
Data de naixement:
Participació en altres estudis:

Origen pares:
Origen avis:

Nom: Data:

SUBSTÀNCIES TÒXIQUES

- Ha pres algun altre tipus de substància tòxica (p.ex. marihuana, cocaïna, heroïna, etc...) en els últims 5 anys?

Sí ☐ No ☐

En el cas de que sí hagi pres alguna substància tòxica, especifiqui quines: _____

	No	Ocasionalment	Regularment
Actualment prenc substàncies tòxiques	☐	☐	☐
En els 12 mesos abans de l'embaràs prenia subst. tòxiques	☐	☐	☐

	No	Abans dels 3 mesos	Entre els 3 i els 6 mesos	Després dels 6 mesos
He he deixat durant l'embaràs durant els mesos	☐	☐	☐	☐

ACTIVITAT FÍSICA (durant l'embaràs)

- Quina activitat física fa en el treball, estudi o feina de casa?

Hores/setmana

- El meu treball és bàsicament d'estar asseguda i caminar poc (estudiant, docent, conductora de vehicles, dependenta, administrativa)..... ☐ _____
- Al meu treball camino força però no faig cap esforç vigorós (mestressa de casa, fàbrica, venedora, cartera...)..... ☐ _____
- El meu treball és bàsicament de molta activitat física (esportista.)..... ☐ _____

- Quina activitat feu en el temps de lleure? (anotar la prioritària si dues activitats coincideixen en hores)

Horas/semana

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

- Durant els últims 12 mesos

	Mai	Esporàdicament	Habitualment
Ha tingut el costum de prendre el Sol?	☐	☐	☐

Nom: Data:

PLANIFICACIÓ DE L'EMBARÀS

- Ha buscat / planificat aquest embaràs? **Sí** ☐ **No** ☐
- Durant els 6 mesos abans de l'embaràs

	Cap	DIU	Anticonceptius orals	Pegat anticonceptiu	Anell vaginal	Preservatiu
Quin mètode d'anticonceptiu ha fet servir?	☐	☐	☐	☐	☐	☐

- Cicles sense prendre anticonceptius orals abans de l'embaràs? _____
(Número de regles des de que va deixar de prendre anticonceptius fins que va quedar embarassada)

DADES SOCIODEMOGRÀFIQUES

- Quina és la seva feina en l'actualitat i quin nivell d'estudis ha completat

	Mare	Pare
Feina actual	☐	☐
Nivell d'estudis	Primaris sense finalitzar ☐ Primaris (ESO, EGB, ...) ☐ Secundaris (BUP, Batxillerat, FP, ...) ☐ Superiors (Universitaris) ☐	Primaris sense finalitzar ☐ Primaris (ESO, EGB, ...) ☐ Secundaris (BUP, Batxillerat, FP, ...) ☐ Superiors (Universitaris) ☐ No aplicable (Família monoparental) ☐

- Nombre de persones que formen la unitat familiar _____
- Ingressos nets anuals totals a la llar

Exemple, si la dona té un sou de 20000 €, l'home un de 18000€ i hi ha un avi que viu amb la família i rep una pensió de 6000 €

Menys de 9000 €	>9000 € - 19000 €	>19000 € - 25000 €	>25000 € - 35000 €	Més de 35000 €
☐	☐	☐	☐	■

Menys de 9000 €	>9000 € - 19000 €	>19000 € - 25000 €	>25000 € - 35000 €	Més de 35000 €
☐	☐	☐	☐	☐

Appendices

UNIVERSITAT ROVIRA I VIRGILI

Reus & Tarragona Birth Cohort

Unitat de Medicina Preventiva i Salut Pública

TP-90

ID ☐☐☐

Inicials ☐☐☐☐

Gènere ☐

Centre ☐

Data de entrevista ☐☐ ☐☐ ☐☐ (d d m m a a)

Data de naixement ☐☐ ☐☐ ☐☐ (d d m m a a)

Pes (kg)	☐☐,☐	☐☐,☐
Alçada (cm)	☐☐☐,☐	☐☐☐,☐
Circumferència cranial(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

ID ☐☐☐ **TP-90**

Inicials ☐☐☐☐

Centre ☐

Data de compleció ☐☐ ☐☐ ☐☐
d d m m a a

(A omplir per l'investigador)

Reus & Tarragona Birth Cohort

Qüestionari d'activitat física i desenvolupament

Aquest quadern l'han d'omplir les mares i/o els pares, o bé el tutor legal amb l'ajuda del nen. Les preguntes fan referència a la activitat física, i al desenvolupament del vostre fill.

Les respostes que figurin dins d'aquest quadern, seran confidencials i mai estaran lligades al nom del nen.

Moltes gràcies per la vostra col·laboració.

UNIVERSITAT ROVIRA I VIRGILI

Qüestionari d'activitat física

Volem conèixer el nivell d'activitat física del vostre fill durant els **darrers 7 dies** (la setmana anterior). Això inclou esports o ball que el fan suar o que provoca que senti les cames cansades, o bé jocs que el fan respirar ràpidament com ara tocar i parar, saltar a la corda, córrer, escalar o d'altres.

Recordeu:

1. No hi ha respostes correctes o incorrectes – No es tracta de cap examen.
2. Si us plau, responguеu totes les preguntes tan honesta i precisament com pugueu.

1. Activitat física durant el temps lliure: El vostre fill ha realitzat alguna de les activitats següents en els **últims 7 dies** (setmana passada)? Si la resposta és SI, quantes vegades? (Marqueu un sol cercle per filera).

	No	1-2	3-4	5-6	7 cops o més
Saltar a la corda	○	○	○	○	○
Patinar	○	○	○	○	○
Jocs: Tocar i parar, ...	○	○	○	○	○
Caminar (exercici)	○	○	○	○	○
Bicicleta	○	○	○	○	○
Córrer/ Footing	○	○	○	○	○
Natació	○	○	○	○	○
Ballar	○	○	○	○	○
Muntar en monopatí	○	○	○	○	○
Futbol	○	○	○	○	○
Hoquei sobre gespa	○	○	○	○	○
Hoquei sobre patins	○	○	○	○	○
Bàsquet	○	○	○	○	○
Patinatge artístic	○	○	○	○	○
Esquí	○	○	○	○	○
Hoquei sobre gel	○	○	○	○	○

Altres:

_____ ○ ○ ○ ○ ○

_____ ○ ○ ○ ○ ○

2. En els **últims 7 dies**, durant les classes d'Educació Física, quantes vegades va estar molt actiu (jugant intensament, corrent, saltant, fent llançaments)? (Marqueu una sola resposta.)

No fa educació física	○
Gairebé mai	○
Alguna vegada	○
Sovint	○
Sempre	○

3. En els **últims 7 dies**, que feia la majoria de vegades a l'hora del pati? (Marqueu una sola resposta.)

Estar assegut (parlar, llegir, fer els deures)	○
Passejar	○
Córrer o jugar una mica	○
Córrer o jugar bastant	○
Córrer o jugar gairebé sempre	○

4. En els **últims 7 dies**, que feia la majoria de vegades a l'hora de dinar (a part de menjar)? (Marqueu una sola resposta.)

Estar assegut (Parlar, llegir, fer els deures)	○
Passejar	○
Córrer o jugar una mica	○
Córrer o jugar bastant	○
Córrer o jugar gairebé sempre	○

5. En els **últims 7 dies**, quants dies just després de l'escola va practicar esport, ball (dansa) o va jugar a jocs en els quals estava molt actiu? (Marqueu una sola resposta.)

- Cap
- Una vegada en la darrera setmana
- 2 o 3 vegades en la darrera setmana
- 4 vegades en la darrera setmana
- 5 vegades en la darrera setmana

6. En els **últims 7 dies**, quants dies per la tarda va practicar esport, ball (dansa) o va jugar a jocs en els quals estava molt actiu? (Marqueu una sola resposta.)

- Cap
- Una vegada en la darrera setmana
- 2 o 3 vegades en la darrera setmana
- 4 vegades en la darrera setmana
- 5 vegades en la darrera setmana

7. En **l'últim cap de setmana**, quants cops va practicar esports, ball (dansa) o va jugar a jocs en els quals estava molt actiu? (Marqueu una sola resposta.)

- Cap
- Una vegada en la darrera setmana
- 2 o 3 vegades en la darrera setmana
- 4 vegades en la darrera setmana
- 5 vegades en la darrera setmana

8. Quina de les següents afirmacions el descriu millor en els **últims 7 dies**? Llegiu les 5 afirmacions abans de decidir quina resposta el descriu.

a. Tot, o gairebé tot el seu temps lliure el va dedicar a activitats que requereixen poc esforç físic ○

b. Alguna vegada (1-2 vegades) ha practicat activitat física en el seu temps lliure (ex.: fer esport, córrer, nedar, bicicleta o aeròbic) ○

c. Sovint (3-4 vegades) ha practicat activitat física en el seu temps lliure ○

d. Bastant sovint (5-6 vegades) ha practicat activitat física en el seu temps lliure ○

e. Molt sovint (7 o més vegades) ha practicat activitat física en el seu temps lliure ○

9. Marqueu quantes vegades ha realitzat alguna activitat física (tals com fer esport, jocs, ballar o qualsevol altre activitat física) per cada dia durant la **darrera setmana**.

	Cap (0)	Poca (1)	Normal (2)	Bastant (3)	Molta (4)
Dilluns	○	○	○	○	○
Dimarts	○	○	○	○	○
Dimecres	○	○	○	○	○
Dijous	○	○	○	○	○
Divendres	○	○	○	○	○
Dissabte	○	○	○	○	○
Diumenge	○	○	○	○	○

10. Ha estat malalt durant aquest **última setmana**, o alguna cosa ha impedit que practiqués alguna activitat física? (Marqueu una casella.)

Si ○
No ○

Si la resposta és si, què ho ha impedit?

11. Com acostuma a anar el nen a l'escola?

- Bicicleta ○
- Cotxe/ moto ○
- Caminant ○
- Autobús ○

12. Quanta estona tarda en arribar a l'escola?

- 1 a 5 minuts ○
- 6 a 10 minuts ○
- 11 a 15 minuts ○
- Més de 15 minuts ○

13. Penseu en una **setmana habitual**. Indiqueu quantes hores al dia el nen realitza les accions següents:

	Entre setmana (promig dels cinc dies)	Caps de setmana (promig dels dos dies)
Mirar la televisió (DVD, vídeos i pel·lícules al ordinador)		
Jugar a l'ordinador (consoles,)		
Fer deures (sense ordinador)		
Dormir		
Llegir		

14. Té televisió a la seva habitació?

- Si ○
- No ○

Desenvolupament físic

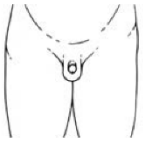
Ens agradaria avaluar el desenvolupament físic del vostre fill utilitzant les figures que es representen en aquesta pàgina.
Aquestes, indiquen diferents estadis de pubertat comunament utilitzats per metges per avaluar el desenvolupament i creixement dels nens.

Els nens passen per els diversos estadis de desenvolupament físic en diferents edats. Alguns d'ells comencen tan aviat com als 6 anys, i d'altres no ho fan fins als 16.

Les figures següents mostren diferents quantitats de pèl púbic masculí. Els nens poden passar per cada un dels diferents estadis representats.

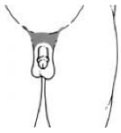
Si us plau, mireu atentament cada una de les diferents figures. És important també que llegiu les descripcions.

Assenyaleu la casella que més concorda amb l'estadi del vostre fill.



○ Lleugera vellositat infantil.

○ Pel escàs, llis i lleugerament pigmentat, usualment arrelat al penis.



○ Pel arrissat, escassament desenvolupat, però obscur, clarament pigmentat i arrelat al penis.

○ No n'estic segur.

Monitoreig Ambulatori de la Pressió Arterial (MAPA)

Es tracta d'una tècnica no invasiva que obté mesures de la pressió arterial durant 24h del dia.

L'ús del MAPA no ha de provocar cap alteració en l'estil de vida del nen, i l'ha de permetre realitzar totes les activitats quotidianes de manera habitual.

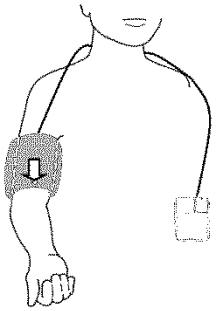
Cal tenir especial cura en el manteniment del MAPA i procurar que no quedi fora de la bandolera.

Per apagar, col·locar la pestanya de la part superior al "0", i per tornar a encendre, moure-la cap a l'altre costat. Sempre que el MAPA estigui posicionat en el braç, ha de romandre encès.

Què faig si...

... el braçalet es mou de lloc?

La fletxa de color blanc ha de quedar uns 2 cm per damunt del plec del braç, i assenyalant al punt mig tal i com es mostra en el dibuix.



...el MAPA pressiona molt fort a l'hora de prendre la mesura?

Serà necessari afluixar el braçalet, treien el "velcro" i col·locant-lo de manera que no pressioni massa, però que no quedi mòbil.

Per a qualsevol dubte, o problema que sorgeixi, 654 660 005.

Moltes gràcies per participar!!!

Unitat de Medicina Preventiva i Salut Pública

UNIVERSITAT ROVIRA I VIRGIL

Reus & Tarragona Birth Cohort

Unitat de Medicina Preventiva i Salut Pública

ID ☐☐☐
Inicials ☐☐☐
Gènere ☐
Centre ☐

TP-90

Data entrevista ☐☐ ☐☐ ☐☐ (d d m m a a)
Data naixement ☐☐ ☐☐ ☐☐ (d d m m a a)

Història clínica

1. Qui respon a la Història Clínica?

☐ Mare
☐ Pare
☐ Altre familiar. Especificar:
☐ Tutor/a Responsable no familiar
☐ Altres. Especificar

2.

	Si	No	Motiu
Extracció sanguínia			
Carnet de Salut			
BIA			
Quadern d'activitat física			
MAPA			
Diari d'activitat física			
Neurodesenvolupament 1			
Neurodesenvolupament 2			

3. Ha estat diagnosticat d'alguna malaltia o malformació congènita?_____

_____

4. Ha estat diagnosticat d'alguna malaltia crònica? _____

_____

5. Ha estat hospitalitzat per algun motiu?

☐ No ☐ Si Especificar:_____

Edat durant l'hospitalització (anys)?_____

6. Se li ha realitzat algun tipus cirurgia?

☐ No ☐ Si

Quina i Quan?_____

ID:
Inicials:

Reus & Tarragona Birth Cohort

Unitat de Medicina Preventiva i Salut Pública

7. Es va alimentar el nen amb llet materna?

☐ No ☐ Si

Durant quants mesos de manera exclusiva (mesos)?_____
Durant quants mesos en total?_____

8. Quina edat tenia quan es van introduir aliments sòlids a la seva dieta (mesos)?____

9. El seu fill té problemes visuals?

☐ No ☐ Si Necessita portar ulleres?_____

10. El seu fill té problemes auditius?

☐ No ☐ Si

11. Ha estat diagnosticat o ha tingut en algun moment:

	No	Si	Abans dels 2 anys	Entre 2 i 5 anys	Més tard dels 5 anys	Nº de vegades els últims 12 mesos
Refredat amb febre	☐	☐	☐	☐	☐	
Infecció d'oïda	☐	☐	☐	☐	☐	
Infecció a la gola	☐	☐	☐	☐	☐	
Pneumònia	☐	☐	☐	☐	☐	
Bronquitis	☐	☐	☐	☐	☐	
Bronquiolitis	☐	☐	☐	☐	☐	
Gastroenteritis	☐	☐	☐	☐	☐	
Anèmia	☐	☐	☐	☐	☐	
Càries	☐	☐	☐	☐	☐	
Xarampió	☐	☐	☐	☐	☐	
Varicel·la	☐	☐	☐	☐	☐	
Rosada	☐	☐	☐	☐	☐	
Altres. Especificar:	☐	☐	☐	☐	☐	

ID:
Inicials:

12. Té alguna al·lèrgia alimentària?

	Si	No
- Llet de vaca	☐	☐
- Ous de gallina	☐	☐
- Soja	☐	☐
- Cacahuets	☐	☐
- Fruits secs	☐	☐
- Blat	☐	☐
- Peix	☐	☐
- Marisc	☐	☐
- Altres:	☐	☐

13. Ha disminuït o suprimit el consum d'algun aliment de la dieta del nen degut a l'al·lèrgia?

☐ No ☐ Si Quin/s?_____

Ha substituït aquet/s aliment/s per algun altre?_____

14. Durant el darrer any ha estat prenent algun medicament?

☐ No ☐ Si Quin?_____

15. En la actualitat, està prenent algun medicament?

☐ No ☐ Si

Quin?_____

16. Algun familiar directe ha estat diagnosticat d'algun trastorn psicopatològic (exemples trastorn psicòtic, depressió, trastorn d'ansietat, retard mental, trastorns per dèficit d'atenció amb hiperactivitat, consum de tòxics, trastorn de la personalitat)?

☐ No ☐ Si Qui?_____Tipus de trastorn?_____

ID:
Inicials:

Reus & Tarragona Birth Cohort

Unitat de Medicina Preventiva i Salut Pública

Qüestionari bàsic de sibilàncies

a. Ha tingut alguna vegada sibilàncies o xiulets al pit durant qualsevol moment del passat? ☐ Si ☐ No

SI HA RESPOST "NO" PASSEU A LA PREGUNTA F

b. Ha tingut alguna vegada sibilàncies o xiulets al pit en els darrers 12 mesos?

☐ Si ☐ No

SI HA RESPOST "NO" PASSEU A LA PREGUNTA F

c. Quants atacs de sibilàncies el vostre fill ha tingut en els darrers 12 mesos?

☐ Cap ☐ 1 a 3 ☐ 4 a 12 ☐ Més de 12

d. En els darrers 12 mesos, quantes vegades, de promig, el somni del vostre fill ha estat pertorbat a causa de les sibilàncies? ☐ Mai s'ha despertat amb sibilàncies

☐ Menys de una nit per setmana ☐ Una nit o més nits per setmana

e. En els darrers 12 mesos, les sibilàncies han estat prou severes per limitar la parla del nen a únicament una o dues paraules entre respiracions? ☐ Si ☐ No

f. El vostre fill ha tingut algun cop asma? ☐ Si ☐ No

g. En els darrers 12 mesos, el tòrax del vostre fill ha sonat sibilant durant o després la realització d'exercici físic? ☐ Si ☐ No

h. En els darrers 12 mesos, el vostre fill ha tingut tos seca per les nits, sense haver estat refredat o amb una infecció de tòrax? ☐ Si ☐ No

Qüestionari bàsic de rinitis

a. Ha tingut algun cop esternuts, el nas "moquejava" o estava tapat quan no tenia la grip ni estava refredat? ☐ Si ☐ No

SI HA RESPOST "NO" PASSEU A LA PREGUNTA F

b. En els darrers 12 mesos, ha tingut algun cop esternuts, el nas "moquejava" o estava tapat quan no tenia la grip ni estava refredat? ☐ Si ☐ No

ID:
Inicials:

SI HA RESPOST "NO" PASSEU A LA PREGUNTA F

c. En els darrers 12 mesos, aquests problemes al nas han estat acompanyats de picors als ulls o estaven plorosos? ☐ Si ☐ No

d. En quin dels darrers 12 mesos es van donar aquests problemes al nas?

☐ Gener ☐ Febre ☐ Març ☐ Abril ☐ Maig ☐ Juny ☐ Juliol ☐ Agost ☐ Setembre ☐ Octubre ☐ Novembre ☐ Desembre

e. En els darrers 12 mesos, quantes vegades aquests problemes al nas han interferit amb les activitats diàries del vostre fill? ☐ Cap vegada ☐ Una mica ☐ Unes quantes vegades ☐ Moltes vegades

f. El vostre fill ha tingut mai al·lèrgia al pol·len? ☐ Si ☐ No

Qüestionari bàsic de èczema

a. Ha tingut alguna erupció cutània amb picors que va estar començant i marxant al menys durant 6 mesos? ☐ Si ☐ No

SI HA RESPOST "NO" PASSEU A LA PREGUNTA G

b. Ha tingut alguna erupció cutània amb picors en algun moment dels darrers 12 mesos? ☐ Si ☐ No

SI HA RESPOST "NO" PASSEU A LA PREGUNTA G

c. Aquesta erupció cutània amb picors ha afectat alguna de les següents zones del cos: els plecs dels colzes, al darrera dels genolls, davant dels turmells, sota les natges o al voltant del coll, orelles o ulls? ☐ Si ☐ No

d. A quina edat va sorgir la primera erupció cutània amb picors? ☐ Abans dels 2 anys ☐ Entre 2-4 anys ☐ 5 anys o més

e. Aquesta erupció es va aclarir completament en qualsevol moment durant els darrers 12 mesos? ☐ Si ☐ No

f. En els darrers 12 mesos, quantes vegades de promig, el vostre fill s'ha quedat despert durant la nit a causa d'aquesta erupció? ☐ Mai en els darrers 12 mesos ☐ Menys de una nit per setmana ☐ Una o més nits per setmana

g. Ha tingut mai èczema? ☐ Si ☐ No

ID:
Inicials:

Appendices

Reus & Tarragona Birth Cohort

Unitat de Medicina Preventiva i Salut Pública

Carnet de Salut

Creixement

Data	Pes (g)	Talla (cm)

Calendari de vacunacions

	DTPa	Poliomelitis	Haemophilus influenza tipus B	Meningitis C conjugada	XRP	Hepatitis B
2m	✓	✓	✓	✓		✓
4m	✓	✓	✓			✓
6m	✓	✓	✓	✓		✓
12m					✓	
15m				✓		
18m	✓	✓	✓			
4a					✓	
4-6a	✓					

ID:
Inicials:

Verificar informació embaràs

17. Hàbit tabàquic durant l'embaràs

Era fumadora activa 3 mesos abans de quedar-se embarassada?

☐ No ☐ Si Va mantenir l'hàbit durant tot l'embaràs?_____

☐ No ☐ Si Si no, quan ho va deixar?_____

Va estar en contacte amb alguna persona que fumés davant seu durant l'embaràs?

☐ No ☐ Si Durant quantes hores diàries aproximadament?_____

18. Hàbit tabàquic en l'entorn del nen

La mare fuma actualment o ha fumat al llarg de la vida del nen?

☐ No ☐ Si

Dins de casa, en quin lloc acostuma a fumar?

Si ha deixat de fumar, edat del nen?____

☐ Fora de casa
☐ A casa però sense estar davant del nen
☐ Davant del nen
☐ Altres

	0 cigs/dia	1-5 cigs/dia	6-10 cigs/dia	> 10 cigs/dia
Quants cigarrets al dia fuma?	☐	☐	☐	☐

19. El pare fuma actualment o ha fumat al llarg de la vida del nen?

☐ No ☐ Si

Dins de casa, en quin lloc acostuma a fumar?

Si ha deixat de fumar, edat del nen?____

☐ Fora de casa
☐ A casa però sense estar davant del nen
☐ Davant del nen
☐ Altres

	0 cigs/dia	1-5 cigs/dia	6-10 cigs/dia	> 10 cigs/dia
Quants cigarrets al dia fuma?	☐	☐	☐	☐

20. Altres persones de l'entorn del nen actualment fumen o han fumat al llarg de la vida del nen?

☐ No ☐ Si

Dins de casa, en quin lloc acostuma a fumar?

Si ha deixat de fumar, edat del nen?____

☐ Fora de casa
☐ A casa però sense estar davant del nen
☐ Davant del nen
☐ Altres

	0 cigs/dia	1-5 cigs/dia	6-10 cigs/dia	> 10 cigs/dia
Quants cigarrets al dia fuma?	☐	☐	☐	☐

ID:
Inicials:

Reus & Tarragona Birth Cohort

Unitat de Medicina Preventiva i Salut Pública

Dades sociodemogràfiques

21. Quina és la seva feina actual i quin nivell d'estudis màxim ha completat

	Mare		Pare	
Feina actual/ darrers 12 mesos				
Nivell d'estudis	☐	Sense estudis	☐	Sense estudis
	☐	Primària incompleta	☐	Primària incompleta
	☐	Primària (ESO, EGB)	☐	Primària (ESO, EGB)
	☐	EGB, batxiller	☐	EGB, batxiller
	☐	Formació professional	☐	Formació professional
	☐	BUP, batxillerat	☐	BUP, batxillerat
	☐	COU, PREU	☐	COU, PREU
	☐	Estudis universitaris de grau mig (Diplomatura)	☐	Estudis universitaris de grau mig (Diplomatura)
	☐	Estudis universitaris de grau superior (llicenciatura, màster, doctorat)	☐	Estudis universitaris de grau superior (llicenciatura, màster, doctorat)

22. Quantes persones formen la unitat familiar? ____

23. Ingressos nets anuals totals a la llar

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

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

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

ID:
Inicials: