THE ONE-CARBON METABOLIC NETWORK, THE L-ARGININE PATHWAY AND HYPERTENSION IN ADULTS AND DURING PREGNANCY

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UNIVERSITAT ROVIRA i VIRGILI

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Doctoral tesis

Thesis supervised by Dr. Michelle Murphy

Department of Basic Medical Sciences

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Resus

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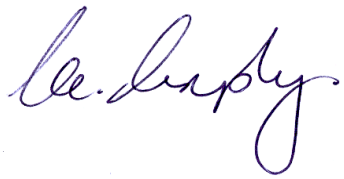
FAIG CONSTAR que aquest treball, titulat “The One-Carbon metabolic network, the L-Arginine pathway and hypertension in adults and during pregnancy”, que presenta Carla Ramos Rodriguez per a l’obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament Ciències Mèdiques Bàsiques d’aquesta universitat.

HAGO CONSTAR que el presente trabajo, titulado “The One-Carbon metabolic network, the L-Arginine pathway and hypertension in adults and during pregnancy”, que presenta Carla Ramos Rodriguez para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento Ciencias Medicas Basicas de esta universidad.

I STATE that the present study, entitled “The One-Carbon metabolic network, the L-Arginine pathway and hypertension in adults and during pregnancy”, presented by Carla Ramos Rodriguez for the award of the degree of Doctor, has been carried out under my supervision at the Department Ciències Mèdiques Bàsiques of this university.

Reus, 31 de Agosto de 2022

La directora de la tesi doctoral
La directora de la tesis doctoral
Doctoral Thesis Supervisor



Dra. Michelle Murphy

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"No temas a las dificultades, lo mejor surge de ellas"
-Rita Levi (Premio Nobel de Medicina)

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Abstract

Endothelial dysfunction is one of the earliest stages of atherosclerosis and cardiovascular disease. Impaired One-carbon metabolism (1CM) has been associated with endothelial dysfunction and a wide range of cardiovascular diseases, such as hypertension. In addition, adverse pregnancy outcomes including hypertension and pre-eclampsia have been reported in mothers with impaired 1CM. The mechanism by which 1CM is associated with hypertension and with pregnancy-induced hypertension is unknown. The 1C metabolic network intersects with the L-Arginine pathway during the conversion of methionine to homocysteine. S-adenosylmethionine (SAM) donates methyl groups to methylate L-Arginine, creating asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA). These L-Arginine analogues can impair the conversion of L-Arginine to nitric oxide (NO) by eNOS. Reduced NO synthesis has been associated with endothelial dysfunction, as NO is a vasodilator.

This thesis investigates whether the L-Arginine pathway is involved in the association between impaired 1CM with hypertension, outside of pregnancy and with pregnancy-induced hypertension. Furthermore, it also investigates whether paternal 1CM is involved in pregnancy-induced hypertension. The thesis is based on 2 studies, a population study and a mother, father, newborn cohort study, *The Reus-Tarragona Birth Cohort.*

Chapter 1: The Population study

The association between 1CM and hypertension was investigated in a representative sample of an adult population, randomly selected from population registers stratified by age and sex, from 3 towns in Tarragona province (2 inland and 1 coastal). From 812 adults that participated in the study (aged 18-75 years old), biological determinations were available for 788. They had a medical check-up in which lifestyle data and fasting blood samples were collected for biomarkers and genotyping determinations. Biomarkers were determined in plasma samples. For both age groups (≤ or > 50 years old) and sexes, predictors of ADMA

and hypertension probability were identified by linear and logistic regressions. The potential participation of ADMA in the association between impaired 1CM and hypertension was assessed by mediation analysis.

Fasting plasma total homocysteine concentration (tHcy) was associated with ADMA (B coefficient, 0.002; SE, 0.001, p <0.001) and SDMA (B coefficient, 0.007; SE, 0.002; p <0.001) in adults not on medication (n=524). After stratifying by age group and sex, tHcy was associated with SDMA in those ≤50 years old (B coefficient, 0.006; SE, 0.002; p, 0.003) and in men (B coefficient, 0.007; SE, 0.003; p, 0.009) but not in women. The *MTHFR* C677T polymorphism was not associated with any L-Arginine pathway metabolites. Mid-high versus low tertiles of ADMA (OR [95% CI] (2.3 [1.1, 5.0]) and low-mid versus high tertiles of the L-Arginine/ADMA Ratio (OR [95% CI] (2.4 [1.1, 5.3]) were associated with increased hypertension risk in adults over 50 years of age. The GT+TT, compared to GG, genotypes of the *NOS* G894T polymorphism were associated with increased hypertension risk only when ADMA was in the mid-high tertile (OR [95% CI] (3.3 [1.2, 9.4]) and in adults over 50. The increased risk of high versus low-mid tHcy on hypertension in these adults was mediated via ADMA tertiles (B, 0.286; CI, 0.045-0.630).

Chapter 2: The Reus-Tarragona Birth Cohort study

810 mothers were recruited from first prenatal clinics at the University hospitals Sant Joan Reus (most participants) and Joan XXIII Tarragona, at <12 weeks' gestation. Lifestyle information was collected throughout pregnancy with questionnaires and from clinical history. Uterine artery Doppler ultrasound was performed at 20 GW to measure arterial pulsatility index and detect the presence notches in the waveform. We defined, as pathological Doppler of the uterine arteries, a mean pulsatility index of left and right arteries was >95 percentile and/or the presence of bilateral notch. Fasting blood was collected for metabolite and genotyping determinations in each trimester of pregnancy and nonfasting blood on admission to hospital with confirmed labour and from the umbilical cord before placenta expulsion.

The evolution of plasma L-Arginine pathway metabolite concentrations during pregnancy was explored according to first trimester tertiles of tHcy and genotypes for the *MTHFR* C667T polymorphism. The probability of having a pathological Doppler of the uterine arteries or pregnancy-induced hypertension (PIH) was explored using multiple logistic regression analysis.

Maternal first trimester plasma ADMA and L-Arginine/ADMA Ratio concentrations (mean ± SD) were higher in mid (ADMA, 0.43 ± 0.07; L-Arginine/ADMA Ratio,104.6 ± 28.4) versus low (ADMA, 0.40 ± 0.05; L-Arginine/ADMA Ratio, 118.79 ± 25.55; p <0.05) first trimester tHcy tertiles. Higher plasma SDMA was observed when tHcy was in the highest tertile (SDMA, 0.39 ± 0.08) compared to the lowest (SDMA, 0.36 ± 0.07; p <0.05). First trimester ADMA (0.40 ± 0.05) and SDMA (0.35 ± 0.06) concentrations were lower in participants with the *MTHFR* TT versus CC genotype (ADMA, 0.44 ± 0.06; SDMA, 0.40 ± 0.08; p, 0.02). Plasma concentrations of L-Arginine pathway metabolite in the first trimester were not associated with increased risk of pathological Doppler of the uterine arteries. First trimester maternal plasma ADMA/SDMA Ratio was associated with increased risk of pregnancy-induced hypertension (OR [95% CI] (1.4 [1.1, 1.9]).

Chapter 3: The Reus-Tarragona Birth Cohort Fathers

From the 810 mothers that completed the study follow-up, 640 fathers were contacted (as mothers had an adverse pregnancy outcome, i.e., a miscarriage, had a sperm donor, were impossible to contact or refuse to provide the fathers' contact). Out of them, 416 fathers participated in the study. Questionnaires were used to collect information on lifestyle and habits and fasting blood samples were collected to determine nutrient and metabolite concentrations and genotypes of the fathers. Paternal plasma L-Arginine pathway metabolite concentrations were compared between tHcy tertiles and *MTHFR* C677T polymorphism genotypes by ANOVA and associated by linear regression. Paternal predictors of probability of pathological Doppler of the uterine arteries and pregnancy-induced hypertension were identified using multiple logistic regression analysis.

Fathers with tHcy in the highest tertile had higher plasma ADMA (0.52 ± 0.07; p, 0.052) and SDMA (0.57 ± 0.2; p, 0.005) concentrations compared to the lowest tertiles (ADMA, 0.50 ± 0.07; SDMA, 0.52 ± 0.1). Having tHcy in the highest tertile was positively associated with SDMA (B, 0.038; SE, 0.014; p, 0.018) compared to the lowest tertile. Paternal L-Arginine pathway metabolite concentrations did not differ between *MTHFR* C677T genotypes. L-Arginine pathway metabolites were not associated with pathological Doppler of the uterine arteries. An average increase of 0.1 mmol/L in paternal plasma ADMA or SDMA was associated with increased risk (OR [95% CI] of pregnancy-induced hypertension (OR 2.0 [1.2,3.3] and 1.6 [1.1,2.4], respectively. However, the risk was no longer significant after adding maternal first trimester plasma ADMA and SDMA concentrations to the fully adjusted model. The paternal *NOS* G894T polymorphism was not associated with pathological Doppler of the uterine arteries or pregnancy-induced hypertension.

Table 0.1: Summary of past, present and future aspects of each chapter.

	What is known	What is unknown	What this thesis adds	Future research
Chapter 1 Population study	Impaired 1CM and L-Arginine pathways have been associated with hypertension, but it is unclear whether they play a shared role in this disease.	The detailed mechanism by which impaired 1CM affects hypertension.	Evidence for a potential mechanism via the L-Arginine pathway in the association of elevated tHcy with hypertension.	Random controlled trials testing whether improving 1CM improves endothelial dysfunction through modification of the L-Arginine pathway.
Chapter 2 The Reus-Tarragona Birth Cohort Study	Maternal folate is crucial for a successful pregnancy and the remethylation of homocysteine. L-Arginine is considered an essential amino acid during development.	How maternal 1CM and the L-Arginine pathway interact during early pregnancy.	No interaction between the first trimester 1CM and the L-Arginine pathway was observed.	Animal models investigating the association between 1CM and the L-Arginine pathway during pregnancy in the absence of the possible effect of folic acid supplementation.
	Impaired 1CM and L-Arginine pathways have been associated with pregnancy-induced hypertension.	If the increased risk of poor placentation and PIH due to impaired 1CM is mediated by its effect on the L-Arginine pathway and the consequent effects on vasculature.	Elevated first trimester maternal L-Arginine analogues are associated with an increased risk of PIH.	Study the L-Arginine pathway as a potential mechanism in the association of impaired 1CM with other adverse pregnancy outcomes.
Chapter 3 The Reus-Tarragona Birth Cohort Fathers	Paternal factors have been associated with some pregnancy complications. Impaired paternal 1CM has been associated with poor placentation and PIH.	Whether impaired paternal 1CM is associated with poor placentation or PIH via the L-Arginine pathway.	The paternal L-Arginine pathway may influence pregnancy-induced hypertension	The association of plasma L-Arginine pathway metabolites with sperm fertility parameters. Sperm samples are being collected in the LED-Fertyl study.

1CM, One-Carbon metabolism; MTHFR, methylenetetrahydrofolate reductase; tHcy, total plasma homocysteine;

Abbreviations

Abbreviation	Definition
1CM	One-Carbon metabolism
5-MTHF	5-methyl-tetrahydrofolate
ACEIs	Angiotensin-containing enzyme inhibitors
ADMA	Asymmetric dimethylarginine
AGXT2	Alanine:glyoxylate amino-transferase 2
ANOVA	Analysis of variance
BH2	6,7-dihydropteridine
BH4	5,6,7,8-tetrahydropteridine
BHMT	Betaine-homocysteine S-methyltransferase
BMI	Body mass index
CAT	Cationic amino acid transporter
CBS	Cystathionine beta synthase
CD11/CD18	Integrin beta 2
cGMP	Cyclic guanosine monophosphate
CI	Confidence interval
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DDAH	Dimethylarginine dimethylaminohydrolase
DHF	Dihydrofolate
DHPR	Dihydrobiopterin reductase
EGRAC	Erythrocyte glutathionine reductase activation coefficient
eNOS	Endothelial nitric oxide synthase
FAD	Flavin adenine dinucleotide
GW	Gestational weeks
HLAs	Human lymphocyte antigens
HPLC-MS/MS	Liquid chromatography–mass spectrometry
HR	Hazard ratio
iNOS	Inducible nitric oxide synthase
IQR	Interquartile range
IUGR	Intrauterine growth restriction
KIR	Killer-cell immunoglobulin-like receptors
LDL	low-density lipoprotein
MMA	Monomethylated arginine
MTHFR	Methylenetetrahydrofolate reductase
MTR	Methionine synthase
MTRR	5-methyltetrahydrofolate-homocysteine methyltransferase reductase
NADPH	Nicotinamide adenine dinucleotide phosphate
NK	Natural killer cells
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NTDs	Neural tube defects
OR	Odd Ratio
PI	Pulsatility index
PIH	Pregnancy-induced hypertension
PRMT	Protein arginine N-methyltransferase

RBCF	Red blood cell folate
RI	Resistance index
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SBP	Systolic blood pressure
SD	Standard deviation
SDMA	Symmetric dimethylarginine
SE	Standard error
tHcy	Plasma total homocysteine
THF	Tetrahydrofolate
TNFa	Tumour necrosis factor alpha
WHO	World Health Organization
y	Years old

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1.INTRODUCTION

1.1 One-Carbon metabolism and L-Arginine pathway summary

One-Carbon metabolism (1CM) is involved in a large range of biological functions. This metabolic network involves an intertwining of enzymes and metabolites that activates and transfers one carbon unit for the biosynthesis of purine and thymidine and the remethylation of homocysteine [1]. During the folate cycle, the first phase of the 1CM, folate plays a key role and folic acid must be reduced to dihydrofolate (DHF) and then transformed into tetrahydrofolate (THF), the biologically active form of folate. THF is free to accept other one carbon units from the folate pool allowing the cycle to continue. Several enzymes act in this cycle, such as Methylenetetrahydrofolate reductase (MTHFR), a vitamin B2 dependent enzyme, that catalyses the formation of THF to be used as cofactor in the methionine cycle for homocysteine remethylation [2, 3]. Methionine is converted to S-adenosylmethionine (SAM), which acts as a methyl group donor in a variety of biological processes through its conversion to S-adenosylhomocysteine (SAH). Subsequently, SAH is converted back to homocysteine to continue the methionine cycle [4]. In this process, SAM donates a methyl group to the enzyme Protein arginine N-methyltransferase (PRMT). This enzyme methylates the amino acid L-Arginine into Asymmetric dimethylarginine (ADMA) and Symmetric dimethylarginine (SDMA). After proteolysis, the endothelial nitric oxide synthase (eNOS) enzyme catalyses the synthesis of nitric oxide (NO) from free L-Arginine. NO regulates vasodilation, vascular tone and blood pressure. However, ADMA and SDMA are competitors of L-Arginine, reducing NO synthesis [5].

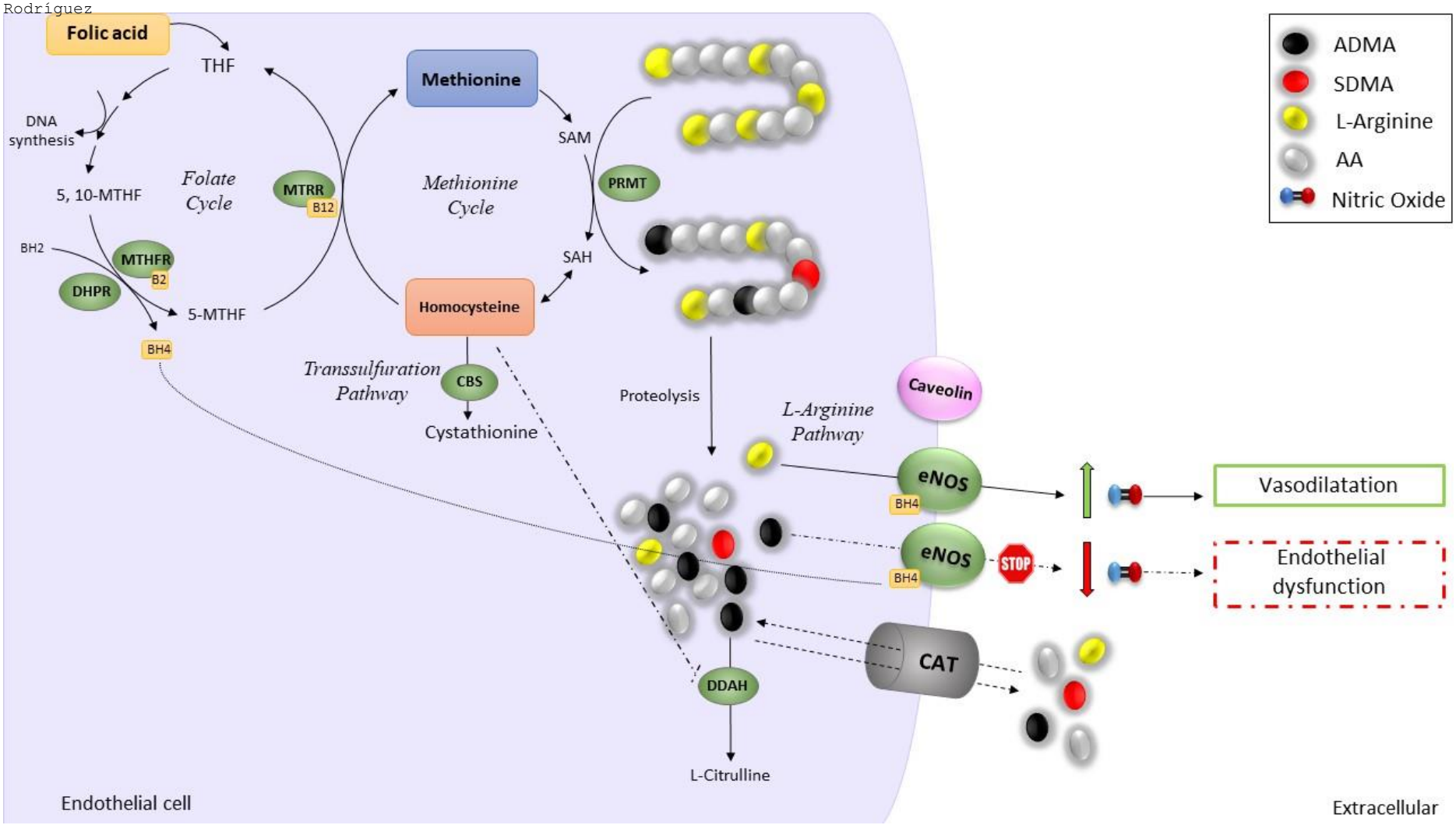


Figure 0.1: Folate cycle, Methionine cycle and L-Arginine pathway representation. 5,10-methylene-THF; 5-MTHF: 5-methyl-THF; AA: general amino acid; ADMA: Asymmetric dimethylarginine; B12: Vitamin B12; B2: Vitamin B2; BH2: 6,7-dihydropteridine; BH4: 5,6,7,8-tetrahydropteridine; CAT: Cationic amino acid transporters; CBS: Cystathionine beta synthase; DDAH: Dimethylarginine dimethylaminohydrolase; DHPR: Dihydrobiopterin reductase; eNOS: Endothelial nitric oxide synthase; MTHFR: Methylenetetrahydrofolate reductase; MTRR: 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; PRMT: Protein arginine N-methyltransferase; SAH: S-Adenosylhomocysteine; SAM: S-Adenosylmethionine; SDMA: Symmetrical Dimethylarginine; THF: tetrahydrofolate. Adapted from [6] and [7].

1.2 One-carbon metabolism

1.2.1 Folate Cycle

Folate is synthesised *de novo* in microorganisms and plants. Mammals lack the ability to synthesise it themselves, thus, it is required in the diet, making it an essential nutrient [8].

THF are polyglutamates that function as coenzymes, carrying 1C units and activating 10-formyl-THF, 5-formyl-THF or 5,10-methenyl-THF [9]**.**

10-formyl-THF is incorporated into the purine ring for purine biosynthesis [10]. In addition, it can be reduced into 5-10-methylene-THF to be the substrate of thymidylate synthase in the biosynthesis of thymidine. This reaction is essential for DNA synthesis [11].

In parallel, 5-10-methylene-THF can participate in the folate cycle in an irreversible reduction catalysed by MTHFR. This reaction requires vitamin B2, FAD and NADPH as cofactors and is the linking point between folate and the methionine cycle.

Folate deficiency

The World Health Organisation indicates that plasma folate values below 4 ng/mL (10 nmol/L) and erythrocyte values below 151 ng/mL (340 nmol/L), imply deficiency, using homocysteine concentrations as metabolic indicator [12]. Deficient folate status can be caused by different factors. The most common is **diet**. As mentioned above, mammals lack the ability to synthesize folate *de novo*, so it must be consumed in the

diet. Consistently low intakes of certain food groups such as green leafy vegetables, legumes and citric fruits can lead to folate deficiency [9].

Substances such as **tobacco**, alcohol or various medications can interfere with folate metabolism. Smokers have been found to have lower plasma folate status than non-smokers. Since tobacco is composed of a large number of substances, the effect of smoking on folate depletion has yet to be elucidated. However, various substances such as nitrous oxide, free radicals, oxidants or inflammation inducers could block part of the folate cycle and homocysteine remethylation [13].

The effect of **alcohol** on folate status is due to impaired absorption of this vitamin after chronic exposure. Heavy consumption of this substance inhibits the expression of the reduced folate carrier and decreases uptake of the vitamin and its storage in the liver and kidney [14].

Drugs that affect folate status mainly include those that are key components of chemotherapy treatments. Among others, methotrexate has a molecular structure very similar to folate and a high affinity for the enzyme dihydrofolate reductase, which traps folate in a non-functional form (DHF). Also, the anticancer drug 5-fluorouracil prevents cancer cell proliferation by inhibiting thymidylate synthetase activity. This enzyme uses folate as a substrate. In the presence of the drug, this enzyme is inhibited, blocking DNA synthesis and cell proliferation, blocking the folate cycle [8]. Polymorphisms in various enzymes of the folate cycle, such as the *MTHFR* C677T polymorphism (explained below), can also reduce plasma folate pool status. Folate deficiency is associated with various diseases such as megaloblastic anaemia, glossitis or even infertility [9].

Importance of folate and folic acid supplementation

The discovery of folate was made in 1931 by Lucy Wills who observed an effect on the treatment of anaemia in pregnant women when they were treated with yeast extract [15]. Over the years, it has been shown that the supply of this vitamin is key to many biochemical processes and to prevent diseases of all kinds.

Initially, the importance of folic acid supplementation was focused on its effect in preventing neural tube defects during *in utero* development. These defects, which can include spina bifida, anencephaly or others, are caused by folate deficiency during pregnancy, especially in the first trimester [16].

In 1991, it was recommended that public health should take measures to make sure that fertile women have the necessary intake of folic acid [17]. In 1998, the U.S. Food and Drug Administration (FDA) mandated fortification of grain products with 1.4 mg/lb of folic acid [18]**.** Since then, countries all over the world have fortified foods with folic acid such as wheat flour, maize flour or rice, to cover the entire populations' needs (**Figure 0.2**).

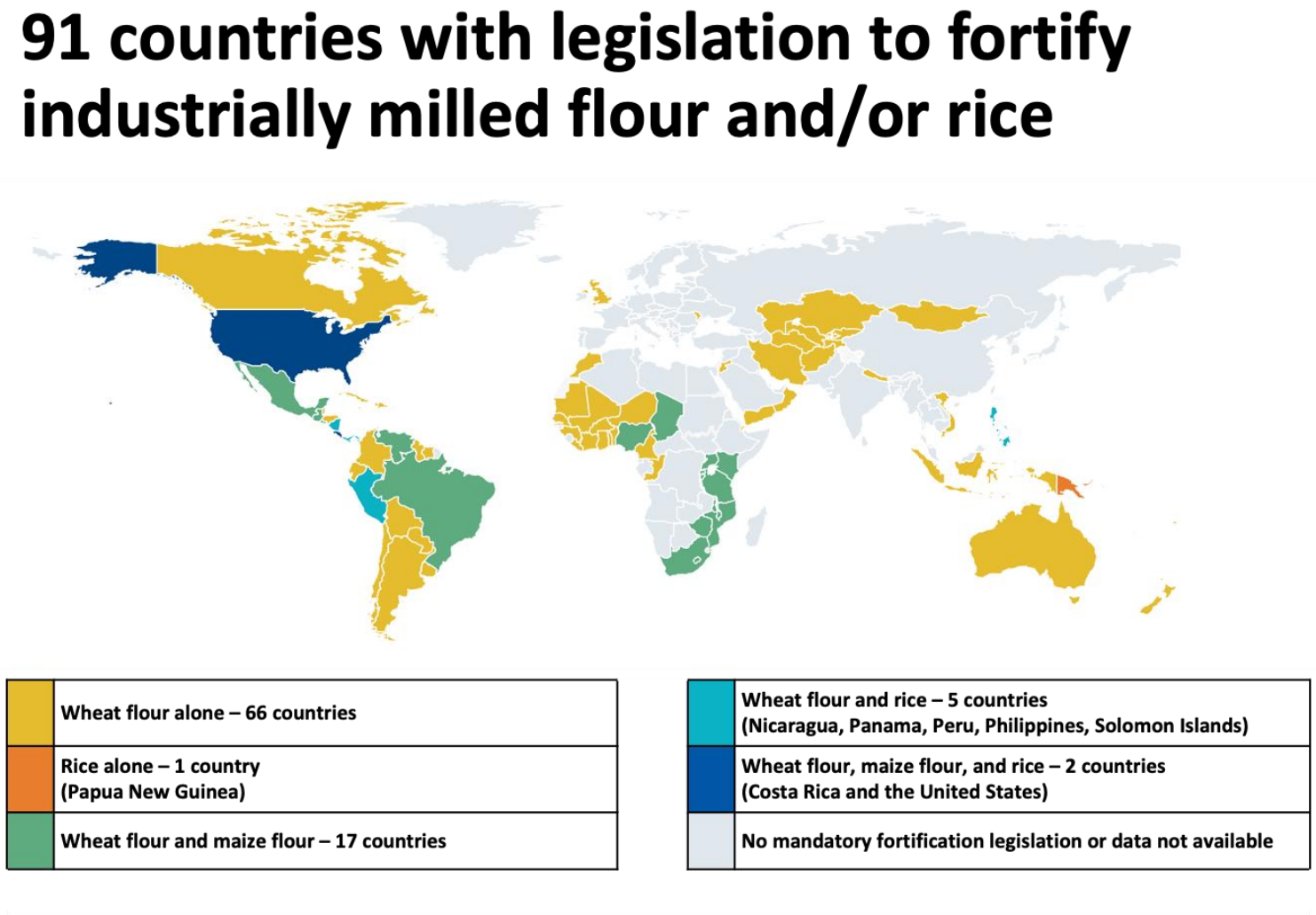


Figure 0.2: Mandatory cereal grain fortification legislation in the World (October 2021). Source: Food Fortification Initiative [19].

Worldwide, there are more than 300.000 babies born with neural tube defects each year and folic acid supplementation can help prevent 150.000-210.000 of them [20]. In addition, in recent years it has been observed that impaired folate status is involved in other diseases associated with homocysteine as a biomarker or vasodilation (further explanation in part 1.4).

1.2.2 Methionine pathway

Methylenetetrahydrofolate reductase (MTHFR) enzyme catalyses the formation of 5-methyl-THF, the most reduced form of folate, to be used as a cofactor in the remethylation of homocysteine into methionine. This reaction also needs vitamin B12 and Zn^{+2} as cofactors and is mediated by Methionine synthase (MTR) [21]. Homocysteine can also be transformed into methionine in a folate independent reaction, mediated by betaine-homocysteine S-methyltransferase (BHMT). However, BHMT is only expressed in the kidney and in the liver [22], while MTR expression is more widespread [23].

Methionine cycle is of crucial importance, as methionine is the precursor of SAM, the reactive methyl carrier. In mammals, SAM serves as methyl donor, playing a major role in epigenetics processes and the synthesis of phosphatidylcholine, creatine and polyamine [24]. Regarding methyl group donation, SAM is converted into SAH. SAH is subsequently cleaved to homocysteine, to be remethylated into methionine. Homocysteine can also enter the transsulphuration pathway, thus removing sulphur from the methionine conservation cycle and forming other products, such as cysteine [25].

Hyperhomocysteinemia

Hyperhomocysteinemia is defined as fasting plasma total homocysteine concentration (tHcy) >15 μM and is usually divided into the following categories: moderate (16-30 μmol/L), intermediate (31-100 μmol/L), and severe (>100 μmol/L) [26].

Diverse factors can determine homocysteine status. Refsum et al. (1998) compiled the major determinants that will be exposed below [27].

Among the **non-modifiable factors**, sex, age and genetic factors may modelate tHcy. Women have been found to have lower tHcy than men. These differences have been attributed mainly to B vitamin status and sex hormones (Deeper reviewed in [28]). Higher tHcy concentration in men may also be associated with creatinine synthesis (the precursor of homocysteine), which is proportional to muscle mass [29]. In addition, since tHcy clearance occurs via the kidneys, renal dysfunction leads to an increased tHcy. The deterioration of renal function due to the effects of age would explain the increase in tHcy with age. Polymorphisms in 1CM enzymes such as MTHFR and CBS are associated with increased plasma tHcy concentration.

Modifiable factors affecting homocysteine concentrations can be separated into three groups: diet, lifestyle and drug use. Two large studies (the Framinghan study and the Hordaland study) found that both food and supplemental intake of B vitamins were inversely associated with tHcy status [30, 31]. Regarding lifestyle habits, smoking, alcohol and coffee consumption may increase tHcy concentrations. It has been observed that smokers have a less varied diet than non-smokers [32], however, after adjusting for folate intake, smoking has been found to have a diet-independent effect on increasing tHcy concentrations [33]. The association between alcohol consumption and tHcy is complex. Alcohol abuse has been associated with increased

in tHcy [34, 35], however, moderate consumption has been associated with decreased tHcy (due to folate content in beer) [36], while others reported no association between homocysteine and alcohol consumption [28]. Coffee consumption has been associated with an increase in homocysteine concentrations, while daily physical exercise has been associated with a reduction [37, 38]. Different types of medications such as sex hormones, insulin, anti-epileptic drugs, cancer treatment drugs (Methotrexate) or proton pump inhibitors have been associated with increased tHcy [28].

MTHFR C677T polymorph*ism (*RS1801133*)*

First described in 1995 [39], the *MTHFR* C677T polymorphism is a nonsynonymous mutation in exon 4 resulting in an alanine to valine substitution in the catalytic domain. This variant encodes a thermolabile enzyme whose specific activity at 37ºC is reduced by ≈55% compared with the wild variant [39]. Heterozygotes for this polymorphism show intermediate values between wild and mutant genotypes. Biochemically, wild and variant enzymes show no differentiation, however, the TT genotype tends to dissociate into monomers, losing its FAD cofactor [40]. The frequency of the TT genotype of the *MTHFR* C677T polymorphism varies between regions and ethnicity. The ALFRED database (The allele frequency database) [41] includes 313 populations and the frequency of the T allele around the world. A representation of the prevalence of the T allele of the *MTHFR C*677T is shown in **Figure 0.3.**

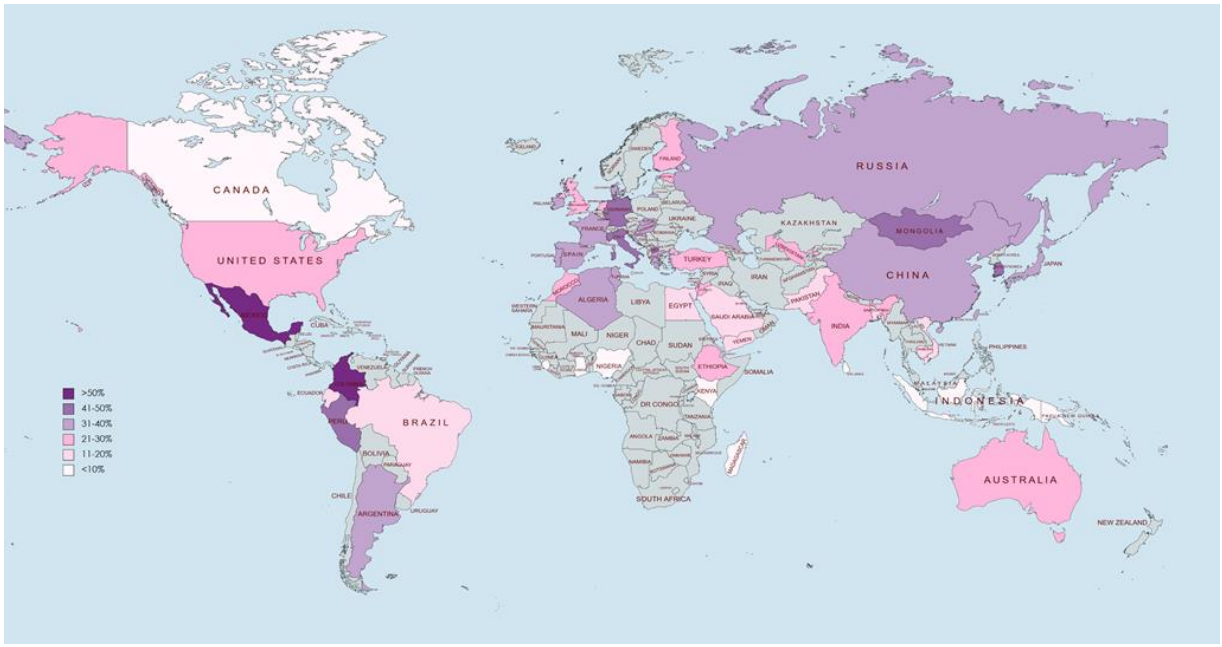


Figure 0.3: *MTHFR* C677T polymorphism T allele frequency in the world (Source: ALFRED database).

In addition, homozygosity for this polymorphism is also variant. As with the T allele, the *MTHFR* 677TT genotype is highly prevalent in the Hispanic population, especially in Mexican (35.7%) and South Europe as Spanish (18.1%), Italian (19.9%) and French (14.2%) populations [42, 43]. In contrast, African ethnicity has the lowest frequency values for this polymorphism, less than 2%.

MTHFR is the only enzyme that catalyses the production of 5-methyl-THF, so a decrease in its activity, due to the polymorphism, impairs the re-methylation of homocysteine. Several studies have associated the presence of the TT genotype of the *MTHFR* C677T polymorphism with high tHcy [44–48]. Moreover, this association occurs mostly when folate [46] or riboflavin [49] statuses are low. This polymorphism results in an imbalance in folate pools, as 5-MTHF status decreases and methylene-THF status increases. Consequently, plasma folate concentrations have been observed to be decreased in the TT genotype [43, 50].

1.3 L-Arginine pathway

L-Arginine

L-Arginine is a basic amino acid used as a precursor for the synthesis of proteins, NO, citrulline, creatinine and others. Although it's blood concentration is affected by protein turnover, supply by the kidney and food intake [51], *de novo* synthesis seems to be insufficient for cellular demands, especially in high-demand processes such as growth or wound healing, leading L-Arginine to be considered as a semi-essential amino acid [52].

Even though L-Arginine concentrations are higher in the extracellular space (50-200 μM) than in the intracellular space (100-800 μM) of endothelial cells, cationic amino acid transporters (CAT) mediate the uptake of L-Arginine across the plasma membranes [53]. These CAT proteins are also carriers for other cationic amino acids such as L-Lysine or L-Arginine competing metabolites such as L-Ornithine, ADMA or SDMA [52].

After oral administration, 40% of L-Arginine is catabolized by arginase in the small intestine [54] into L-Ornithine and urea in order to eliminate toxic ammonia in the urea cycle [51]. Once inside the cell, the half-life of L-Arginine is between 1 to 2 hours [55] given the many pathways in which L-Arginine is involved, among others, protein synthesis.

Nitric Oxide synthesis and functions

After proteolysis, free L-Arginine is the substrate for NOS to form NO. So far, three isoforms of this enzyme have been found in mammals. The NOS1, NOS2 and NOS3

genes encode the neuronal (nNOS), inducible (iNOS) and endothelial nitric oxide synthase (eNOS) enzymes, respectively [56]. Unlike nNOS and eNOS, iNOS isoform is calcium- and calmodulin-independent under physiological conditions and acts is crucial for innate immune system and inflammatory response [57]. nNOS is present in the central and peripheral nervous systems and intervenes in neuronal signalling and communication [58]. The eNOS isoenzyme is mostly expressed in endothelial cells and is involved in several anti-atherosclerotic and vasoprotective processes [59]. Located in the caveolae (invaginations in cell membranes), eNOS is bound to a resident coat protein, caveolin [60]. The regulation of the enzyme depends on the cellular localization and the interaction with proteins as caveolin [61]. eNOS knockout mice, in addition to being hypertensive, had poor vasorelaxant activity, developmental growth problems, impaired wound healing capacity and endothelium dependent vasodilation, among other conditions [60].

NO released by eNOS dilates all types of blood vessels by stimulating guanylyl cyclase and increasing cyclic guanosine monophosphate (cGMP) in smooth muscle cells, inhibiting platelet aggregation and adhesion. This protects against thrombosis and prevents platelet-derived growth factors that stimulate smooth muscle proliferation [59]. Endothelial NO is also a modulator of leukocyte adherence to vascular endothelium, interfering with CD11/CD18 (the adhesion molecule of leukocytes) [62]. In addition, NO directly interferes with caspases (key in apoptosis signalling), counteract apoptosis by proinflammatory or proatherosclerotic factors such as TNFa, reactive oxygen species, angiotensin II or oxidised LDL [63].

However, arginase (the enzyme that hydrolyses L-Arginine in the urea cycle) may compete with NOS for L-Arginine, which could reduce NO [64]. In addition, L-Arginine analogues (ADMA or SDMA) may also impair NO synthesis.

Methylated L-Arginines

In the homocysteine remethylation process, SAM donates methyl groups to the Protein arginine N-methyltransferase (PRMT). In normal protein turnover, PRMTs catalyse the posttranslationally methylation of L-Arginine, transferring methyl groups to the guanidino nitrogen atoms [5]. Depending on the type of methylated arginine they generate, different PRMTs have been described. PRMT type I and type II are capable of monomethylation at the N-terminal region of L-Arginine residues incorporated into proteins, forming monomethylated arginine (MMA). In addition, both types can add a second methyl group to the same (type I) or opposite (type II) nitrogen, forming asymmetrically (ADMA) or symmetrically (SDMA) dimethylated arginine, respectively. L-Arginine and its methylated analogues are released as free amino acids after proteolysis [65].

ADMA is an endogenous inhibitor that competes with L-Arginine for the active site of eNOS [66], reducing NO synthesis. The Dimethylarginine Dimethylaminohydrolase (DDAH) enzyme is the main enzyme responsible for metabolising ADMA into L-Citrulline and dimethylamine. In humans, it has been estimated that about 300 µl of ADMA are generated per day, of which 250 µl are metabolised by DDAH [67]. An inhibitory effect of ADMA on NOS was discovered in 1970 [68], and it was believed that SDMA did not inhibit NOS. However, it was proposed that CAT transporters substrates, such as SDMA, compete with L-Arginine for its transfer in and out of the

cell. In addition, these L-Arginine analogues may drive out intracellular L-Arginine, probably limiting its supply to the enzyme [69, 70].

NOS G894T polymorphism (RS1799983)

This missense variant within exon 7 of eNOS was discovered in 1998 in patients with coronary spastic angina [71]. The G to T conversion at nucleotide position 894 results in the substitution of glutamic acid for aspartic acid at codon 298. This polymorphism is also known as Glu298Asp.

In healthy patients, the T allele of the *NOS* G894T has been associated with reduced basal NO production [72]. However, studies of recombinant eNOS showed no discernible difference in the Michaelis constant Km, nor the Vmax, nor the Ki for ADMA, of the two forms of the enzyme. This polymorphism does not contact the active site as it is located within a loop on the outer surface of the enzyme [73], suggesting that the polymorphism would have to exert its effect by a mechanism independent of NO synthase catalysis [74]. The regulation of the enzyme depends on the cellular localization and the interaction with proteins such as caveolin [61]. Studies that processed intracellular eNOS isoforms observed a cleavage of proteins, of 100- and 35-kDa fragments, with the amino acid substitution of aspartate versus glutamate due to the G>T polymorphism [75, 76]. However, studies using buffers to prevent proteolysis, in order to limit acidic hydrolysis of Aspartic acid–Proline bonds, did not observe such cleavage, which may indicate that this is an artefact of Western blotting [61, 77]. Shaheen et al. (2021), in a *in silico* deep structural analysis, saw that the Glu298Asp substitution resulted in the loss of flexibility and deformities that alter the conformation and configuration of the protein and ultimately decrease protein stability [78].

1.4 Alterations in 1CM and L-Arginine pathway and vascular disease

Endothelial cells are located between circulating blood, blood cells and the vascular smooth muscle. Initially, it was thought that denudation of the endothelium triggered atherosclerosis. However, in 1986 Ludmer et al. (1986) first described endothelial dysfunction in atherosclerotic arteries. The current hypothesis proposed that atherosclerosis may be due to endothelial dysfunction rather than denudation [79, 80]. The endothelium is indispensable for the regulation of vascular tone and the maintenance of vascular homeostasis, dependent on the balance between vasodilators and vasoconstrictors. When this balance is disturbed, endothelial dysfunction occurs, which is considered an early marker for atherosclerosis. Mostly, this endothelial dysfunction is due to a decreased bioavailability of vasodilators while vasocontraction factors increase [81].

Atherosclerosis is an inflammatory disease in which there is an accumulation of fatty, especially LDL cholesterol, and fibrous material in the intima (the inner layer of the arteries). Prolonged accumulation can lead to atherosclerotic plaques invading the lumen of the arteries, preventing optimal blood flow and resulting in various cardiovascular diseases [79, 82].

Endothelial dysfunction is formed in most cardiovascular diseases (CVDs), including renal failure [83]. CVDs are the leading cause of death globally. Several cardiovascular risk factors such as smoking, age, hypercholesterolaemia, hypertension, hyperglycaemia, and a family history of premature atherosclerotic disease are all associated with altered endothelial function [84].

In 2020, Song et al., provided an estimation of the prevalence of carotid atherosclerosis in the general population. The global prevalence of increased carotid intima-media thickness, carotid plaque and carotid stenosis was estimated to be 27.6%, 21.1% and 1.5%, respectively. In all cases, a percentage change of more than 57% was observed in comparison with data obtained in 2000 [85].

Lifestyle changes associated with cardiovascular disease such as smoking cessation, physical exercise or lowering cholesterol also have an impact on endothelial health [86]. In addition, it has been observed that pharmacological interventions may also have an effect on endothelial function.

One-carbon metabolism

As mentioned above, **folate,** in the form of 5-methyl-THF is a substrate for the remethylation of homocysteine to methionine. In adult cardiovascular cells, neither BHMT nor cystathionine-β synthase (CBS) activity is expressed [87]. In other cells, homocysteine clearance may occur via the betaine or trans-sulphuration pathway. However, in cardiovascular cells, removal of homocysteine, which is cytotoxic at high concentrations, must occur via remethylation by methionine synthetase. This reaction is determined by adequate status of both folate and vitamin B12 [9]. Therefore, folate deficiency has been associated with hyperhomocysteinemia and cardiovascular disease.

SAM is a major donor of methyl groups, which are essential for gene expression and epigenetics. However, SAM depends on an adequate supply of dietary folate. Low dietary folate intake leading to folate deficiency decreases DNA methylation [88], and this becomes more significant in the presence of the TT genotype of the *MTHFR*

C677T polymorphism [89]. Abnormal methylation may result in undesirable activation of gene expression, apoptosis or developmental malformations [88].

Elevated **homocysteine** status has been recognised as a cardiovascular biomarker and has been associated with several diseases. Cross-sectional and longitudinal studies have found an association between elevated tHcy and the risk of hypertension. These associations have been observed in the general population [90, 91], in adolescents [92] and the elderly [48, 93], and in different ethnicities [94].

In addition, a meta-analysis of 40,173 participants concluded that an increase in 5 μmol/L of plasma homocysteine is associated with an increased risk of hypertension by 30% [95]. Renal failure has also been closely associated with elevated homocysteine status. Renal tubular cells catabolize the reduction of the fraction of homocysteine nonprotein-bounded so that it can be used in the remethylation or trans-sulphuration pathways. In patients with renal failure, the usual elimination of the 70% of homocysteine in plasma is impaired, however, the mechanism of this association remains unknown [96].

Several studies have associated the ***MTHFR* 677 TT** genotype with an increased risk of various cardiovascular diseases such as hypertension [48, 97–99], coronary artery disease [100–102] or stroke [103, 104], in different populations, ethnicities and ages. However, in a meta-analysis of 12,513 genotyped participants, the association between *MTHFR* C677T and homocysteine was not causally related to the pathogenesis of the vascular disease [44], which could indicate that the mechanism by which it acts is a different one.

L-Arginine pathway

Due to their effect on NO reduction, ADMA and SDMA are emerging risk factors for cardiovascular diseases. High plasma **ADMA** concentrations have been associated with several CVDs such as hypertension [105–107], stroke [108] or coronary artery disease [109]. In addition, two prospective studies, the Framingham Offspring Study and the Population Study of Women in Gothenburg, observed an increased risk of 30% of high plasma ADMA concentrations with all-cause mortality and incident cardiovascular risk, respectively [110, 111].

Only 20% of ADMA is eliminated by renal extraction, while **SDMA** is mainly catabolized by this system and its association with renal failure has been more studied [112]. Even so, higher plasma concentration of SDMA in participants with hypertension has been observed in children, adolescents and elderly [113, 114].

L-Arginine/ADMA and ADMA/SDMA Ratio have been postulated to be better predictors than the metabolites separately [115, 116]. The L-Arginine/ADMA Ratio reflects the substrate and inhibitor of eNOS and is postulated to be a better indicator of eNOS activity [115]. SDMA is not a substrate for DDAH, thus, its status is affected by changes in protein methylation, proteolysis and cell transporters. An increase in the ADMA/SDMA Ratio may indicate impaired DDAH activity and may also reflect relative changes in the two classes of PRMT enzyme (type 1 and type 2) that methylate ADMA and SDMA, respectively [116].

A decreased L-Arginine/ADMA Ratio in patients compared to controls has been seen in a variety of hypertensive disorders such as intima media thickness [117], high blood pressure [118], pulmonary artery disease [119] or essential hypertension [120]. Lower

ADMA/SDMA Ratio have been observed in children and adolescents with chronic kidney disease [121].

The GT+TT versus GG genotype of the ***NOS* G894T** polymorphism have been associated with increased risk of premature coronary artery disease [122] and ischemic stroke [123]. In addition, in a meta analysis of 14,185 cases and 13,407 controls, the presence of the T allele of the *NOS* G894T polymorphism was associated with a 40% increased risk of hypertension compared to those with the GG genotype (OR [95% CI] (1.42 [1.25, 1.63]) [124].

1CM, L-Arginine pathway and other mechanisms' association with endothelial dysfunction

It has been suggested that L-Arginine methylates, more specifically ADMA, may act as mediator of the endothelial dysfunction associated with hyperhomocysteinemia [125]. Homocysteine is the substrate for the CBS enzyme, which produces H_2S. This compound has been shown to be involved in the NO synthesis pathway. In addition, NO synthesis is also diminished by high concentrations of homocysteine, as this inhibits the enzyme DDAH, responsible for the degradation of ADMA, which inhibits eNOS [126].

Supplementation with compounds from both the 1CM and L-Arginine pathway has been observed to have a beneficial effect on the endothelium. Supplementation of 5-10 mg/day of folic acid improved endothelial function in patients with coronary artery disease [127], hyperhomocysteinemia [128] and coronary heart disease [129]. While reduction of homocysteine seems to be the general hypothesis, the mechanism is not yet clear. L-Arginine has been proposed as a therapeutic option for the treatment of cardiovascular diseases. L-Arginine supplementation has been shown to restore NO

production and improve endothelial function in cases of hypercholesterolemia or coronary artery disease [130, 131]. Furthermore, in healthy elderly people (mean age 73.2 ± 2.7 years), supplementation with L-Arginine would be beneficial as it may improve L-Arginine/ADMA Ratio status [132]. In the resting endothelial cell, the formation of an inhibitory eNOS–caveolin heteromeric complex may serve to ensure the latency of the NO signal until calcium-mobilising extracellular stimuli destabilise this complex and activate the enzyme [133].

Antihypertensive treatments are very varied and act on different target pathways. Angiotensin-containing enzyme inhibitors (ACEIs) increase the bioavailability of NO. ACEIs increase the concentration of bradykinin, which stimulates NO release and also causes endothelium-dependent hyperpolarization [134], key in the endothelium-dependent relaxation.

Oxidised LDL cholesterol increases caveolin-1 synthesis, and the subsequent inhibition of NO production [135].

2. HYPOTHESES AND AIMS

Hypotheses

Chapter 1

High tHcy concentrations and the *MTHFR* C677T polymorphism are associated with hypertension via the L-Arginine pathway. Increased risk of hypertension associated with tHcy might be through the inhibition of eNOS activity by high concentrations of ADMA and SDMA.

Chapter 2

Impaired first trimester maternal impaired One-Carbon metabolism is associated with L-Arginine pathway metabolites during pregnancy. Elevated L-Arginine competitors are associated with an increased risk of adverse outcomes of placental origin.

Chapter 3

The association between impaired One-Carbon metabolism and the L-Arginine pathway also occurs in fathers. Paternal L-Arginine pathway metabolites are associated with an increased risk of adverse outcomes of placental origin not explained by maternal factors.

Aims

Chapter 1

GENERAL AIMS

To explore whether there is evidence for the involvement of the L-Arginine pathway in the 1CM-hypertension link, in a representative sample of an adult population.

SPECIFIC AIMS

- To investigate the association of high tHcy and the *MTHFR* 677TT genotype with L-Arginine pathway metabolites according to age group and sex differences.
- To explore the association of impaired L-Arginine pathway metabolites and the *NOS* G894T polymorphism on the increased risk of hypertension in adult men and women.
- To study whether the L-Arginine pathway plays a mediating role in the association between elevated tHcy, the *MTHFR 677*TT genotype and hypertension risk.

Chapter 2

GENERAL AIMS

To explore the association between impaired One-Carbon metabolism and the L-Arginine pathway and to investigate the association of the L-Arginine pathway with adverse outcomes of pregnancy.

SPECIFIC AIMS

- To describe plasma L-Arginine pathway metabolite fluctuations throughout the three trimesters of pregnancy, at labour and in cord.
- To assess the association between elevated first trimester tHcy and *MTHFR* C677T genotype with L-Arginine pathway metabolites during pregnancy.

- To determine the association between impaired first trimester L-Arginine pathway metabolites and risks of impaired placentation (diagnosed by pathological Doppler of the uterine arteries at 20 GW) and pregnancy-induced hypertension.

Chapter 3

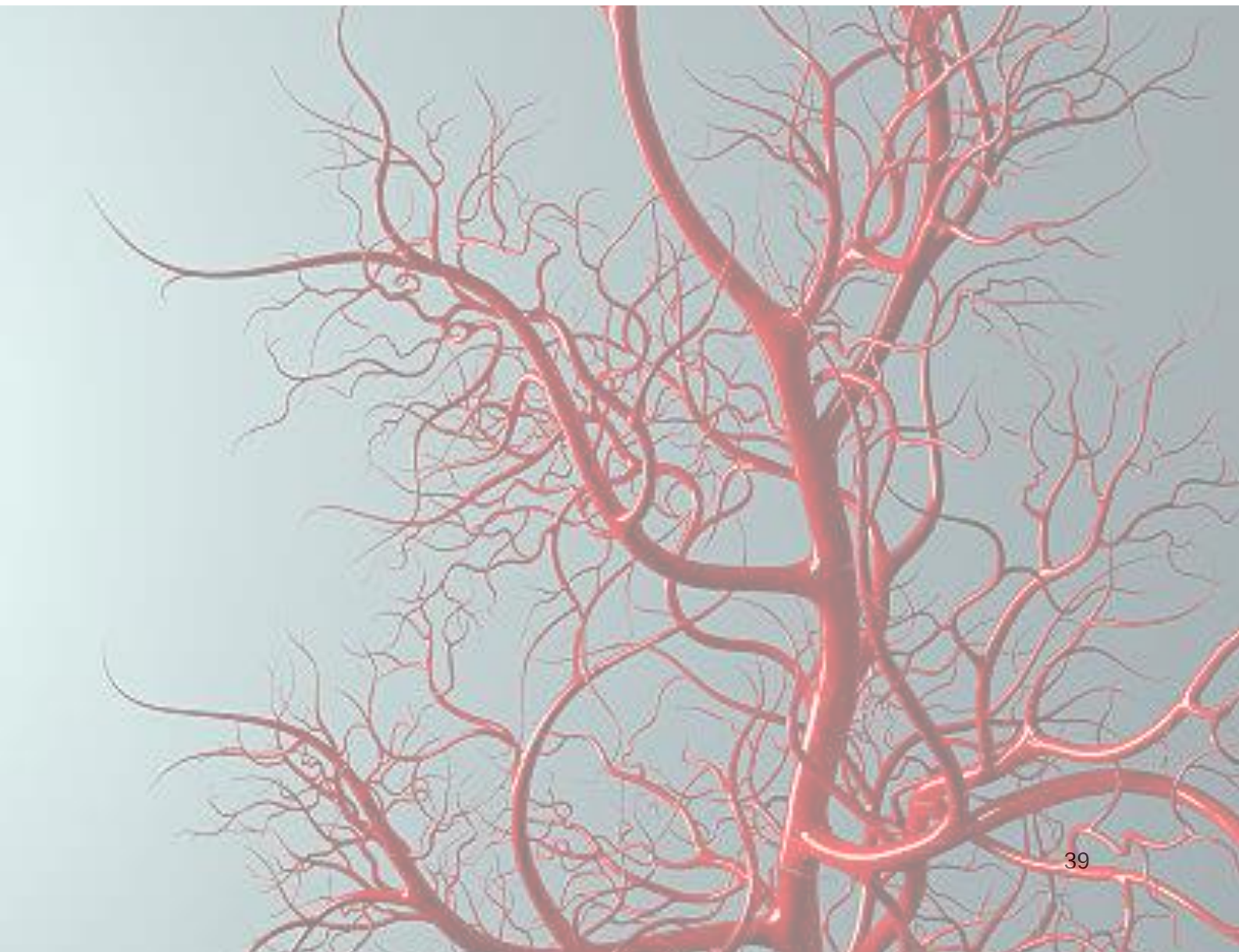
GENERAL AIMS

To explore the involvement of paternal L-Arginine pathway metabolites with adverse pregnancy outcomes in fathers.

SPECIFIC AIMS

- To investigate the association of impaired paternal One-carbon metabolism with L-Arginine pathway.
- To study the associations between impaired paternal L-Arginine pathway metabolites, the *NOS* G894T polymorphism and poor placentation and pregnancy-induced hypertension.

Chapter 1
The Population study



3. CHAPTER 1: THE POPULATION STUDY

3.1 Introduction

Endothelial dysfunction is an early event in the pathogenesis of atherosclerosis and hypertension [83]. Non-modifiable factors (age, sex and genetic factors) and modifiable factors (smoking habits, alcohol consumption and physical activity) have been shown to increase the risk of having hypertension [136].

Impaired 1CM has also been associated with hypertension. The *MTHFR* C677T polymorphism, that can lead to increased tHcy, has been reported to be associated with hypertension, in situations of compromised 5-methyltetrahydrofolate production due to low folate or riboflavin intake, leading to impaired remethylation of homocysteine to methionine [137]. In the Tarragona area, 17.5% of the population have the homozygous variant genotype [43]. In a random control trial with hypertensive adults with the T variant of the *MTHFR* C677T polymorphism, 92% of participants were prescribed multiple classes of antihypertensive drugs, but less than 40% achieved a blood pressure ≤140/90 mm Hg. However, riboflavin supplementation, together with their antihypertensive medication, reduced systolic blood pressure in 16 weeks [138].

Our group recently reported that elevated tHcy is associated with an increased risk of hypertension in adults aged 50 years or more (from a study of a representative sample of the adult population). The association was not observed in adults under 50 years. However, in this group, an increased risk of hypertension was observed in the TT genotype of *MTHFR* 677 versus CC [48]. However, the mechanisms by which 1CM

components are associated with hypertension remains unknown. Among others, one of the hypotheses that could explain this association is the L-Arginine pathway and its involvement in NO synthesis.

ADMA [70] and the T allele of the *NOS* G894T polymorphism have previously been associated with an increased risk of hypertension [139]. SDMA has mainly been associated with renal dysfunction, since it is eliminated via the kidneys [112]. However, studies investigating its possible association with cardiovascular disease are scarce and its role is not entirely clear. A study by Kiechl et al. (2009), postulated that both ADMA and SDMA can similarly predict cardiovascular risk [140].

Here, we set out to investigate whether 1CM is associated with the L-Arginine pathway and whether the 1CM – hypertension association is mediated by this pathway, in a representative sample of adults from a population unexposed to mandatory fortification with folic acid or to B vitamin supplement use.

3.2 Material and methods

Subjects and procedure

The regional population-based cross-sectional study including 812 adults aged 18-75 years, from two towns in Tarragona province (Spain), has been described previously [43, 141, 142]. The population records for those towns were stratified according to sex and age group. Participants were randomly selected in accordance with these strats so that the population distribution was represented in our study. The study was carried out between 1998 and 2002 and was approved by the Sant Joan University Hospital,

Reus and Jordi Gol Gorina Foundation ethics committees. After recruitment, 24 participants taking B-vitamin supplements or medication known to affect folate or cobalamin status were excluded from the study.

Additionally, 5 participants with impaired renal function (plasma creatinine >97 mmol/L for women and >124 mmol/L for men) and 59 participants whose blood samples were not processed in less than 2 hours after their collection were excluded from all analyses to prevent artefacts in tHcy determinations.

Clinical history and lifestyle data (including sex, age, BMI, previous and current illness, medication use, smoking habits, socioeconomic status, alcohol and illegal drug use) were recorded at the medical check-up, and blood pressure measured, as previously described [142].

Blood pressure was measured following a standardized protocol and by a trained clinician. At least 15 minutes before the measurements, the participants remained seated. During the measurement, the participants kept their back supported, their feet on the floor and their arm upside down, so that the cubital fossa was at the level of the heart. Using a mercury column sphyg-momanometer (Riester), the mean of two measurements (2 minutes apart) was recorded. Participants with no previously detected hypertension and a blood pressure measurement >140/90 mmHg, at the check-up, were recommended to consult their doctor and were excluded from the analysis of hypertension risk, to avoid misclassification. Participants with no history of hypertension and with normal blood pressure (<140/90 mmHg) at the check-up were categorised as controls. All controls had a SBP/DBP ≤136/88 mmHg. Participants with normal blood pressure but taking antihypertensives medication were classified as cases. Participants with previously diagnosed hypertension were classified as

hypertension cases, even if they were normotensive at the visit due to medical control of their condition.

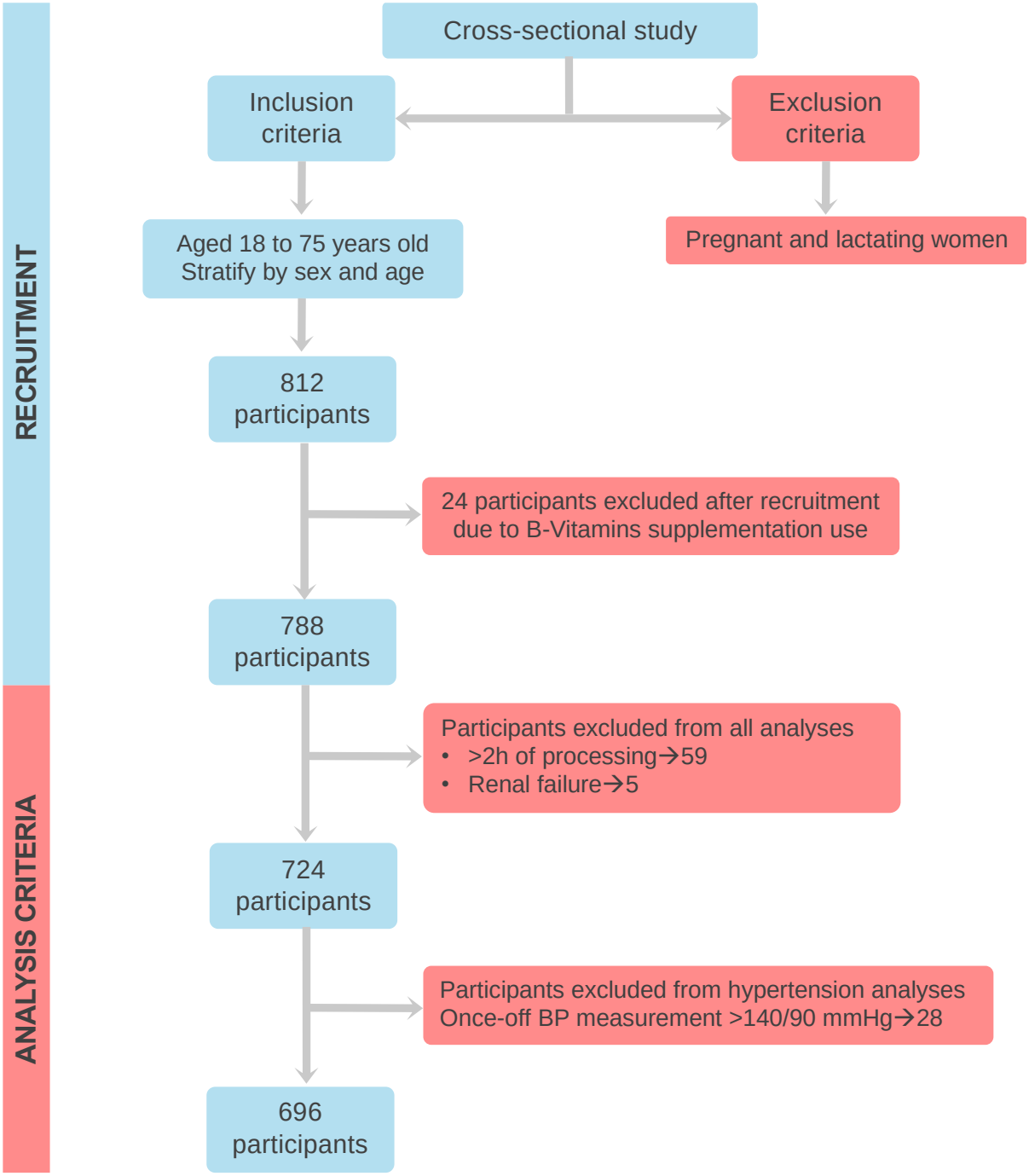


Figure 1.1: Flow charts of the participants in the Population study.

Collection and classification of data on medication use

Medication use recorded at the check-up were coded according to the WHO ATC/DDD index [143]. A PubMed search of literature linking the active ingredients of any medication recorded by clinicians with components of the L-Arginine pathway was carried out. Treatments were classified into three categories (Supplementary **Table 1**): Group 1) none or sporadic, Group 2) chronic, not affecting the L-Arginine pathway (i.e., pain treatment, insulin, depression, asthma) and Group 3) medical treatment known to affect the L-Arginine pathway (i.e. anti-hypercholesterolemia, anti-hypertensive or thyroid treatments).

Blood samples

Fasting venous blood samples were collected into EDTA-K3 vacutainers and kept chilled until they were processed, including plasma separation, in less than 2 hours after collection. Plasma whole blood diluted in 1% ascorbic acid solution for Red Blood Cell Folate, washed erythrocytes and Leukocyte fractions, were kept at -80ºC for posterior determinations as previously described [43, 144].

Total plasma homocysteine was determined by fluorescence polarisation immunoassay using the IMx autoanalyzer (Abbott laboratories). L-Arginine, ADMA and SDMA were measured by HPLC-MS/MS [145]. Plasma folate and red blood cell folate were measured by microbiological assay with *Lactobacillus casei* [146] and plasma cobalamin with *L. leichmannii* [147]. Functional riboflavin status (erythrocyte glutathionine reductase activation coefficient (EGRAC) [144]) and plasma creatinine concentration (Jaffé reaction (Quimica Clinica Aplicada) [43, 144]) were determined using the COBAS MIRA autoanalyzer (Roche Diagnostics). Plasma total and HDL-

cholesterol and triglycerides were determined as previously described [148] and the Friedewald equation used to estimate LDL-cholesterol [149].

MTHFR C677T (rs1801133) and *NOS* G894T (rs1799983) genotypes were determined from leukocyte extracted DNA by matrix-assisted laser desorption/ionisation/time-of-flight mass spectrometry [150].

Statistical analysis

Descriptive data are reported as means and 95% confidence interval (CI) for normally distributed variables and as geometric means and 95% CI in variables with skewed distributions that were ln-transformed for the application of parametric statistical tests. Categorical variables are reported as percentages and 95% CI. Comparison between age groups and between sexes were by ANOVA for continuous variables and χ^2 for categorical variables.

The association between 1) tHcy, 2) *MTHFR* 677CT versus CC and TT versus CC genotypes, and L-Arginine pathway metabolites were assessed using multiple linear regression analysis. All models were adjusted for age, sex, body mass index (BMI), smoking (cigarettes/day), category of regular alcohol intake (moderate (<16 g/d in women and <24 g/d in men) versus none; high versus none (≥16 g/d in women and ≥24 g/d in men)), low versus mid-high socio-economic status, plasma LDL cholesterol and medication use (reference: none/sporadic). The *MTHFR* genotype models were further adjusted for plasma folate, plasma cobalamin, EGRAC and plasma creatinine. The models included the interaction terms tHcy x medical treatment category or *MTHFR* genotype x medical treatment category, as appropriate. Stratified analyses by age group (≤ 50 years, > 50 years) or sex were also performed, adjusting for the same variables as the total population models except age or sex, respectively.

The probability of having hypertension was explored using multiple logistic regression analysis. Basic models explored L-Arginine pathway metabolite status (low-mid tertile versus highest tertile for L-Arginine and L-Arginine/ADMA Ratio and mid-high tertile compared with lowest tertile for ADMA, SDMA and ADMA/SDMA Ratio) as predictors of hypertension. All tertiles were sex and age group specific (≤ 50 years, > 50 years). Models were adjusted for BMI, smoking (cigarettes/day), category of regular alcohol intake (moderate (<16 g/d in women and <24 g/d in men) versus none; high versus none (≥16 g/d in women and ≥24 g/d in men)), low versus mid-high socio-economic status, plasma creatinine, plasma total cholesterol and diabetes. The *NOS* 894 TT and GT versus GG genotype models were stratified by ADMA or SDMA low or mid-high tertiles and adjusted for the same variables as above. Again, stratified model by age group were not adjusted by age and sex stratified model were not adjusted by sex.

To test whether the relationship between impaired 1CM and diagnosed hypertension was via the L-Arginine pathway, we performed three mediation analyses following the principles of Hayes [151]. 1) The potential association of the *MTHFR* 677 TT versus CC genotype with ADMA via tHcy, the mediation effect of ADMA tertiles in the association of 2) *MTHFR* 677 TT versus CC genotype and 3) highest versus low-mid tHcy tertiles with hypertension.

All mediation models were stratified by age group or sex and adjusted for BMI, smoking (cigarettes/day), category of regular alcohol intake (moderate (<16 g/d in women and <24 g/d in men) versus none; high versus none (≥16 g/d in women and ≥24 g/d in men)), low versus mid-high socio-economic status and medication category (reference: none/sporadic). Models including the *MTHFR* 677 TT versus CC genotype were also adjusted for plasma folate, plasma cobalamin and EGRAC. Models

including diagnosed hypertension were also adjusted for plasma total cholesterol, diabetes and *NOS* GT+TT versus GG genotypes.

All models met the requirements for assumptions in linear regressions (normality of errors, multicollinearity, homogeneity of variance (homoscedasticity)). Bonferroni corrected p-values to account for multiple comparisons in linear regressions are reported. Logistic regression diagnostics were performed by examining box plots to identify outliers and Cook's distance (>4/√n) to identify influential cases. The difference in the number of participants included in each analysis was due to missing data for some of the biochemical or clinical variables. Data was analysed using SPSS software version 27.0. The mediation analyses were performed using macro PROCESS software version 3.5.3. Significance was accepted at $p < 0.05$ and the indirect effects of mediation analysis were considered as statistically significant when the 95% CI did not include the value 0.

3.3 Results

Characteristics and lifestyle habits of participants stratified by age group and sex are described in **Table 1.1**. BMI was higher in the older compared to the younger age group and frequency of heavy alcohol consumption was higher in men compared to women. 42.5% of the population under 50 years of age were smokers, compared to 14.2% in those of 50 or over, but smoking habits did not differ between men (36.5%) and women (30.5%).

Table 1.1: Habits and lifestyle characteristics according to age group and sex

	All participants	≤ 50 years old	>50 years old	Women	Men
Women[1]	51.6 (48.2, 55.1) [788]	51.7 (47.5, 56.0) [534]	51.6 (45.5, 57.7) [254]	-	-
Age[2]	43.01 (41.9, 44.1) [788]	-	-	43.0 (41.5, 44.5) [407]	43.0 (41.5, 44.5) [381]
BMI[2]	27.1 (26.7, 27.4) [773]	25.7 (25.3, 26.1) [524]	29.9 (29.3, 30.5)^{aaa} [249]	26.7 (26.1, 27.2) [399]	27.5 (27.0, 28.0)^{b} [374]
Smokers[1]	33.4 (30.2, 36.7) [788]	42.5 (38.4, 46.7) [534]	14.2 (10.4, 19.0)^{aaa} [254]	30.5 (26.5, 35.1) [407]	36.5 (31.8, 41.4) [381]
Alcohol consumption[†]					
None[1]	59.4 (55.9, 62.8) [468]	59.9 (55.7, 64.0) [320]	58.3 (52.1, 64.2) [148]	80.3 (76.2, 83.9) [327]	37.0 (32.3, 42.0) [141]
Moderate[1]	24.9 (22.0, 28.0) [196]	26.4 (22.8, 30.3) [141]	21.7 (17.0, 27.1) [55]	14.7 (11.6, 18.5) [60]	57.7 (31.1, 40.6)^{bbb} [136]
High[1]	15.7 (13.4, 18.4) [124]	13.7 (11.0, 16.8) [73]	20.1 (15.6, 25.4)^{a} [51]	4.9 (3.2, 4.7) [20]	27.3 (23.1, 32.0)^{aaa} [104]
Low socioeconomic status[1‡]	37.6 (34.3, 41.0) [788]	21.7 (18.4, 25.4) [534]	70.9 (65.0, 76.1)^{aaa} [254]	47.4 (42.6, 52.3) [407]	27.0 (22.8, 37.7)^{bbb} [381]
Diagnosed hypertension[1]	16.9 (14.3, 20.0) [656]	5.2 (3.5, 7.6) [466]	45.8 (38.9, 52.9)^{aaa} [190]	17.3 (13.8, 21.6) [358]	16.4 (12.7, 21.1) [298]
Diabetes	4.2 (3.0, 5.8) [788]	1.5 (0.8, 2.9) [534]	9.8 (6.8, 14.1)^{aaa} [254]	3.4 (2.1, 5.7) [407]	5.0 (3.2, 7.7) [381]

BMI, body mass index. [1]Percentage, 95%CI, [n]. [2]Means, 95%CI, [n]. Comparison between age groups and sex was with ANOVA for continuous variables and χ^2 for categorical variables. Twenty-four participants were excluded after the medical check-up due to declared B vitamin supplement use. [†]Category of habitual alcohol intake: moderate (<16 g/d in women and <24 g/d in men) and high (≥16 g/d in women and ≥24 g/d in men). [‡]Socioeconomic status was based on household income and maternal and paternal educational level and occupation. [a] Significant difference between age groups, p <0.05. [aa] Significant difference between age groups, p <0.01. [aaa] Significant difference between age groups, p <0.001. [b] Significant difference between sex, p <0.05. [bb] Significant difference between sex, p <0.01. [bbb] Significant difference between sex, p <0.001.

The prevalence of hypertension in this population is 16.9% and four out of five participants with diagnosed hypertension were over 50 years of age. In addition, this group was more likely to be of lower socio-economic status. More women were in the low socioeconomic status category than men.

Table 1.2 shows plasma and red blood cell concentrations of the studied nutrients and metabolites. Participants aged over 50 years had better status in folate, cobalamin and riboflavin compared to younger participants. THcy, plasma ADMA, SDMA and plasma cholesterol (total and LDL) were all higher in the older compared to the younger group. THcy, plasma SDMA, creatinine and plasma LDL cholesterol were higher and plasma folate status lower, in men compared to women. The older population and women show lower L-Arginine/ADMA Ratio compared to the youngest one and men, respectively. Men had lower ADMA/SDMA Ratio compared to women.

Table 1.2: Plasma and red blood cell nutrient and metabolite concentrations according to age group and sex

	All participants	≤ 50 y	>50 y	Women	Men
One-Carbon metabolism					
Folate (nmol/L)[1]	11.5 (11.1, 11.9) [787]	10.0 (9.5, 10.4) [533]	15.5 (14.5, 16.5)[aaa] [254]	12.3 (11.6, 12.9) [407]	10.7 (10.2, 11.3)[bbb] [380]
RBCF (nmol/L)[1]	812.3 (790.9, 834.4) [787]	747.5 (724.5, 771.2) [533]	967.4 (926.0, 1010.7)[aaa] [254]	800.3 (770.4, 790.3) [407]	824.8 (794.6, 856.3) [380]
B12 (pmol/L)[1]	346.7 (337.8, 355.7) [786]	339.7 (329.4, 350.3) [533]	361.8 (345.0, 379.4)[a] [253]	349.7 (336.7, 363.4) [406]	343.4 (331.7, 355.6) [380]
EGRAC[1]	1.35 (1.34, 1.7) [776]	1.4 (1.37, 1.41) [526]	1.29 (1.26, 1.31)[aaa] [250]	1.36 (1.33, 1.38) [401]	1.35 (1.33, 1.37) [375]
tHcy (µmol/L)[1]	9.6 (9.4, 9.8) [788]	9.3 (9.1, 9.3) [534]	10.2 (9.9, 10.1)[aaa] [254]	8.8 (8.6, 9.0) [407]	10.5 (10.2, 10.8)[bbb] [381]
L-Arginine pathway					
L-Arginine (µmol/L)[2]	65.8 (64.5, 67.2) [784]	66.6 (64.9, 68.2) [531]	64.3 (62.3, 66.4) [253]	64.8 (63.0, 66.7) [405]	66.9 (65.0, 68.8) [379]
ADMA (µmol/L)[2]	0.53 (0.52, 0.54) [783]	0.52 (0.51, 0.52) [530]	0.57 (0.56, 0.58)[aaa] [253]	0.54 (0.53, 0.55) [404]	0.53 (0.52, 0.54) [379]
SDMA (µmol/L)[2]	0.51 (0.50, 0.52) [783]	0.49 (0.48, 0.50) [530]	0.56 (0.54, 0.58)[aaa] [253]	0.49 (0.47, 0.50) [404]	0.53 (0.52, 0.55)[bbb] [379]
L-Arg/ADMA[2]	126.0 (122.3, 127.6) [783]	130.2 (126.9, 133.5) [530]	114.1 (110.1, 118.1)[aaa] [253]	121.9 (118.4, 125.5) [404]	128.2 (124.3, 132.2)[b] [379]
ADMA/SDMA[2]	1.10 (1.09, 1.12) [782]	1.11 (1.09, 1.14) [529]	1.09 (1.05, 1.13) [253]	1.15 (1.12, 1.18) [403]	1.06 (1.03, 1.08)[bbb] [379]
Other metabolites					
Creatinine (µmol/L)[2]	71.9 (70.8, 72.9) [784]	72.0 (70.7, 73.2) [531]	71.7 (69.8, 73.6) [253]	62.8 (61.9, 63.8) [405]	81.5 (80.2, 82.8)[bbb] [379]
Total cholesterol (mmol/L)[2]	5.3 (5.2, 5.4) [786]	5.1 (5.0, 5.2) [533]	5.7 (5.6, 5.9)[aaa] [253]	5.2 (5.1, 5.3) [406]	5.3 (5.2, 5.4) [380]
LDL cholesterol [2]	3.2 (3.1, 3.2) [774]	3.0 (2.9, 3.1) [525]	3.5 (3.4, 3.7)[aaa] [249]	3.1 (3.0, 3.2) [403]	3.3 (3.2, 3.4)[bb] [371]

tHcy, total plasma homocysteine; ADMA, Asymmetric Dimethylarginine; SDMA, Symmetric Dimethylarginine; EGRAC, erythrocyte glutathione reductase activation coefficient; RBCF, Red blood cell folate; B12, plasma cobalamin. Comparison between age groups and sex was done using ANOVA for continuous variables. Twenty-four participants were excluded after the medical check-up due to declared B vitamin supplement use. A further fifty-one participants were excluded from all analyses involving tHcy because their blood samples were not processed within 2 h of collection and five participants because they had suspected altered renal function (plasma creatinine >97 mmol/L for women and >124 mmol/L for men). Two participants were also excluded for having outlier values of ADMA and SDMA. [1]Geometric mean. [2]Arithmetic mean. [a] Significant difference between age groups, p <0.05. [aa] Significant difference between age groups, p <0.01. [aaa] Significant difference between age groups, p <0.001. [b] Significant difference between sex, p <0.05. [bb] Significant difference between sex, p <0.01. [bbb] Significant difference between sex, p <0.001.

MTHFR C677T and *NOS* G894T polymorphisms were in Hardy-Weinberg equilibrium and 17.9% and 17.4% of the population presented the mutant genotype, respectively (**Table 1.3**).

Table 1.3: Genetics characteristics according to age group and sex

	All participants	≤ 50 y	>50 y	p	Women	Men	p
MTHFR 677							
CC	35.7 (32.4, 39.2) [278]	35.0 (31.0, 39.1) [185]	37.3 (31.6, 43.5) [93]		35.8 (31.2, 40.6) [143]	35.7 (31.1, 40.7) [135]	
CT	46.4 (42.9, 49.9) [361]	47.4 (43.2, 51.7) [251]	44.2 (38.1, 50.4) [110]	0.693	47.8 (42.9, 52.6) [191]	45.0 (40.0, 50.0) [170]	0.533
TT	17.9 (15.3, 20.7) [139]	17.6 (14.6, 21.19 [93]	18.5 (14.1, 23.8) [46]		16.5 (13.2, 20.5) [66]	19.3 (15.7, 23.6) [73]	
NOS 894							
GG	36.3 (32.9, 39.8) [273]	35.4 (31.4, 39.6) [182]	38.1 (32.2, 44.4) [91]		37.0 (32.4, 42.0) [143]	35.4 (30.7, 40.4) [130]	
GT	46.3 (42.8, 49.9) [349]	47.3 (43.0, 51.6) [243]	44.4 (38.2, 50.7) [106]	0.731	44.6 (39.7, 49.6) [172]	48.2 (43.2, 53.3) [177]	0.567
TT	17.4 (14.9, 20.3) [131]	17.3 (14.3, 20.8) [89]	17.6 (13.3, 22.9) [42]		18.4 (12.1, 19.1) [71]	16.3 (12.9, 20.5) [60]	

MTHFR, methylenetetrahydrofolate reductase; NOS, Nitric oxide synthase. *MTHFR* 677CT and *NOS* 894GT polymorphisms were in Hardy-Weinberg equilibrium. Comparison between age groups and sex was by χ^2.

The categories created according to medication use are shown in **Figure 1.2**. Three quarters of the population took none or sporadic medication (Group 1). In the remainder, half were on medication that did not affect the L-Arginine pathway (Group 2). Medication Group 3 consisted of participants on medication that has been shown to affect the L-Arginine pathway (33.3%) and the remaining group was on antihypertensive medication.

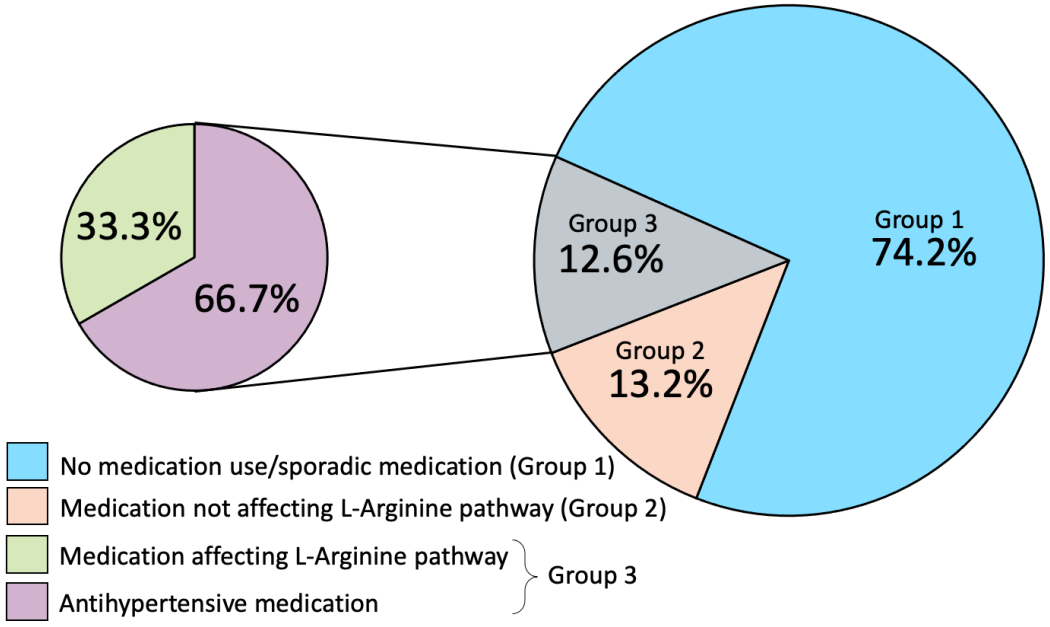


Figure 1.2: Medication category frequency.

Figure 1.3 shows the association of tHcy and L-Arginine pathway metabolites in participants not taking medication/sporadic medication (Group 1). The interaction term for tHcy x medication category were significant in the ADMA model for adults over 50 (B, 0.008; SE, 0.002; p <0.001) and in the SDMA model for adults ≤ 50 (B, -0.013; SE, 0.004; p, 0.002), so stratified analyses by medication category were carried out. Plasma ADMA was positively associated with tHcy in the total population, however, significance was lost after stratifying by age group or sex. On the other hand, the significant positive association between tHcy and SDMA that we observed in the whole population was also observed after stratifying by age group and sex, but when we performed the Bonferroni test to adjust the corrections for multiple comparisons, this association was only maintained in the younger age group and in men.

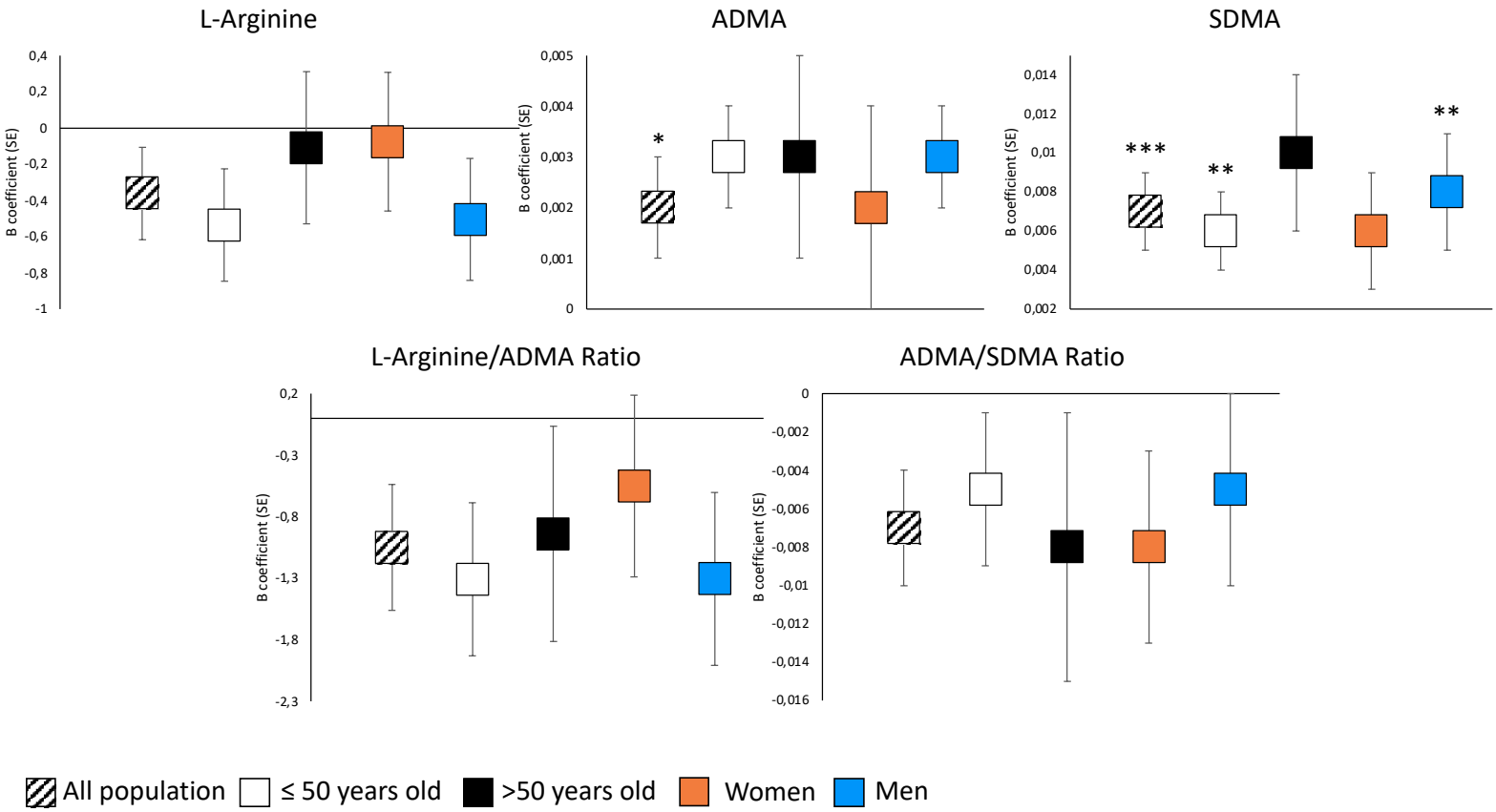


Figure 1.3: Multiple linear regressions analyses testing the association between tHcy and L-Arginine pathway metabolites, stratified by age group and sex. tHcy, total fasting plasma homocysteine; BMI, body mass index; ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine. All models were adjusted for age (in the total population models and sex-stratified models), sex (in the total population and age group-stratified models), BMI, smoking habits (cigarettes/day), category of regular alcohol intake (moderate (<16 g/d in women and <24 g/d in men) versus none; high versus none (≥16 g/d in women and ≥24 g/d in men)), low versus mid-high socio-economic status and plasma LDL cholesterol. Only participants not taking medication/ sporadic medication (Group 1) are represented. Unstandardized B-coefficients and standard error. *p <0.05; **p <0.01; ***p <0.001. L-Arginine N= 524 (Total population: R2, 0.011; p , 0.095; ≤50 y: R2, 0.028; p, 0.015; >50 y: R2, 0.013; p, 0.299; Women: R2, 0.024; p, 0.075; Men: R2, 0.056; p, 0.004. ADMA N= 524 (Total population: R2, 0.120; p <0.001; ≤50 y: R2, 0.063; p <0.001; >50 y: R2, 0.000; p, 0.442; Women: R2, 0.127; p <0.001; Men: R2, 0.100; p <0.001. SDMA N=523 (Total population: R2, 0.106; p <0.001; ≤50 y: R2, 0.035; p , 0.005; >50 y: R2, 0.087; p, 0.015; Women: R2, 0.082; p <0.001; Men: R2, 0.091; p <0.001. L-Arginine/ADMA Ratio N= 523 (Total population: R2, 0.055; p <0.001; ≤50 y: R2, 0.052; p <0.001; >50 y: R2, -0.023; p, 0.733; Women: R2, 0.049; p, 0.008; Men: R2, 0.087; p <0.001. ADMA/SDMA Ratio N=522 (Total population: R2, 0.062; p <0.001; ≤50 y: R2, 0.052; p <0.001; >50 y: R2, 0.105; p, 0.006; Women: R2, 0.017; p, 0.140; Men: R2, 0.042, p, 0.015.

As shown in **Table 1.4**, the *MTHFR* C677T polymorphism was not associated with any of the metabolites of the L-Arginine pathway after adjusting for cofounders in the linear regression and correcting with the Bonferroni test for multiple comparisons in any of the age groups or sexes.

Table 1.4: Multiple linear regression analysis of *MTHFR* C677T polymorphism and L-Arginine pathway metabolites, stratified by age group and sex

				CT versus CC			TT versus CC		
L-Arginine	n	R^2	B coefficient[1]	SE	p	B coefficient	SE	p
All	677	0.050***	-0.311	1.570	2.529	-1.740	2.039	1.182
<50 y	463	0.046**	0.435	1.976	2.568	-1.976	2.608	1.347
>50 y	214	0.073*	-3.053	2.524	0.684	-2.058	3.144	1.542
Women	348	0.037*	-1.001	2.255	1.962	-1.444	2.963	1.878
Men	329	0.087***	0.473	2.174	2.484	-0.008	2.826	2.994
ADMA								
All	676	0.137***	-0.013	0.007	0.129	-0.013	0.008	0.351
<50 y	462	0.051***	-0.010	0.008	0.609	-0.018	0.010	0.255
>50 y	214	0.016	-0.021	0.012	0.255	-0.004	0.015	2.370
Women	347	0.170***	-0.025	0.016	0.321	-0.006	0.021	2.355
Men	329	0.235***	-0.009	0.017	1.824	0.003	0.022	2.715
SDMA								
All	676	0.216***	-0.018	0.011	0.375	-0.004	0.015	2.313
<50 y	462	0.102***	-0.029	0.013	0.096	-0.010	0.018	1.758
>50 y	214	0.264***	0.006	0.022	2.373	0.007	0.028	2.412
Women	347	0.170***	-0.025	0.016	0.321	-0.006	0.021	2.355
Men	329	0.235***	-0.009	0.017	1.824	0.003	0.022	2.715
L-Arg/ADMA Ratio								
All	676	0.106***	2.602	3.094	1.203	0.495	4.027	2.706
<50 y	462	0.076***	3.942	3.892	0.936	1.218	5.152	2.439
>50 y	214	0.044	-1.962	5.013	2.088	-2.214	6.244	2.169
Women	347	0.087***	0.731	4.259	2.592	1.581	5.624	2.337
Men	329	0.120***	5.164	4.523	0.762	2.701	5.878	1.938
ADMA/SDMA Ratio								
All	675	0.195***	0.009	0.021	2.052	0.001	0.027	2.889
<50 y	461	0.159***	0.030	0.025	0.708	0.007	0.034	2.538
>50 y	214	0.236***	-0.033	0.039	1.191	-0.009	0.048	2.553
Women	346	0.143***	0.013	0.031	2.025	0.021	0.041	1.812
Men	329	0.192***	0.004	0.029	2.658	-0.017	0.038	1.977

Multiple linear regressions testing the association between *MTHFR* C677T polymorphism and L-Arginine pathway metabolites, stratified by age group and sex. BMI, body mass index; ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine. All models were adjusted for age (in the total population models and sex-stratified models), sex (in total population and age group-stratified models), BMI, smoking habits (cigarettes/day), category of regular alcohol intake (moderate (<16 g/d in women and <24 g/d in men) versus none; high versus none (≥16 g/d in women and ≥24 g/d in men)), low versus mid-high socio-economic status, plasma LDL cholesterol and medication category. [1]Unstandardized B-coefficients and standard error. *p <0.05; **p <0.01; ***p <0.001.

Mid-high tertile of plasma ADMA compared with lowest tertile, increases hypertension risk in participants over 50 years of age (OR [95% CI] (2.3 [1.1, 5.0]). In addition, men (OR [95% CI] (2.8 [1.3, 6.2]) and participant over 50 years of age (OR [95% CI] (2.4 [1.1, 5.3]) lowest L-Arginine/ADMA Ratio tertiles have a more than twofold increased risk of hypertension compared with those in the highest L-Arginine/ADMA Ratio tertile (**Table 1.5**).

When ADMA is in the lowest tertile, no significant association is observed between the T allele of the *NOS* G894T polymorphism and hypertension risk compared to the GG genotype (**Table 1.6**). However, the *NOS* 894 TT and GT genotypes compared to GG, are associated with triple the risk of having hypertension in participants with ADMA in the mid or highest tertiles in people over 50 years of age (OR [95% CI] (3.3 [1.2, 9.4]).

Table 1.5: Association between L-Arginine pathway metabolites and diagnosed hypertension stratified by age group and sex

		All participants			≤ 50 y			> 50 y			Women			Men		
	Model	n	R^2	OR (95%CI)	n	R^2	OR (95%CI)	n	R^2	OR (95%CI)	n	R^2	OR (95%CI)	n	R^2	OR (95%CI)
L-Arginine	1	599	0.002	1.2 (0.7, 1.9)	430	0.003	1.4 (0.6, 3.2)	169	0.004	1.3 (0.7, 2.5)	323	0.005	1.4 (0.7, 2.5)	276	0.000	1.0 (0.5, 2.0)
	2		0.193***	1.3 (0.8, 2.2)		0.087**	1.4 (0.6, 3.4)		0.054*	1.4 (0.7, 2.7)		0.338***	1.5 (0.8, 3.1)		0.090***	1.1 (0.5, 2.4)
	3		0.428***	1.4 (0.8, 2.4)		0.308***	1.9 (0.7, 5.2)		0.308***	1.3 (0.6, 2.8)		0.559***	1.3 (0.5, 3.1)		0.320***	1.6 (0.7, 3.5)
ADMA	1	599	0.011	1.6 (1.0, 2.5)	430	0.003	0.7 (0.3, 1.9)	169	0.082**	3.0 (1.5, 5.9)	323	0.012	1.6 (0.9, 2.9)	276	0.009	1.5 (0.8, 3.0)
	2		0.196***	1.5 (0.9, 2.4)		0.089**	0.6 (0.2, 1.6)		0.132***	3.2 (1.6, 6.3)		0.339***	1.6 (0.8, 3.1)		0.096***	1.5 (0.7, 3.0)
	3		0.426***	0.9 (0.5, 1.7)		0.344***	0.2 (0.1, 0.7)		0.334***	2.3 (1.1, 5.0)		0.558***	1.0 (0.4, 2.4)		0.315***	1.1 (0.5, 2.5)
SDMA	1	598	0.004	1.3 (0.8, 2.0)	429	0.000	1.1 (0.4, 2.6)	169	0.017	1.6 (0.9, 3.1)	322	0.006	1.4 (0.7, 2.5)	276	0.002	1.2 (0.6, 2.4)
	2		0.195***	1.4 (0.8, 2.)		0.085**	1.1 (0.5, 2.8)		0.061*	1.6 (0.8, 3.0)		0.341***	1.5 (0.8, 3.1)		0.091***	1.2 (0.6, 2.5)
	3		0.426***	1.0 (0.6, 1.9)		0.299***	0.8 (0.3, 2.3)		0.309***	1.3 (0.6, 2.8)		0.561***	1.5 (0.6, 3.5)		0.314***	1.0 (0.4, 2.3)
L-Arginine/ADMA Ratio	1	599	0.016*	1.7 (1.1, 2.7)	430	0.011	1.7 (0.7, 4.1)	169	0.058**	2.5 (1.3, 5.0)	323	0.004	1.3 (0.7, 2.4)	276	0.042**	2.5 (1.3, 4.8)
	2		0.205***	1.9 (1.1, 3.0)		0.031**	1.6 (0.7, 3.9)		0.110**	2.7 (1.4, 5.5)		0.335***	1.4 (0.7, 2.7)		0.132***	2.6 (1.3, 5.3)
	3		0.434***	1.8 (1.0, 3.1)		0.303***	1.6 (0.6, 4.2)		0.333***	2.4 (1.1, 5.3)		0.559***	1.2 (0.5, 3.0)		0.347***	2.8 (1.3, 6.2)

BMI, body mass index; ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine. Multiple logistic regression analysis was used. Tertiles were sex and age specific. Cut-offs for low-mid L-Arginine tertiles were ≤73.3 µmol/L in women ≤50 years, ≤72.3 µmol/L in women >50, ≤ 75.6 µmol/L in men ≤50 years, ≤ 70.3 µmol/L in men >50. Cut-offs for mid-high ADMA tertiles were ≥0.485 µmol/L in women ≤50 years, ≥0.546 µmol/L in women >50, ≥0.489 µmol/L in men ≤50 years, ≥0.382 µmol/L in men >50. Cut-offs for mid-high SDMA tertiles were ≥0.404 µmol/L in women ≤50 years, ≥0.450 µmol/L in women >50, ≥0.442 µmol/L in men ≤50 years, ≥0.484 µmol/L in men >50. Cut-offs for low-mid L-Arginine/ADMA Ratio tertiles were ≤142.89 in women ≤50 years, ≤130.38 in women >50, ≤ 151.30 in men ≤50 years, ≤ 127.74 in men >50. Cut-offs for mid-high ADMA/SDMA Ratio tertiles were ≥1.03 in women ≤50 years, ≥1.03 in women >50, ≥0.95 in men ≤50 years, ≥0.89 in men >50. Participants without diagnosed hypertension but with point blood pressure measurements >140/90mmHg, at the study check-up, were referred for blood pressure monitoring and excluded from the analysis. Model 1: L-Arginine or L-Arginine/ADMA Ratio low-mid versus high tertile; ADMA, SDMA or ADMA/SDMA Ratio mid-high versus low tertile. Model 2: included the same variables as model 1 as well as low versus mid-high socio-economic status. Model 3: included the same variables as model 2 as well as BMI, smoking habits (cigarettes/day), category of regular alcohol intake (moderate (<16 g/d in women and <24 g/d in men) versus none; high versus none (≥16 g/d in women and ≥24 g/d in men)), plasma creatinine, plasma total cholesterol and diabetes. Nagelkerke R^2. *p <0.05; **p <0.01; ***p <0.001.

Table 1.6: Association between the *NOS* G894T polymorphism and diagnosed hypertension stratified by age group and sex according to ADMA or SDMA tertiles

		ADMA						SDMA					
		Low tertile			Mid-high tertiles			Low tertile			Mid-high tertiles		
	Model	n	R^2	OR (95%CI)	n	R^2	OR (95%CI)	n	R^2	OR (95%CI)	n	R^2	OR (95%CI)
All	1	205	0.005	1.4 (0.5, 3.7)	368	0.001	1.1 (0.6, 1.9)	187	0.001	1.2 (0.5, 2.8)	386	0.001	1.2 (0.6, 2.1)
	2		0.360***	1.7 (0.5, 5.2)		0.435***	1.3 (0.6, 2.5)		0.360***	1.4 (0.5, 3.8)		0.436***	1.3 (0.6, 2.6)
	3		0.529***	1.5 (0.4, 6.1)		0.524***	1.4 (0.7, 3.0)		0.609***	0.9 (0.2, 3.2)		0.561***	1.4 (0.6, 3.0)
≤50 y	1	145	0.000	1.1 (0.2, 6.2)	270	0.028	0.4 (0.2, 1.1)	136	0.013	0.6 (0.1, 2.3)	279	0.013	0.5 (0.2, 1.5)
	2		0.001	1.1 (0.2, 6.2)		0.055	0.4 (0.2, 1.1)		0.141*	0.5 (0.1, 2.0)		0.013	0.5 (0.2, 1.5)
	3		0.375	1.6 (0.1, 18.8)		0.396***	0.5 (0.2, 1.8)		0.491**	0.3 (0.1, 3.1)		0.515***	0.7 (0.2, 2.7)
>50 y	1	60	0.006	1.4 (0.4, 5.1)	98	0.065*	2.6 (1.1, 6.0)	51	0.029	1.9 (0.6, 6.6)	107	0.029	1.9 (0.8, 4.4)
	2		0.031	1.4 (0.4, 5.1)		0.097*	3.0 (1.2, 7.2)		0.099	2.6 (0.7, 9.6)		0.043	2.0 (0.8, 4.5)
	3		0.485**	1.1 (0.2, 6.5)		0.283***	3.3 (1.2, 9.4)		0.498*	1.1 (0.1, 8.1)		0.321**	1.9 (0.7, 5.1)
Women	1	108	0.001	1.1 (0.3, 4.0)	200	0.002	1.3 (0.6, 2.7)	98	0.002	0.8 (0.3, 2.6)	210	0.006	1.4 (0.6, 3.1)
	2		0.388***	1.7 (0.4, 7.9)		0.586***	2.1 (0.7, 6.2)		0.540***	1.8 (0.4, 9.0)		0.524***	2.0 (0.7, 5.7)
	3		0.633***	1.7 (0.3, 10.9)		0.717***	2.4 (0.6, 8.8)		0.837***	2.5 (0.1, 50.5)		0.686***	3.4 (0.9, 13.0)
Men	1	97	0.015	1.9 (0.4, 9.7)	168	0.000	0.9 (0.4, 2.1)	89	0.013	1.7 (0.4, 6.8)	176	0.000	0.9 (0.4, 2.2)
	2		0.321***	1.7 (0.3, 10.3)		0.280***	0.9 (0.3, 2.2)		0.204**	1.4 (0.3, 6.0)		0.336***	0.9 (0.3, 2.3)
	3		0.495**	1.8 (0.2, 20.2)		0.391***	0.8 (0.3, 2.5)		0.514***	0.8 (0.1, 5.2)		0.483***	0.9 (0.3, 2.8)

BMI, body mass index; NOS, Nitric oxide synthase; ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine. Multiple logistic regression analysis was used. Cut-offs for mid-high ADMA tertiles were ≥0.485 μmol/L in women ≤50 years, ≥0.546 μmol/L in women >50, ≥0.489 μmol/L in men ≤50 years, ≥0.382 μmol/L in men >50. Cut-offs for mid-high SDMA tertiles were ≥0.404 μmol/L in women ≤50 years, ≥0.450 μmol/L in women >50, ≥0.442 μmol/L in men ≤50 years, ≥0.484 μmol/L in men >50. Participants without diagnosed hypertension but with point blood pressure measurements >140/90mmHg, at the study check-up, were referred for blood pressure monitoring and excluded from the analysis. Model 1: NOS 894 GT+TT versus NOS 894 GG. Model 2: included the same variables as model 1 as well as age (in the total population models and sex-stratified models) and sex (in total population and age group-stratified models). Model 3: included the same variables as model 2 as well as BMI, smoking habits (cigarettes/day), category of regular alcohol intake (moderate (<16 g/d in women and <24 g/d in men) versus none; high versus none (≥16 g/d in women and ≥24 g/d in men)), low versus mid-high socio-economic status, plasma creatinine, plasma total cholesterol and diabetes. Nagelkerke R^2. *p <0.05; **p <0.01; ***p <0.001

Figure 1.4 illustrates the analysis testing the association between the *MTHFR* 677 TT versus CC genotype with ADMA, and homocysteine as a mediator. The analysis is stratified by age group or sex. In the whole population as well as in age and sex groups, there is a negative direct association (only significant in the whole and youngest age group) between the TT genotype and ADMA. When tHcy is included as a mediator, a significant positive association between the variant genotype and tHcy and between tHcy and ADMA is observed in all models. Thus, both when stratifying by age group and sex, there is also an indirect effect via tHcy between the association of *MTHFR* 677 TT genotype and ADMA compared to the wild-type variant. Although the direct effect is in the opposite sense to the indirect effect, it is stronger than the indirect effect.

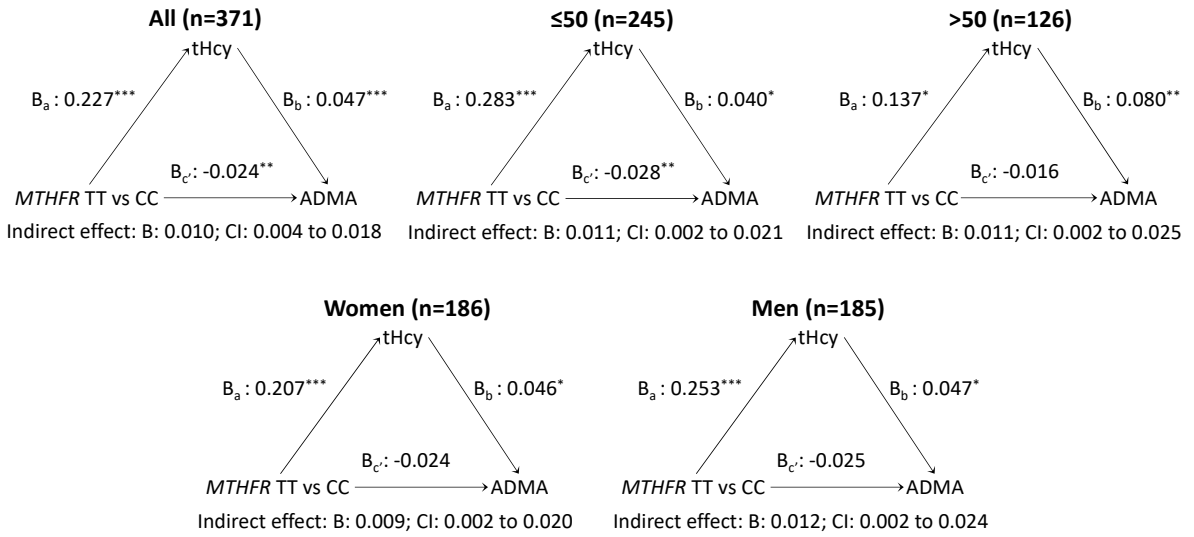


Figure 1.4: Mediation analysis to test tHcy as a mediator of the relationship between *MTHFR* 677 TT versus CC genotype with ADMA. MTHFR, methylenetetrahydrofolate reductase; EGRAC, erythrocyte glutathione reductase activation assay; tHcy, total fasting plasma homocysteine; BMI, body mass index; ADMA, Asymmetric dimethylarginine. All mediation models were adjusted for BMI, smoking habits (cigarettes/day), alcohol intake (moderate (<16 g/d in women and <24 g/d in men) versus none; high versus none (≥16 g/d in women and ≥24 g/d in men)), low versus mid-high socio-economic status, plasma folate, plasma cobalamin, EGRAC, plasma total cholesterol and medication category. Models were adjusted for sex when stratifying by age group and for age when stratifying by sex. $^{*}p < 0.05$; $^{**}p < 0.01^{***}p < 0.001$.

Our group previously observed an increased risk of hypertension in people younger than 50 years when they had the TT genotype of the *MTHFR* C677T polymorphism

compared to CCs, this was not observed in the older population [48]. Here we investigate whether ADMA is a mediator in this association.

Figure 1.5 illustrates the association of the TT genotype and hypertension mediated by ADMA. As in the previous figure, the age- and sex-stratified models are represented. In all cases, no significant indirect effect is observed in the association between *MTHFR* 677 TT compared to CC and hypertension mediated by ADMA. The previous positive association seen by our group between *MTHFR* TT vs CC with hypertension is maintained in the younger age group and as previously reported, there was no association in the older age group. When stratifying by sex, we did not observe any significant association (either direct or indirect) between the polymorphism and outcome in either men or women.

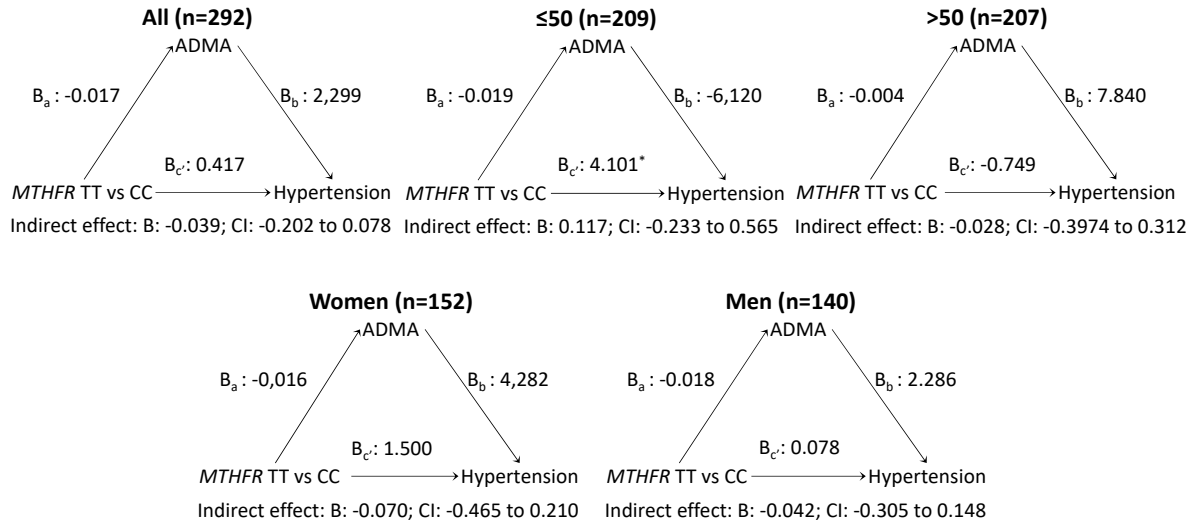


Figure 1.5: Mediation analysis to test ADMA as a mediator in the association between the *MTHFR* 677 TT versus CC genotype and diagnosed hypertension. MTHFR, methylenetetrahydrofolate reductase; EGRAC, erythrocyte glutathione reductase activation assay; BMI, body mass index; ADMA, Asymmetric dimethylarginine. All mediation models were adjusted for BMI, smoking habits (cigarettes/day), alcohol intake (moderate (<16 g/d in women and <24 g/d in men) versus none; high versus none (≥16 g/d in women and ≥24 g/d in men)), low versus mid-high socio-economic status, plasma folate, plasma cobalamin and EGRAC. In models with ADMA as the dependent variable, plasma LDL cholesterol and medication category were included as covariables. In models with hypertension as a dependent variable, models were also adjusted for plasma total cholesterol, diabetes and *NOS* GT+TT versus GG genotypes. Models were also adjusted for sex when stratifying by age group and for age when stratifying by sex. Direct and indirect effects on hypertension are on a log-odds metric. $^{*}p < 0.05$; $^{**}p < 0.01$ $^{***}p < 0.001$.

Previously our group observed an increased risk of hypertension in those over 50 years of age when tHcy was in the highest tertile compared to the low-mid tertile. This was not observed in the younger age group [48]. **Figure 1.6** shows the association between highest versus low-mid tertiles of tHcy and hypertension, mediated by tertiles of ADMA.

The older age group model showed an indirect effect on the association between homocysteine and ADMA-mediated hypertension. In the youngest group, in men and in women, neither high tHcy nor ADMA are associated with an increased risk of hypertension.

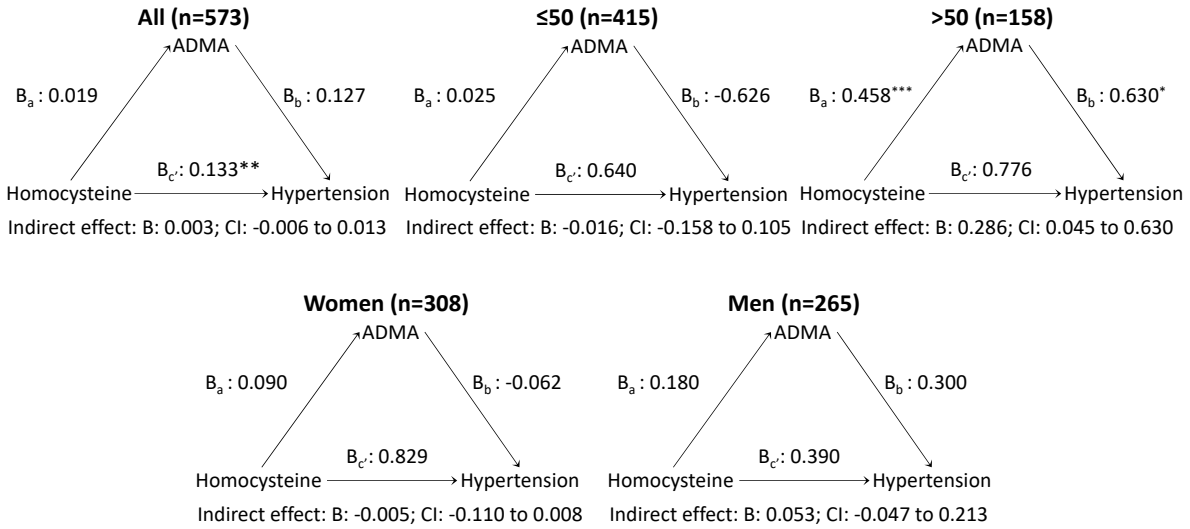


Figure 1.6: Mediation analysis to test ADMA as a mediator of the association between highest versus low-mid tertiles of tHcy with diagnosed hypertension. tHcy, total fasting plasma homocysteine; BMI, body mass index; ADMA, Asymmetric dimethylarginine. All mediation models were adjusted for BMI, smoking habits (cigarettes/day), alcohol intake (moderate (<16 g/d in women and <24 g/d in men) versus none; high versus none (≥16 g/d in women and ≥24 g/d in men)) and low versus mid-high socio-economic status. In models with ADMA as the dependent variable, plasma LDL cholesterol and medication category were included as covariables. In models with hypertension as a dependent variable, models were also adjusted for plasma total cholesterol, diabetes and the *NOS* GT+TT versus GG polymorphism. Models were also adjusted for sex when stratifying by age group and for age when stratifying by sex. Direct and indirect effects on hypertension are given in log-odds metric. $^{*}p < 0.05$; $^{**}p < 0.01$ $^{***}p < 0.001$.

3.4 Discussion

Major findings

In the no medication use/sporadic medication group, tHcy was positively associated with both ADMA and SDMA. When stratified by age group and sex, the association with SDMA was only maintained in the population under the age of 50 years and in men. The *MTHFR* C677T polymorphism was not associated with any of the metabolites of the L-Arginine pathway. Having ADMA in the mid-high versus lowest tertile and L-Arginine/ADMA Ratio in the low-mid versus highest tertile was associated with a twofold greater risk of hypertension in adults over 50. The GT+TT versus GG genotype of the *NOS* G894T polymorphism was associated with an increased risk of hypertension only in participants with ADMA in the mid-high tertile. In the mediation analysis, the association between the *MTHFR* TT genotype and plasma ADMA showed a direct negative effect but a positive indirect effect via tHcy. The association between mid-high tHcy tertile and hypertension was mediated by ADMA in the older population.

One-Carbon metabolism and L-Arginine pathway relationship

Our results regarding the association between tHcy and ADMA or SDMA are in line with other studies. In monkeys with diet-induced hyperhomocyst(e)inemia, mean ADMA concentrations were threefold higher in those with hyperhomocyst(e)inemic diet compared with controls ($p < 0.05$), however, SDMA concentrations did not differ between hyperhomocysteinemic monkeys and to controls [152]. In other observational studies in humans, such as the Framingham study, a positive correlation between tHcy with ADMA and SDMA had been observed in patients and in the general population

[153–155]. A positive association between tHcy and ADMA has been reported in several studies [156].

Few studies have examined the association between the *MTHFR* C677T polymorphism and L-Arginine pathway metabolites. Dimitroulas et al. (2016), reported higher serum ADMA concentrations in rheumatoid arthritis patients with the *MTHFR* 677 TT genotype compared to the CT or CC genotypes in a univariate analysis. However, when adding tHcy and other covariables in the multivariate analysis, serum ADMA concentrations did not differ among *MTHFR* genotypes [157]. In a study of epileptic patients, plasma ADMA concentrations were higher and L-Arginine/ADMA Ratio was lower in patients (but not in controls) with the *MTHFR* 677CT compared to CC genotype [158]. To the best of our knowledge, the possible association between the *MTHFR* C677T polymorphism and SDMA has not been studied previously. Interestingly, the mediation analysis indicates that there is a direct inverse association between *MTHFR* TT genotype with ADMA but that there is a positive association via tHcy.

The association between *MTHFR* 677TT genotype and DNA hypomethylation reported by some authors may explain the inverse association between the *MTHFR* 677TT genotype and ADMA in our population. This genotype may limit the availability of methyl groups from 5-methyltetrahydrofolate for SAM synthesis, which may reduce cellular capacity to methylate, decreasing ADMA and SDMA concentrations [159, 160]. In the positive association between tHcy and ADMA, tHcy has been shown to inhibit the enzyme DDAH, the enzyme responsible for the degradation of ADMA into L-Citrulline. In humans, 80% of ADMA is degraded by this enzyme [67, 161]. Inhibition of L-Citrulline synthesis by tHcy would increase intraendothelial ADMA concentrations.

This might suggest that although the *MTHFR* 677TT genotype may present a mechanism of ADMA reduction, its effect on increasing tHcy and the consequent inhibition of the ADMA degrading enzyme (DDAH), might explain the higher ADMA concentrations found in patients (compared with our random population) of the above-mentioned studies among the TT compared to the wild-type genotype.

THcy was already known to be higher in men than in women [162]. Furthermore, the rate of homocysteine re-methylation and trans-sulphuration is higher in women than in men [163]. In addition, the higher homocysteine in men has also been suggested to be due to lower plasma vitamin status [30]. In our population, men had lower plasma folate status than in women. In addition, as mentioned above, the main degradation of SDMA is via the kidney. Several studies, both in humans and in other mammals, have observed an effect of sexual hormones on renal damage. Testosterone has been found to have a pro-inflammatory and pro-apoptotic effect on the kidney, while oestrogens are protective against renal injury [164, 165]. This could explain why the association between tHcy and SDMA is only observed in the men of our study. However, it should be noted that stratification by sex reduces the statistical power and that, in addition, a significant positive association (p, 0.029) between tHcy and SDMA was observed in women before performing the Bonferroni test for multiple comparisons corrections.

In addition, homocysteine has been shown to induce apoptosis through activation of endoplasmic reticulum stress, which may also trigger an increase in protein proteolysis [116]. Nonetheless, there is an alternative pathway of ADMA degradation, via the enzyme alanine:glyoxylate amino-transferase 2 (AGXT2). This mitochondrial enzyme, which is mainly expressed in the kidney, can also catabolise SDMA [166]. The

potential target of this enzyme is poorly studied and the possible involvement of 1CM is not known.

L-Arginine pathway and hypertension association

We reported an increased risk of hypertension when plasma ADMA was in the mid or highest tertile, only in the oldest population. This is consistent with studies that observed a relationship between high concentrations of plasma and serum ADMA, hypertension and ageing [167, 168]. However, our results are in disagreement with those of Sonmez et al. (2010), who observed higher plasma ADMA concentrations in hypertensive patients compared to controls, in a population of young men (mean age 24.4 ± 1.9 years) [105]. In a random population sample, ADMA and age were positively correlated, leading the authors to suggest that ADMA may reflect a vascular degenerative process associated with ageing [168]. In fact, endothelium-dependent coronary microvascular dysfunction was associated with ageing in 34 patients (27 to 73 years old) with no coronary risk factors [169]. In the Bruneck study, both ADMA (HR, 3.94) and SDMA (HR, 8.21) were associated with increased risk of cardiovascular disease in a general population cohort after adjusting for several cofounders [140]. Like in our population, the L-Arginine/ADMA Ratio has been associated with blood pressure [118], and even though it has been postulated to be a better predictors than the metabolites separately [115], the results of our study show that plasma ADMA and the L-Arginine/ADMA Ratio were very similar predictors of hypertension risk.

In addition to ADMA, SDMA has also been associated with age. The Study of Health in Pomerania and The Framingham Offspring Cohort observed in their reference intervals for SDMA an increase in its plasma and serum concentrations with increasing

age [153, 170]. Unlike in our study, Hov et al. (2007) observed no differences in plasma SDMA concentrations (determined by high-performance liquid chromatography) between men and women in a study of 238 participants. In the multivariate analysis, age, L-Arginine, ADMA and estimated glomerular filtration rate predicted SDMA [171]. In a study designed to investigate NO production by insulin in participants with a wide range of age and diseases (including hypertension), age was correlated with ADMA (R^2, 0.36) and SDMA (R^2, 0.3). Age and ADMA (but not SDMA) were associated with a decreased production of whole-body NO *in vivo* [172]. The Framingham study postulated that younger age explains why pre-menopausal women have lower plasma SDMA concentrations than post-menopausal women [153].

In other observational studies, such as the Framingham study, that enrolled 1126 participants, plasma ADMA concentrations did not vary between men (median (IQR) 0.51 (0.45–0.60)) and women (median (IQR) 0.50 (0.45–0.58) µmol/L) [173]. However, estrogens have been shown to have a protective effect against high concentrations of ADMA, as estradiol has been shown to increase the enzymatic activity of the ADMA-degrading enzyme, DDAH [174]. Furthermore, in our population, men have higher plasma LDL cholesterol and creatinine plasma concentrations than women, and these are associated with increased ADMA synthesis and reduced renal excretion, as mentioned above. These factors might explain the sex differences we observed in the association between ADMA and hypertension.

Few studies take into account not only the potential lowering effect on NO of the *NOS* G894T polymorphism, but also the reduced NO synthesis due to low production by the enzyme in the presence of high ADMA (its inhibitor) concentrations. In patients with renal disease, the combination of the *NOS* 894T allele and ADMA above the 75th percentile doubled the risk of cardiovascular mortality (HR [95% CI] (2.7 [1.4, 5.4]),

compared to the GG genotype and plasma ADMA below the 75th percentile [175]. Others concluded that both the *NOS* G894T polymorphism and ADMA are independent factors for the development of atherosclerosis [176]. It has been suggested that, as ADMA competes with L-Arginine for eNOS, the accumulation of ADMA (its inhibitor) could amplify the genetic defects caused by the polymorphism in NO synthesis [175].

One possible mechanism by which ADMA and age are associated is the effect of plasma LDL cholesterol on ADMA accumulation. In our population, the highest concentrations of plasma LDL cholesterol are found in the group over 50 years of age. In human endothelial cells, plasma LDL cholesterol increased PRMT gene expression [177] and DDAH enzyme activity was decreased to almost 60% of baseline values [178]. Moreover, the effect of LDL on the eNOS enzyme *in vitro*, illustrated that in the presence of high LDL concentrations, the production of NO is decreased by upregulating the abundance of the structural protein caveolin and promoting its inhibitory interaction with eNOS [135, 179]. This may contribute to increased ADMA synthesis in hypercholesterolemia by increasing its synthesis by PRMT and decreasing its degradation by DDAH.

As we have observed, gender and age are factors to be taken into account when using the L-Arginine pathway as a predictor of cardiovascular disease. Estrogens trigger a signalling cascade that induces eNOS phosphorylation to increase NO production. In rats, females have been observed to have higher basal expression of eNOS than males and, in addition, females produce higher NO [165].

1CM-hypertension link via L-Arginine pathway

Our group recently reported, from the same population study, that the *MTHFR* 677TT genotype is associated with hypertension in people under 50 years of age, and

moderately elevated tHcy was associated with hypertension in those over 50. [142] The mediation analyses, carried out here, support the L-Arginine pathway as a mediator in the development of hypertension by hyperhomocysteinemia but not by the presence of mutant genotype of the *MTHFR* C677T polymorphism.

NO synthesis can be also altered by tetrahydrobiopterin (BH4). The MTHFR enzyme produces 5-methyltetrahydrofolate, which interacts with BH4 (cofactor of the eNOS enzyme) to reduce the uncoupling of eNOS (**Figure 0.1**). [180] It has been proposed that smokers with the *MTHFR* TT genotype have reduced NO due to eNOS uncoupling and the higher affinity of the enzyme for O_2 rather than L-Arginine. [181] However, neither NO nor BH4 values were available in this study.

3.5. Strengths and limitations

Associations tested in cross-sectional studies can be affected by residual confounding and by reverse causation. To counteract the former, we adjusted for multiple confounding factors. Regarding reverse causation, in which the timing of development of the outcome cannot be determined in relation to the exposure and the possible influence of the outcome on the exposure cannot be discarded.

Maxwell and Cole (2007) outlined the different biases that could be encountered when conducting mediation analysis in cross-sectional studies [182]. In summary, they stated that there is an overestimation of the fact that mediation analyses attempt to explain causality and that the fact that mediation processes develop over time is not taken into account. These biases have been taken into account in our study and while establishing causality is of great importance, it is not the aim of this study. Performing mediation analysis in cross-sectional studies on a preliminary basis could help in subsequent longitudinal or experimental studies where the role of time is present. However, this is not the case in the associations tested with the polymorphisms, as

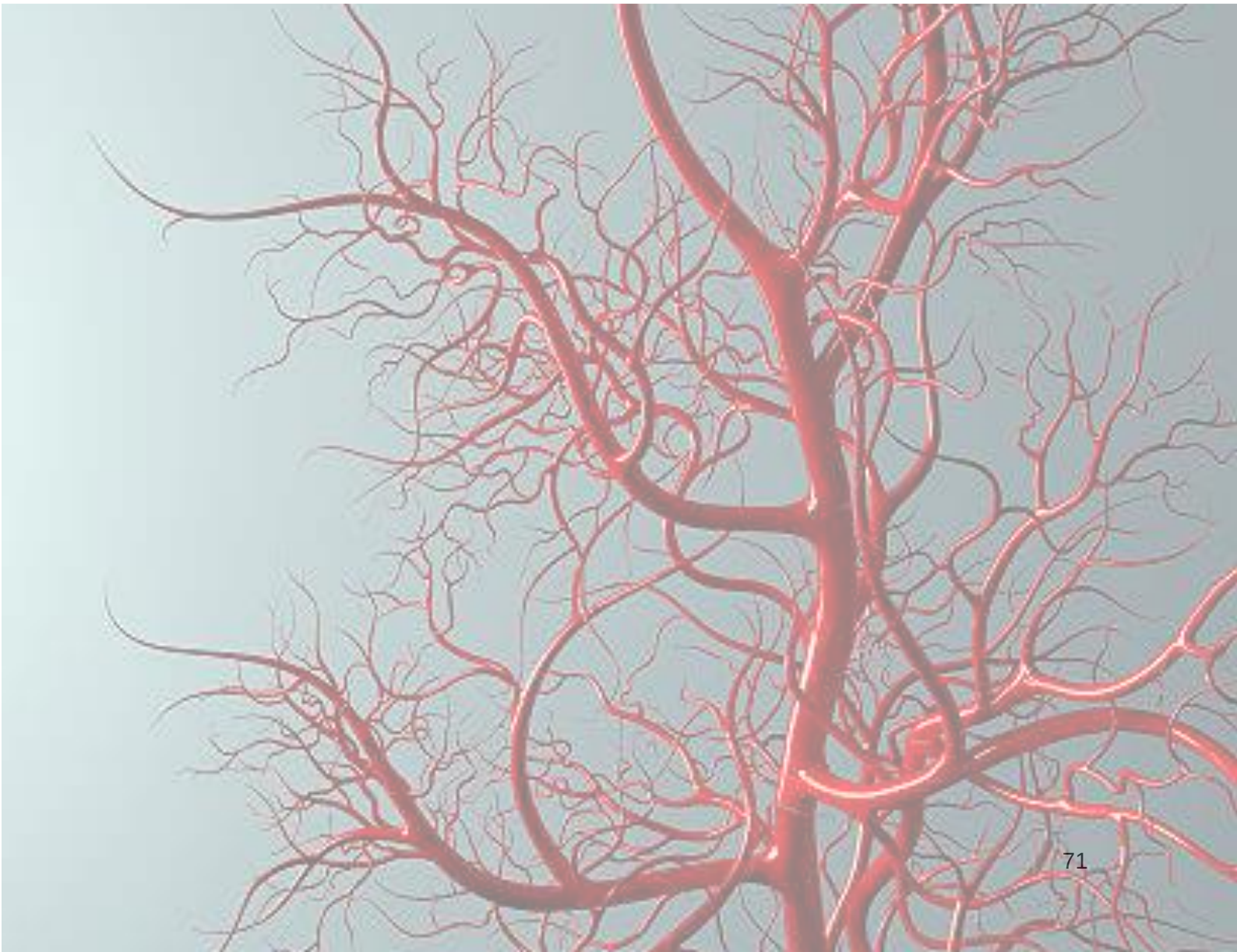
this factor did not change over time. In addition, in an attempt to reduce these biases, the mediation analyses in this study have been adjusted for a wide variety of factors, both their association with risk factors and outcome, as well as mediators.

Importantly, in Spain there is no mandatory fortification with folic acid, so the population was not influenced by B-vitamin supplementation nor mandatory fortification, avoiding their confounding effect in the association between the *MTHFR* C677T polymorphism and tHcy.

Future perspectives

Random control trials should be designed to test whether the reduction of endothelial dysfunction is improved following improvement of 1CM parameters and via changes in the L-Arginine pathway.

Chapter 2
The Reus-Tarragona Birth Cohort Study



4. CHAPTER 2: THE REUS-TARRAGONA BIRTH COHORT STUDY

4.1 Introduction

4.1.1 The importance of 1C metabolism and L-Arginine pathway during pregnancy

During pregnancy, the demand for nutrients is higher, as there is an increase in the number and size of cells and tissues, due to foetal and organ growth. One-carbon metabolism (1CM), and in particular folate, is crucial in this process because different compounds of the folate pathway are used for the synthesis of purines or thymidine, which is the basis of DNA biosynthesis [11]. Therefore, folate status is so important during pregnancy, for both maternal and foetal cell growth and proliferation to occur properly [183].

A decrease in plasma, serum and red blood cell folate concentration over the course of normal pregnancies has been observed in women not using folic acid supplements [184, 185]. It has been considered that folate decrease during pregnancy may be due to low folate intake, haemodilution, folate bioavailability, folate catabolism and excretion [183]. However, studies investigating folate transport between the mother and foetus in humans are scarce. While this process should be optimised for sufficient supply for foetal growth and development, this mechanism is not well known. It has been possible to determine maternal, cord and newborn folate status. Folate concentrations were higher in the cord [186] and newborns [186, 187] than in maternal plasma, indicating that folate enters the foetal circulation against a concentration gradient [188].

Homocysteine is another factor to consider during pregnancy. Homocysteine status depends on status in folate and other B vitamins [189]. However, although plasma folate concentrations decrease during pregnancy, tHcy is also low [190]. This may be due to an increased demand for methionine by the foetus, for protein synthesis. Remethylation of homocysteine to methionine would increase, thus decreasing homocysteine concentrations [191]. In rat embryos cultured in media low in methionine, a hypomethylation of proteins, specifically those located in the neural tube, was observed. This might indicate that methylation plays an important role in neural tube closure [192] and that homocysteine status could be an important marker to take into account [183].

Blood cell abnormalities do not occur when the MTHFR enzyme is deficient, as folate availability for purines and thymidine synthesis is not limited [192]. However, in addition to its effect on increasing homocysteine concentrations, the C677T polymorphism of the MTHFR enzyme decreases the availability of 5-MTHF for methylation, resulting in decreased DNA methylation [159]. It has been observed that the status in most essential amino acids is higher in the foetus than in the mother, indicating that active amino acid transport is essential during gestation [193]. Amino acids provide carbon and nitrogen substrates, serve as constituents of proteins and are precursors of non-protein substances, such as NO, polyamines, neurotransmitters or nucleotides [194].

As previously mentioned, L-Arginine is a semi-essential amino acid. However, it is considered a nutritionally essential amino acid in mammalian reproduction [195]. While L-Arginine is important for protein synthesis, its role as a substrate for NO synthesis is

key. NO is crucial for the development and function of the placenta [196] (more developed in the placental section).

Nitric oxide synthase (NOS), the enzyme that converts L-Arginine to NO, has been shown to have increased activity during gestation in guinea pigs [197]. In addition, impaired NOS expression has been observed in pre-eclampsia or gestational diabetes [198]. NOS activity can be decreased by ADMA, the competitive inhibitor of L-Arginine. ADMA has been observed to be significantly lower in pregnant women than in non-pregnant women [199] and its concentrations increase during the course of pregnancy [200]. However, higher concentrations of plasma and serum ADMA have been observed in women with pregnancy complications, such as pre-eclampsia, compared with those with uncomplicated pregnancies [201, 202].

4.1.2 The process of placentation and the role of 1CM and L-Arginine pathway

The blastocyst (fifth day after conception) is composed of inner and outer cells. The inner cells mass will generate the different tissues of the foetus, and outer cells (the trophoblast) will develop into the placenta and associated membranes [203]. By week 4 after fertilisation, the basic structure of the mature placenta will have been established. A functional placenta transports nutrients, respiratory gases, and the products of their metabolism between the maternal and foetal circulations, crucial for foetal survival, growth, and development [204].

The implantation of the blastocyst starts with trophoblast invasion of the maternal uterus, and during the development of the placenta, the trophoblast invades the decidualized endometrium and migrates into the uterine spiral arteries, a process completed at about 20 – 22 weeks of gestation [205]. After this, the uterine arteries

undergo a series of physiologic transformations, essential for the development of the pregnancy including dilatation of the lumen, loss of smooth muscle cells and replacement of elastic lamina by foetal trophoblast cells [206]. This transforms the spiral uterine arteries from high-resistance, low-flow vessels into large dilated vessels with an increased blood flow at a much reduced pressure [207], maximising the delivery of maternal blood to the intervillous space ensuring adequate foetal oxygen and nutrient supply. The uterus and the placenta increase the vascularity of the endometrium through angiogenesis and vasculogenesis and activate genes for nutrient transport in the uterine lumen [204] as they affect utero-placental flow. Placental maturation and function depend on optimal NO as it is crucial for placental vasculogenesis and angiogenesis [196].

As discussed in the section of NO, this compound regulates blood flow in tissues, including in the uterus and placenta. cGMP (second messenger of NO) activates a cascade that relaxes vascular smooth muscle cells and mitochondrial biogenesis. Nitric oxide also inhibits endothelin-1 release, thus preventing platelet aggregation. The role of NO in angiogenesis and placental growth is crucial [204].

The conceptus (foetus and associated membranes) needs amino acids and other nutrients for growth and development. L-Arginine supplementation during pregnancy was associated with increased birth weight, reduced incidence of small for gestational age and improved foetal growth in foetuses with intrauterine growth retardation [204].

As mentioned above, the vasculature plays a crucial role in the implantation process. Homocysteine toxicity in endothelial cells may have key implications. Homocysteine induces apoptosis in endothelial cells [208] and in the presence of a medium with

physiological concentrations of homocysteine, placental cells (cytotrophoblast) were reported to undergo apoptosis [209].

Folate intake during pregnancy is crucial for embryonic development. Growth during pregnancy requires high DNA synthesis and epigenetic alterations. 1CM provides a supply of methyl groups from SAM, essential mechanisms for foetal and placental growth and development [210].

Poor placentation has been associated with a number of complications in pregnancy, which will be discussed in more detail below.

4.1.3 Doppler ultrasound

As mentioned above, the spiral arteries change from small diameter and high resistance to larger diameter and lower resistance. If trophoblast invasion is not successful, the uteroplacental circulation will maintain this high resistance, causing damage to endothelial cells and impaired production of vasoactive substances, which has been associated with high blood pressure in pregnancy [211].

Appropriate blood flow of uterine arteries is assessed by Doppler ultrasound. Two of the most important variables obtained from the Doppler ultrasound evaluation of the uterine arteries are the pulsatility index (PI) and the presence or absence of a notch in the waveform. PI is currently the most commonly used index for the evaluation of uterine arteries. Doppler waveform patterns and a notch is defined as a persistent decrease in blood flow velocity in early diastole, below the diastolic peak velocity. Mean PI (mean PI of left and right arteries) shows a progressive decrease during pregnancy and the notch tends to disappear. Thus, an abnormal uterine artery Doppler

pattern is defined as the presence of a bilateral notch (left and right arteries) and/or a mean PI>95th percentile [212, 213].

Higher uterine artery PI of Doppler ultrasound has been observed in women with gestational hypertension [214] and pre-eclampsia [215] compared to controls, since the blood flow meets resistances and the necessary nutrients and oxygen cannot reach the foetus.

Homocysteine has a toxic effect on the endothelium [216]. As mentioned above, placental artery remodelling and placental function are key to proper foetal development. Both plasma homocysteine concentrations and the Doppler pulsatility index have been investigated in order to predict adverse pregnancy outcomes. Onalan et at., in a study of 459 pregnant women saw that second trimester (15-19 GW) tHcy above the 95th centile (tHcy>6.3 mol/l) increased the risk of intrauterine growth restriction (IUGR) (OR [95% CI] (11.3 [5.5, 27.7]) and pre-eclampsia (OR [95% CI] (13.8 [3.1, 26.9]). This increased risk was also seen with Doppler ultrasound bilateral notches with a mean resistance index (RI) >0.55 (50th centile), all unilateral notches with a mean RI >0.65 (80th centile), and absence of notches with a mean RI >0.7 (95th centile), for pre-eclampsia (OR [95% CI] (10.3 [2.5, 42.7]) and IUGR (OR [95% CI] (6.7 [2.9, 15.3]). The combination of tHcy and adverse Doppler measurements were more strongly associated with pre-eclampsia (OR [95% CI] (15.7 [5.8, 52.3]) and IUGR (OR [95% CI] (9.2 [3.1, 26.9]) [217]. Maged et al. (2017) also reported that pregnant women with pre-eclampsia and IUGR had higher tHcy and uterine artery resistance index values than those with uncomplicated pregnancies [218]. Conversely, another study (of 94 pregnant women) concluded that the addition of tHcy in the second trimester does not improve pathological uterine Doppler prediction [219].

Our group recently investigated the association of high homocysteine (tHcy >90th percentile) with high pulsatility index and pathological Doppler of the uterine arteries in a cohort of 811 pregnant women. No significant association between tHcy and pulsatility index or pathological Doppler of the uterine arteries was observed. However, it is important to mention the masking effect that folic acid supplementation during pregnancy may have on this association.

Few studies have investigated the possible association between the L-Arginine pathway and pathological Doppler of the uterine arteries. In an *in vitro* study, NOS activity in placental tissue of women with abnormal umbilical artery Doppler flow velocity waveforms was lower compared to those with normal umbilical artery Doppler flow velocity waveforms [220]. In a study of 89 pregnant women, plasma ADMA and ADMA/SDMA Ratio were significantly higher in those bilateral uterine artery notches at 23-25 GW that develop pre-eclampsia during pregnancy, compared with pregnant women without bilateral notches and no adverse pregnancy outcomes. In addition, the L-Arginine/ADMA Ratio was significantly lower in women with bilateral uterine artery notches compared to controls [221]. In a study of 65 Greek pregnant women, the second trimester (20-24 GW) mean uterine artery pulsatility index and plasma ADMA concentrations were independent predictors of pre-eclampsia along with BMI [222]. When 5 women were treated with a NO-generating drug (Glyceryl trinitrate), a decrease of 17% from baseline was seen in umbilical artery systolic/diastolic velocity ratio (p, 0.006) [223].

4.1.2 Hypertensive disorders of pregnancy

While alterations in the 1CM have been associated with neural tube defects [224], placental abruption or miscarriage [225], we will focus on pregnancy-induced hypertension and pre-eclampsia as they are the most common hypertensive disorders of pregnancy [226].

Hypertensive disorders of pregnancy are present in 5-10% of women and they are the second largest cause of maternal mortality worldwide [227]. Both pregnancy-induced hypertension and Pre-eclampsia are defined as SBP ≥140 mmHg or DBP ≥90 mmHg for the first time after 20 GW [228]. However, if protein in urine is ≥0.3 g in 24 hours, pre-eclampsia is diagnosed, while if it is <0.3, the mother is diagnosed with pregnancy-induced hypertension [229].

These hypertensive disorders of pregnancy can be classified into mild (SBP ≥140 mmHg or DBP ≥90 mmHg) [230] or severe (SBP ≥160 mmHg or DBP ≥110 mmHg) [231].

When the normal physiological adaptive changes in the spiral arteries do not occur during pregnancy, increased blood pressure may act as a compensatory mechanism to maintain an adequate blood supply [232]. In addition, poor maternal spiral artery modification by the invading trophoblast may lead to a hypoperfused placenta which releases factors into the maternal circulation which eventually leads to maternal endothelial dysfunction [233]. Among others, risk factors for pregnancy-induced hypertension and pre-eclampsia include maternal age, high BMI, nulliparity, genetic factors and previous history of pre-eclampsia or diabetes [234, 235].

Physiological changes including increased blood volume, increased cardiac output by 40-50% and a decrease in total peripheral resistance and blood pressure occur, in

order to meet metabolic needs. However, women with pregnancy-induced hypertension have been shown to have a significant increase in total peripheral resistance and vascular responsiveness to angiotensin II and a decrease in the production of endothelial-derived relaxing factors, compared to controls, suggesting abnormal endothelial function [236].

While periconceptional folic acid supplementation is recommended to prevent the onset of neural tube defects, the use of multivitamin supplements throughout pregnancy has been found to have a protective effect (OR [95% CI] (0.55 [0.39, 0.79]) on the risk of pregnancy-induced hypertension [237]. As mentioned above, imbalanced folate cycling is associated with impaired methylation and hyperhomocysteinemia, which in turn are associated with hypertension. Folic acid supplementation has been shown to improve endothelial function in adults with hyperhomocysteinemia by lowering tHcy concentrations [128].

Observational studies [238, 239] and meta-analyses that included more than 200 studies [240–242], have reported an increased risk by 7 to 387% of pregnancy-induced hypertension in women carrying the T allele of the *MTHFR* C677T polymorphism. Furthermore, it was observed that when supplemented with folic acid, this association lost significance [238]. In contrast, other authors did not observe any relevance of this polymorphism with regard to pregnancy-induced hypertension [243–245]. However, these studies either only looked at gene frequency among participants, or did not adjust for any covariates. The effect of the homozygous *MTHFR* C677T variant on hypertension appears to be associated with its effect on increasing homocysteine concentrations. Since hyperhomocysteinemia is also influenced by the

status in B group vitamins, environment-genetic interaction may explain the heterogeneity between studies [240, 241].

These findings add to the evidence that high homocysteine status is associated with pregnancy-induced hypertension, however, there are still discrepancies among studies that have searched for the association between hyperhomocysteinemia and pregnancy-induced hypertension. Women with pregnancy-induced hypertension have been found to have higher tHcy than women without complications in the first [246], second [247] and third [248, 249] trimesters of pregnancy. However, some studies report no increased risk of pregnancy-induced hypertension in patients with hyperhomocysteinemia in early pregnancy (tHcy >90 percentile (OR [95% CI] (0.6 [0.3, 1.2]) [250]) and postpartum (tHcy >15µmol/L (OR [95% CI] (1.6 [0.6, 4.0]) [251]).

As mentioned above, homocysteine is a potential biomarker of cardiovascular risk. Its possible involvement in the aetiology of hypertension is hypothesised to be via the L-Arginine pathway [252]. Since homocysteine inhibits DDAH, the enzyme responsible for degrading ADMA, the endothelial dysfunction leading to hypertension would be caused not by low NO synthesis by eNOS from L-Arginine, but increased inhibition of the enzyme due to the accumulation of ADMA [253].

Some authors have observed a fourfold increased risk of pregnancy-induced hypertension when L-Arginine concentrations were below 70 µM [246, 254]. However, L-Arginine/ADMA Ratio measurements have been considered a better indicator of endothelial dysfunction and have been found to be increased in women with pre-eclampsia [233]. In addition, 5-day supplementation with 20 g/500 mL of L-Arginine significantly reduced diastolic and systolic blood pressure values in women with gestational hypertension in randomised placebo-controlled trials [255, 256]. One

hypothesis is that maternal L-Arginine concentrations may be dependent on foetal intake and the increased need for NO to support adaptive vasodilation [254].

Measured plasma ADMA concentrations were found to be lower in normotensive pregnancies than in non-pregnant or pre-eclamptic women [199]. In normal pregnancies, ADMA concentrations decreased along with blood pressure. However, in cases of pre-eclampsia, these concentrations were increased, suggesting that ADMA might play a role in hemodynamic adaptation in pregnancy [199].

The main pathways of elimination of ADMA and SDMA are metabolised by DDAH and eliminated by renal excretion, respectively. Tsikas et al. (2018), saw a higher ADMA/SDMA Ratio in pre-eclamptic women with abnormal placental perfusion (mean ± SD, 6.8 [3.92–8.72]) compared with normal pregnancies (mean ± SD, 1.27 [0.97–1.68]; $p < 0.001$) [257]. An increased ADMA/SDMA Ratio suggests an impaired DDAH activity. DDAH is highly expressed in the placenta and its activity was almost undetectable in the pre-eclamptic placentas [258]. It is suggested that placental dysfunction of this enzyme may be one of the initiating events in the development of pre-eclampsia [221].

In this study we explore the association between impaired One-Carbon metabolism and the L-Arginine pathway and the association of the L-Arginine pathway with adverse outcomes of pregnancy in a longitudinal cohort of Spanish pregnant women.

4.2 Material and methods

Study population

The longitudinal cohort study, The Reus-Tarragona Birth Cohort (RTBC), was carried out by the Area of Preventive Medicine and Public Health from the Universitat Rovira i Virgili in cooperation with the Obstetrics and Gynaecology Units of the University Hospitals of Sant Joan Reus and Joan XXIII Tarragona, respectively.

This study followed-up pregnant women from less than 12 GW throughout pregnancy to investigate early nutritional, physiological and environmental factors associated with adverse pregnancy outcomes.

The Areas of Obstetrics and Gynaecology of the participating hospitals recruited women at their first prenatal check-up. Those with singleton pregnancies, a viable foetus, less than 12 GW pregnant at the first check-up and free of chronic diseases, recent major surgical interventions that could affect their nutritional status or on medication affecting folate or vitamin B12 status were eligible to participate in.

They were informed of the study and signed informed consent was obtained from those that agreed to participate. 821 women were recruited in the study between the years 2005 and 2020 (**Figure 2.1**).

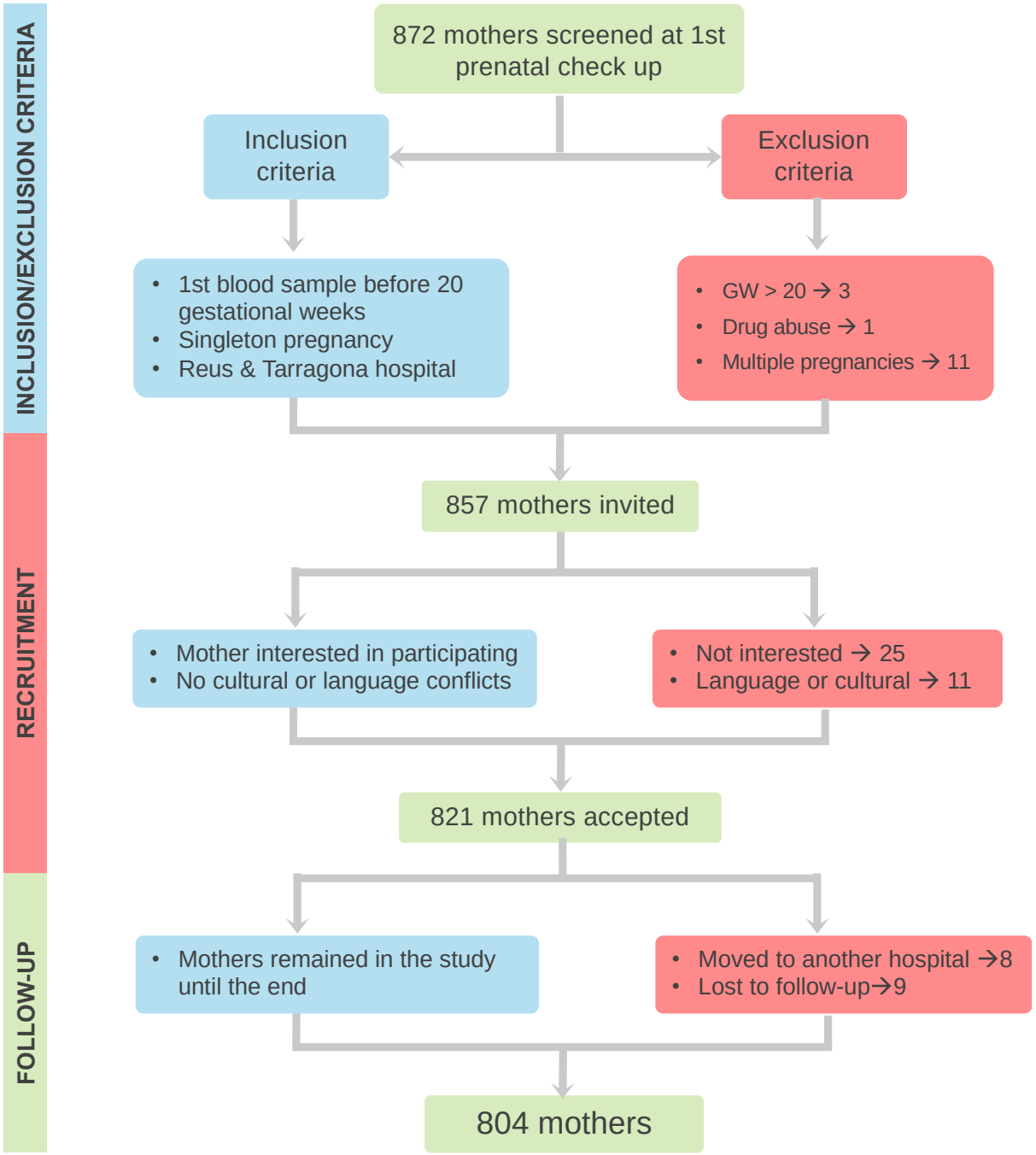


Figure 2.1: Flow charts of the participants in the pregnancy study.

The study population was not exposed to mandatory folic acid fortification since Spain does not have such legislation. However, the Ministry of Health recommends that pregnant women should be supplemented at periconception with at least 400 µg per day regardless of whether they are at a high or low obstetric risk. It is recommended that this supplementation should start 1-2 months before pregnancy and last at least

until the end of the first trimester (12 GW) [259]. The recommended supplements can be multivitamin, so that they include not only folic acid, but also cyanocobalamin, iron, zinc or riboflavin. For the Reus Tarragona Birth Cohort, participants were recommended to take a combined supplement of 400 µg/ of folic acid and 2 µg/d of cyanocobalamin) until the end of the first trimester. They were then recommended to take 40 mg (from the 12th week of gestation on) or 80 to 120 mg of iron per day if they were anaemic.

Data collection

Blood samples

Maternal fasting blood samples were collected at various weeks during gestation: ≤12 GW, 15 GW, 24-27 GW and 34 GW. Another blood sample was taken on admission to hospital with confirmed labour. Not all of these samples were given on fasting. Before placental expulsion, umbilical cord blood was also collected.

Blood samples were collected into ethylenediaminetetraacetic acid (EDTA)-K2 vacutainers (5 and 10 ml) for the processing of whole blood, plasma, erythrocytes and leukocytes extractions; and in dry vacutainers for serum determinations.

To avoid artefacts in the 1CM metabolites, the samples were immediately kept at 4ºC and were processed within one hour after extraction. This procedure was carried out in the *Institut d'Investigació Sanitària Pere Virgili Biobank* and in the research laboratories of the participating hospitals Universitat Rovira i Virgili University (Faculty of Medicine).

Red Blood cell folate

50 µl of whole blood from an EDTA-K2 tube were diluted in 450 µl of fresh 1% ascorbic acid solution [260] and kept at room temperature for 30 minutes in order to haemolyse red blood cells to release gamma glutamyl hydrolase and cellular folates. Subsequently, it was stored at -80ºC in separate aliquots of 250 µl. RBCF was not determined in labour or cord blood samples.

Plasma and Serum

After sample reception at the biobank/ laboratories, the dry vacutainer was kept at room temperature for 20-30 minutes for clot formation. The EDTA and dry vacutainers were centrifuged at 1800 g for 15 minutes at 4ºC in order to obtain plasma and serum, respectively. Samples were aliquoted into 1 ml cryotubes and stored at -80ºC.

Leukocytes

Once the plasma was removed from the EDTA tube, the remaining pellet contained buffy coat and erythrocytes. This pellet was mixed with phosphate buffered solution (PBS) by inversion, and the mixture was added to 30 ml of haemolysis solution in a Falcon tube. The Falcon tube was incubated for 20 minutes at room temperature for erythrocyte lysing. Subsequently it was centrifuged at 2200 g for 5 minutes. The supernatant was discarded and the remaining pellet was washed with haemolysis solution and resuspended in 20 ml of haemolysis solution. Following centrifugation and disposal of the supernatant the previous steps were repeated. After finishing this process, the leukocyte cells were separated from the rest of the cell types. The pellet was resuspended in 900 µl of PBS. 450 µl were aliquoted and stored at -80ºC. To the remaining 450 µl, 10 ml of Cell Lysis Solution was added. The latter Falcon tube was

kept at room temperature (protected from the light), for subsequent DNA extractions.

DNA extraction and quantification

The Falcon tube was stored between 1-6 months. After that, 3.33 ml of Protein Precipitation Solution were added and mixed by vortex. The mixture with leukocytes was incubated on ice for 15 minutes and centrifuged for 12 minutes at 2000 g and 4ºC. 10 ml of cold 100% isopropanol was added to the supernatant and mixed by inversion. When the precipitated DNA was visible, the Falcon tube was centrifuged for 5 minutes at 2000 g and 4ºC. The supernatant was discarded, and the pellet containing the DNA was left in the tube to dry for 30-40 minutes in absorbent paper. The rehydration of the DNA was carried out by incubation with 1200µl of DNA Hydration Solution at room temperature for 3-4 days. After that, a Nanodrop 1000 spectrophotometer at 260 nm was used for DNA quantification of 2 µl of hydrated DNA.

Plasma and leukocytes from cord blood were processed identically as previously described for maternal blood.

Lifestyle, dietary and clinical history

Throughout pregnancy, we collected information directly from participants as well as that recorded in the clinical history, provided by the hospital clinical team.

The following questionnaires were administered:

- 12 GW: food frequency questionnaire on dietary habits of the mother in the 9 months before pregnancy.
- 20 GW: questionnaire on supplement use, with specific questions regarding folic acid and iron, the year before and during the first 20 weeks of pregnancy

(brand, dose, frequency and duration of intake), lifestyle and habits (physical activity, consumption of toxic substances (cigarettes, alcohol and illegal substances)), socioeconomic status (based on education, profession and salary). Based on the guidelines for socioeconomic measurements, the categories were categorised as low, medium or high [261].

- 32 GW: the same questionnaire as at 20 GW, but focused on the 2nd and 3rd trimesters of pregnancy.
- After delivery before discharge from the hospital a final FFQ focusing on dietary habits during pregnancy.

All questionnaires are included in the appendix.

At the first medical check-up, the participant's age, height, weight and parity among other clinical variables, were recorded. Parity is coded using four numerical values (x-x-x-x), which indicate in the following order: 1) the number of previous pregnancies carried to term, 2) the number of previous preterm pregnancies, 3) the number of previous miscarriages and 4) the number of live births. Gestational weeks of pregnancy and probable birth date were calculated based on the date of last menstruation and confirmed or corrected with the first ultrasound echography.

Systolic and diastolic blood pressure was measured in accordance with routine protocol throughout pregnancy. Pregnancy-induced hypertension was defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90mmHg occurring for the first time after 20 GW in previously normotensive women, and at least two measurements of 6 hours apart. If this was accompanied with proteinuria (≥0.3 g of protein/24h in urine), pre-eclampsia was diagnosed [262].

At 24-28 GW, pregnant women underwent the O'Sullivan test (glucose challenge test) for the detection of gestational diabetes following the protocols of both hospitals [263]. Women at high risk, i.e., gestational diabetes in previous pregnancies, family history of diabetes or obesity, underwent this test at the first check-up, at 24-28 GW and at 30-32 GW.

Pregnancy ultrasounds

To monitor blood flow in the uterine and umbilical arteries, pulsed-wave Doppler ultrasound testing was performed [264]. The pulsatility indices of the left and right uterine arteries were recorded and the average calculated for classification of pathological or normal flow. In these Doppler ultrasounds, the waveforms are assessed and if a notch is detected, it indicates a decrease in diastolic flow in the uterine artery. It can be unilateral (only in one artery) or bilateral (in both arteries) [265]. We defined, as pathological Doppler of the uterine arteries as a mean pulsatility index of left and right arteries was ≥95 percentile and/or the presence of a bilateral notch.

Determinations

Plasma samples were sent to BeVital A/S laboratories (Bergen, Norway) in 500 µl aliquots by courier on dry ice for the determination of different metabolites and 120 ng of lyophilized DNA for genetic determinations in CEGEN (Santiago de Compostela, Spain). Samples from the same pregnancy and cord were analysed in the same batch in less than 18 months after collection.

Biological determinations

Plasma cotinine was measured by liquid chromatography–tandem mass spectrometry at <12 GW, at 24-27 GW and in the cord [266]. Since cotinine is present in tobacco and is a metabolite of nicotine, it is a good blood biomarker for tobacco exposure for

the corroboration of self-reported cigarettes smoked per day [267]. Plasma cotinine values ≥10 ng/mL, indicate active smoking smokers.

If the mother reported never smoking and had negative plasma cotinine, she was classified as a never smoker. Mothers that reported giving up smoking before pregnancy and that had negative plasma cotinine at each cotinine check-up were classified as non-smokers. Mothers that reported giving up smoking during the first trimester but that had positive plasma cotinine at <12 GW, followed by negative cotinine in the remaining 2 samples were classified as first trimester only smokers. Mothers that had positive plasma cotinine twice or at all 3 times it was measured were classified as smokers throughout pregnancy. Mothers that self-reported active smoking were classified as smokers, even if all of their plasma cotinine concentrations did not confirm this because plasma cotinine is a more precise measurement in the hours after smoking.

Statistical analysis

Percentages and 95% CI are used to report categorical variables. Continuous variables are reported as arithmetic means and 95% CI for those variables with a normal distribution and geometric means and 95% CI for those variables with skewed distributions that were ln-transformed for the application of parametric statistical tests. Maternal metabolite statuses during pregnancy were compared with first trimester (<12 GW) status and with the previous gestational time point using ANOVA for repeated measures. Cord plasma nutrient and metabolite concentrations were compared with maternal plasma concentrations at labour by paired Student t-test. Gestational weeks at the moment of the blood sample was used as an intrasubject factor and highest plasma folate tertile versus low-mid tertiles in the first trimester was used as the intersubject factor when comparing plasma metabolites concentrations

during pregnancy with the first trimester. For the comparisons of third trimester and labour with previous time points, highest tertile of plasma folate versus low-mid tertiles at 4 to 7 months of pregnancy was used as the intersubject factor.

As L-Arginine pathway plasma metabolite concentration measurements at 15 GW were less than 4%, concentrations at first (<12GW), second (24-27GW), third (32GW) trimesters and labour were considered.

To observe the evolution of L-Arginine pathway metabolisms according to first trimester tertiles of tHcy and *MTHFR* C667T polymorphism genotypes, the intra- and intersubject factors were the same as previously mentioned. In addition, in each trimester the middle and lowest tertiles were compared with highest tertiles of tHcy using ANOVA analysis. This same analysis was used to compare the concentrations of the metabolites in the CT and TT genotypes with the CC for the *MTHFR* C667T polymorphism.

As the frequency of pre-eclampsia pregnancies was low, pregnancy-induced hypertension and pre-eclamptic pregnancies were combined in the assessment of pregnancy-induced hypertension. Means of mean pulsatility index, prevalence of pathologic Doppler and pregnancy-induced hypertension were analysed according to L-Arginine pathway metabolites tertiles. For L-Arginine and L-Arginine/ADMA Ratio, the reference tertile was the highest tertile, whereas for ADMA, SDMA and ADMA/SDMA Ratio was the highest tertile. The two tertiles were compared respectively with the reference using ANOVA analysis for the mean pulsatility index and with chi-square for the outcomes of pathological Doppler of the uterine arteries and pregnancy-induced hypertension.

The evolution of the metabolites of the L-Arginine pathway adjusted by GW was studied in controls and cases of pathological Doppler of the uterine arteries or

pregnancy-induced hypertension. Metabolite concentrations in each trimester of pregnancy and at birth were compared with cases and controls using both ANOVA as well as ANCOVA (adjusting for maternal age, BMI, smoking habits (never, first trimester only and throughout pregnancy), low versus mid-high socio-economic status, previous pregnancies >20 GW and plasma creatinine) at the moment of each trimester.

The probability of having pathological Doppler or pregnancy-induced hypertension was explored using multiple logistic regression analysis. The unadjusted model explored predictors of the L-Arginine pathway and was also adjusted for gestational age as continuous variables. Since the observed changes in plasma ADMA and SDMA metabolites did not reach the change of 1 µmol/L unit, the concentrations of all metabolites were multiplied by 10, for a better interpretation of the results. The adjusted model included maternal age, BMI, smoking habits (never, first trimester only and throughout pregnancy), low versus mid-high socio-economic status and previous pregnancies >20 GW. First trimester maternal plasma creatinine concentration was also included as a covariable when studying pregnancy-induced hypertension as the outcome.

Due to missing information for some clinical or biochemical variables, the number of participants included in the different analyses varied. The post-hoc Bonferroni test for multiple comparison was used to correct p-values. Significance was accepted at $p < 0.05$. The software SPSS (version 28.0) was used for data analysis.

4.3 Results

Maternal characteristics, adverse medical history, and lifestyle habits are shown in **Table 2.1.** Regarding pregnancy history, more than half of the mothers had a previous pregnancy and more than one-third had a previous miscarriage. More than a quarter of mothers (26.2%) smoked in the first trimester only and of these smokers 16.3% of all of the mothers continued smoking until the end. Only 35% of the women took a folic acid supplement before pregnancy.

Table 2.1: Maternal adverse medical history, lifestyle habits and first trimester characteristics

	n	Mean/Frequencies (95% CI)
Previous pregnancies[1]	793	54.1 (50.6, 57.5)
Previous miscarriages[1]	689	33.8 (30.4, 37.4)
Age (years)[2]	801	32.3 (32.0, 32.6)
Low socioeconomic status[1,3]	764	12.2 (10.0, 14.7)
BMI (kg/m^2)[2]	736	24.3 (24.0, 24.7)
Smoking[1]		
Never	569	74.3 (71.1, 77.2)
First trimester only	72	26.2 (22.3, 29.4)
Throughout pregnancy	125	16.3 (13.9, 19.1)
Supplementation use[1]		
Before pregnancy	249	35.9 (35.2, 39.6)
First trimester		
No sup/<400 µg/day	171	26.8 (23.5, 30.3)
≥ 400 ug/day	468	73.2 (69.7, 76.5)
4-7 months		
No sup/<400 µg/day	452	63.8 (60.2, 67.3)
≥ 400 ug/day	256	36.2 (32.7, 39.8)
***MTHFR* C677T polymorphism**[1]		
CC	249	33.7 (30.4, 37.2)
CT	364	49.3 (45.7, 52.99
TT	126	17.1 (14.5, 19.9)

BMI, body mass index, MTHFR, methylenetetrahydrofolate reductase, No sup, not supplemented. [1] Percentage, 95% confidence interval. [2] Mean, 95% confidence interval. [3] Socioeconomic status was based on household income and maternal and paternal educational level and occupation. *MTHFR* C677T polymorphism was in Hardy-Weinberg equilibrium.

During the first trimester, 26.8% of the women either took no supplement at all or did not take the recommended daily dose of folic acid (400ug) until the end of the first trimester. Only 36.2% of the women continued to take folic acid, in the form of folic acid or multivitamin supplements, after the first trimester. The *MTHFR* homozygote variant TT genotype was present in 17.1% of the mothers.

Table 2.2 shows the 1CM and L-Arginine pathway metabolites at the different points of pregnancy and in the umbilical cord. At 15 GW we observe an increase in RBCF and a decrease in tHcy compared to the first trimester. After this, tHcy continues to increase until the end of pregnancy but RBCF remains higher than at <12 GW and does not return to near <12 GW values until late pregnancy. Additionally, both plasma folate and cobalamin decrease throughout pregnancy. Plasma L-Arginine and ADMA did not significantly change their concentration throughout pregnancy, however, plasma ADMA concentrations in cord were significantly higher compared to maternal plasma ADMA concentrations at labour. Nonetheless, by the third trimester (32GW) the L-Arginine/ADMA Ratio was significantly reduced compared with the first trimester. As pregnancy progresses maternal plasma SDMA increases, and the concentrations found in the cord are almost three times the values found in maternal plasma at labour. Since plasma ADMA concentrations do not vary during pregnancy, but SDMA increases, the ADMA/SDMA Ratio in the third trimester and at labour is lower than in the first trimester.

Table 2.2: Maternal plasma 1CM and L-Arginine pathway metabolites during pregnancy and in cord.

	<12 GW	15 GW	24-27 GW	32 GW	Labour	Cord
GW[1]	9.4 (9.3, 9.5) [734]	14.6 (14.5, 14.7) [446]	24.4 (24.3, 24.5) [684]	33.4 (33.3, 33.5) [654]	39.1 (39.0, 39.2)[741]	39.1 (39.0, 39.2) [741]
One-Carbon metabolism						
tHcy (µmol/L)[2]	5.3 (5.2, 5.40) [770]	4.5^{ac} (4.4, 4.6) [455]	4.6^{abcd} (4.5, 4.7) [667]	5.2^{bcd} (5.1, 5.3) [636]	6.1^{abcd} (6.0, 6.3) [615]	4.8*** (4.7, 4.9) [580]
RBCF (nmol/L)[2]	943.2 (906.7, 981.1) [770]	1236.5^{ac} (1180.3, 1295.2) [455]	1147.8^{abcd} (1103.6, 1193.8) [667]	972.8^{bcd} (929.5, 1018.2) [636]	-	-
Folate (nmol/L)[2]	27.1 (25.8, 28.5) [750]	24.9^{ac} (23.3, 26.5) [453]	15.2^{abcd} (14.4, 16.1) [653]	12.8^{abcd} (12.0, 13.7) [618]	12.1^{acd} (11.3, 12.9) [614]	26.0*** (24.8, 27.2) [580]
B12 (pmol/L)[2]	359.1 (350.5, 367.9) [770]	323.6^{a} (313.6, 333.9) [455]	273.9^{abc} (266.7, 281.2) [667]	249.5^{abcd} (242.8, 256.5) [636]	230.7^{abcd} (223.6, 238.0) [609]	315.4*** (298.8, 332.9) [553]
Creatinine (µmol/L)[1]	52.7 (51.9, 53.5) [733]	49.8^{ac} (49.3, 50.4) [447]	49.0^{abcd} (48.5, 49.4) [660]	49.5^{acd} (48.9, 50.1) [624]	52.1^{bcd} (51.3, 52.9) [607]	64.1*** (62.1, 66.2) [557]
L-Arginine pathway						
L-Arginine (µmol/L)[1]	47.0 (44.9, 49.1) [166]	48.0 (43.1, 52.9) [29]	45.2 (42.9, 47.5) [137]	43.4 (41.5, 45.2) [120]	45.7 (42.0, 49.4) [140]	51.2 (46.7, 55.8) [108]
ADMA (µmol/L)[1]	0.42 (0.41, 0.43) [166]	0.41 (0.39, 0.43) [29]	0.43 (0.42, 0.44) [137]	0.43 (0.42, 0.44) [120]	0.45 (0.43, 0.46) [140]	1.13*** (1.08, 1.19) [108]
SDMA (µmol/L)[1]	0.38 (0.37, 0.40) [165]	0.34 (0.31, 0.36) [29]	0.40^{a} (0.39, 0.41) [137]	0.45^{ab} (0.43, 0.47) [120]	0.51^{ab} (0.48, 0.53) [140]	1.42*** (1.33, 1.52) [108]
L-Arginine/ADMA[1]	112.1 (106.8, 117.4) [166]	116.6 (104.3, 128.9) [29]	105.7 (101.1, 110.3) [137]	102.5^{a} (97.5, 107.5) [120]	103.5 (94.9, 112.1) [140]	45.3*** (41.8, 48.7) [108]
ADMA/SDMA[1]	1.14 (1.10, 1.18) [165]	1.24 (1.17, 1.31) [29]	1.10 (1.06, 1.14) [137]	0.97^{ab} (0.93, 1.00) [120]	0.91^{ab} (0.87, 0.94) [140]	0.82*** (0.79, 0.86) [108]

Hcy, total plasma homocysteine; ADMA, Asymmetric Dimethylarginine; SDMA, Symmetric Dimethylarginine; RBCF, Red blood cell folate; B12, plasma cobalamin; GW, gestational weeks. [1]Arithmetic mean, 95% confidence interval, [n]. [2]Geometric mean, 95% confidence interval, [n]. Comparisons were by ANOVA for repeated measures: Intrasubject factor: GW, ^{a}p <0.05 compared with <12GW; ^{b}p <0.05 compared with previous GW. Intersubject factor: folic acid supplement use. ^{c}p <0.05 compared with <12GW; ^{d}p <0.05 compared with previous GW. Cord plasma nutrient and metabolite concentrations were compared with maternal plasma concentrations at labour by paired Student t-test, *p <0.05, **p <0.01, ***p <0.001. Post-hoc Bonferroni correction for multiple comparisons was applied.

One-carbon metabolism and L-Arginine pathway association

The evolution throughout pregnancy of the L-Arginine pathway metabolites according to first trimester tHcy tertiles is shown in **Figure 2.2.** Participants in the mid tHcy tertile had higher plasma ADMA concentrations (mean ± SD, 0.43 ± 0.07) and a lower L-Arginine/ADMA Ratio (mean ± SD, 104.6 ± 28.4) in the first trimester compared to those with tHcy in the lowest tertile (ADMA, 0.040 ± 0.052; L-Arginine/ADMA Ratio, 118.79 ± 25.55; p <0.05). However, plasma ADMA increased and the L-Arginine/ADMA Ratio decreased as pregnancy progressed, only in women in the lowest tHcy tertile. In addition, the plasma concentration of SDMA was higher in the first trimester (mean ± SD, 0.39 ± 0.08) in those women with first trimester tHcy in the highest tertile compared to the lowest (mean ± SD, 0.35 ± 0.07; p, 0.026). However, SDMA concentrations increased significantly throughout pregnancy, regardless of the mothers' tHcy tertile. In parallel, the ADMA/SDMA Ratio decreased throughout pregnancy in all three tHcy tertiles.

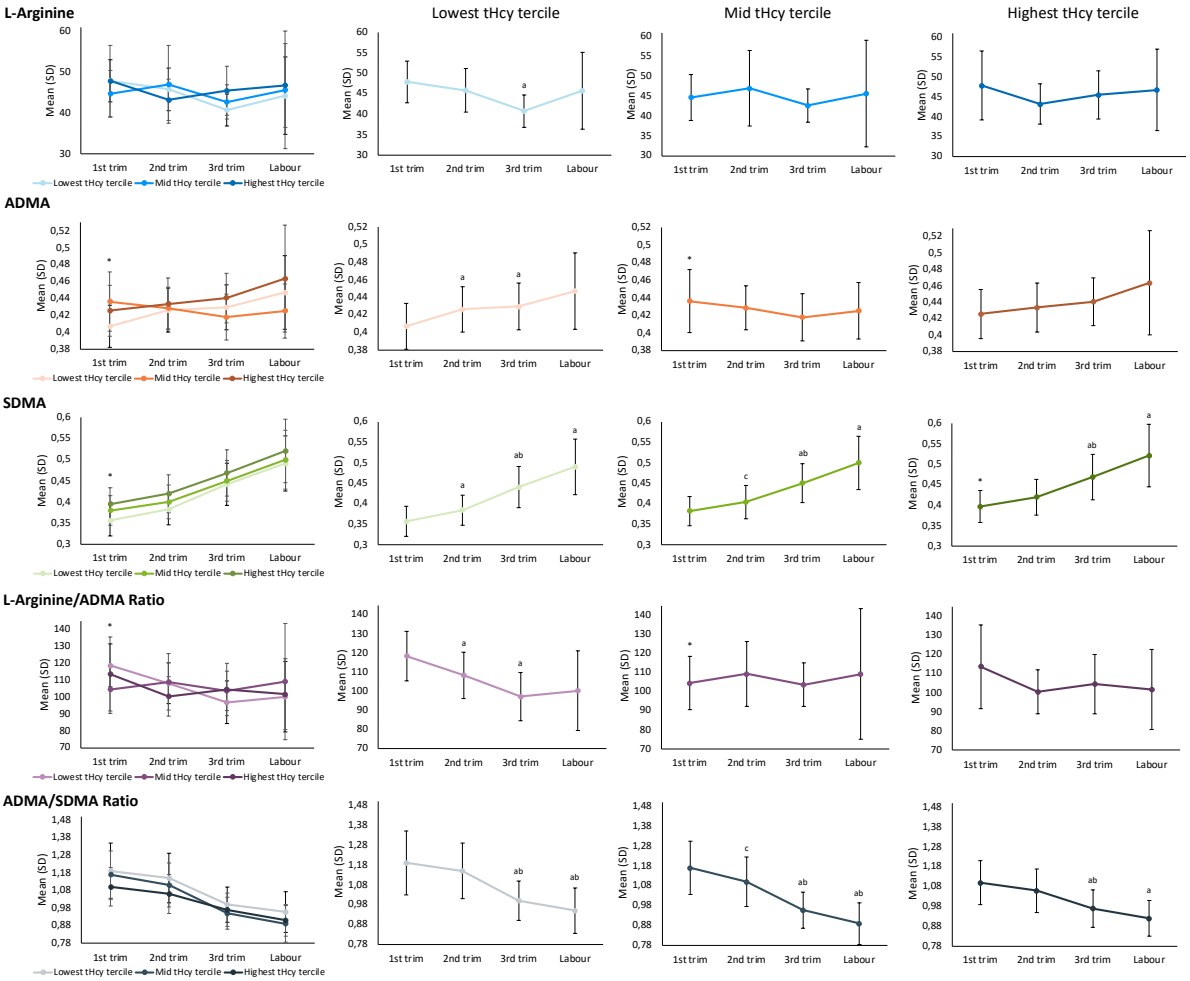


Figure 2.2: Maternal plasma L-Arginine pathway metabolites throughout pregnancy according to first trimester tHcy tertiles. tHcy, total plasma homocysteine; ADMA, Asymmetric Dimethylarginine; SDMA, Symmetric Dimethylarginine; GW, gestational weeks; trim, trimester. Comparisons were by ANOVA for repeated measures: Intrasubject factor: GW, [a]p <0.05 compared with <12GW; [b]p <0.05 compared with previous GW. Intersubject factor: folic acid supplement use. [c]p <0.05 compared with <12GW; [d]p <0.05 compared with previous GW. [*]p <0.05 compared with lowest tHcy tertiles. Post-hoc Bonferroni correction for multiple comparisons was applied. First trimester tHcy tertiles were adjusted for GW. tHcy tertiles cut-offs: Lowest tertile ≤ 4.80 µmol/L, mid tertile >4.80 µmol/L and ≤ 5.73 µmol/L and highest tertile > 5.73 µmol/L. Lowest tHcy tertile: first trimester n=42, second trimester n=34, third trimester n=32, Labour n=33. Mid tHcy tertile: first trimester n=56, second trimester n=44, third trimester n=38, Labour n=44. Highest tHcy tertile: first trimester n=59, second trimester n=54, third trimester n=47, Labour n=60.

Plasma ADMA concentrations at <12 GW were lower in mothers with the *MTHFR* 677 CT (mean ± SD, 0.41 ± 0.06; p, 0.05) and TT (mean ± SD, 0.40 ± 0.06; p, 0.02) genotypes compared to the CC (mean ± SD, 0.44 ± 0.06) (**Figure 2.3**). This was also seen for first trimester plasma SDMA concentrations in TTs (mean ± SD, 0.35 ± 0.06; p, 0.017) and at labour (mean ± SD, 0.50 ± 0.16; p, 0.031) compared to the CC

genotype (First trimester, 0.40 ± 0.08; labour, 0.52 ± 0.03). Parallelly, the ADMA/SDMA Ratio was higher in TTs compared with CCs at labour.

No changes in plasma L-Arginine and ADMA concentrations or the L-Arginine/ADMA Ratio were observed throughout pregnancy according to *MTHFR* C677T genotypes, except for higher ADMA concentrations in the third trimester compared to the second in heterozygous participants. SDMA concentrations increased in all genotypes, however, compared to the first trimester, the significant increase does not occur in the CC genotype until labour, while in the CT and TT the SDMA concentrations are already higher in the third trimester. The decrease in the ADMA/SDMA Ratio compared to first trimester values is noticeable in the third trimester in all genotypes.

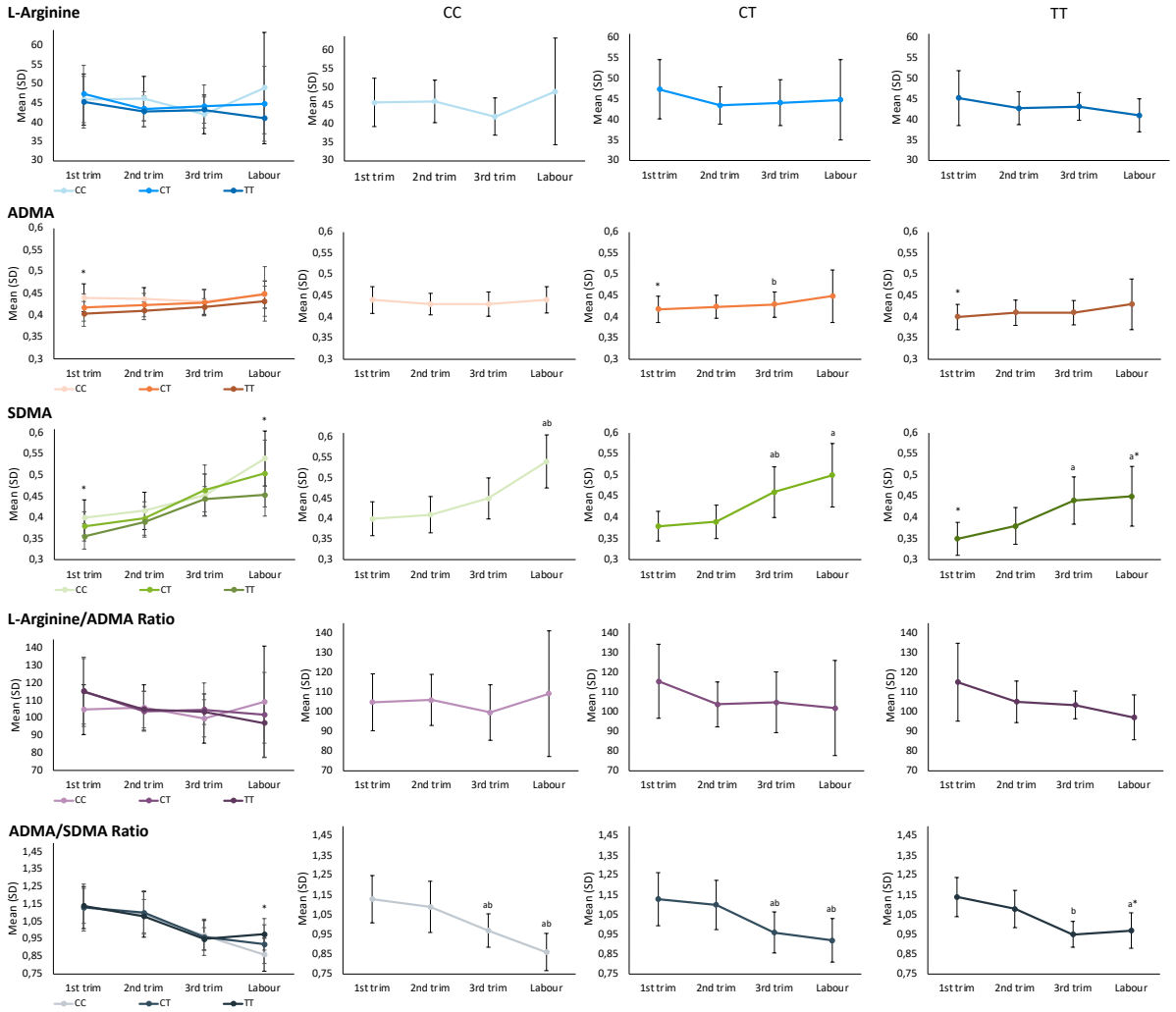


Figure 2.3: Maternal plasma L-Arginine pathway metabolites throughout pregnancy according to *MTHFR* C677T polymorphism genotypes. ADMA, Asymmetric Dimethylarginine; SDMA, Symmetric Dimethylarginine; GW, gestational weeks; trim, trimester. Comparisons were by ANOVA for repeated measures: Intrasubject factor: GW, [a]p <0.05 compared with <12GW; [b]p <0.05 compared with previous GW. Intersubject factor: folic acid supplement use. [c]p <0.05 compared with <12GW; [d]p <0.05 compared with previous GW. [*]p <0.05 compared with *MTHFR* 677 CC genotype. Post-Hoc Bonferroni correction for multiple comparisons was applied. *MTHFR* 677CC: first trimester n=49, second trimester n=43, third trimester n=38, Labour n=46. *MTHFR* 677CT: first trimester n=83, second trimester n=68, third trimester n=61, Labour n=74. *MTHFR* 677TT: first trimester n=24, second trimester n=20, third trimester n=17, Labour n=18.

L-Arginine pathway and adverse pregnancy outcomes

The adverse pregnancy outcomes of placental origin selected for this thesis were pathological Doppler of the uterine arteries, pregnancy-induced hypertension and pre-eclampsia. The prevalence in our population of these outcomes is represented in **Table 2.3.** At 20 GW, 15.7% of mothers had a mean pulsatility index ≥ percentile 95

or had a bilateral notch in the uterine artery waveform (pathological Doppler of the uterine arteries). In addition, 13.9% of the mothers had systolic or diastolic blood pressure ≥140/90 mmHg after 20 GW. Of these, 2.6% of mothers also had proteinuria (pre-eclampsia) while 11.6% of mothers had less than 0.3 g of protein/24h in urine (pregnancy-induced hypertension).

Table 2.3: Adverse pregnancy outcomes prevalence.

	n	% (95% CI)
Pathological Doppler[1]	105/668	15.7 (13.2, 18.7)
Pregnancy-induced hypertension[2]	61/524	11.6 (9.2, 14.7)
Pre-eclampsia[3]	14/538	2.6 (1.6, 4.5)

[1] Mean pulsatility index of the right and left uterine arteries mean pulsatility index ≥percentile 95 or the presence of a bilateral notch in the uterine artery waveforms at 20 GW.
[2] Systolic blood pressure ≥140mmHg and/or Diastolic Blood Pressure ≥90mmHg in the absence of proteinuria (<0.3 g of protein/24h in urine), occurring for the first time after 20 GW.
[3]Systolic blood pressure ≥140mmHg and/or Diastolic Blood Pressure ≥90mmHg in the presence of proteinuria (≥0.3 g of protein/24h in urine), occurring for the first time after 20 GW.

Adverse pregnancy outcomes prevalence is also shown according to first trimester plasma L-Arginine pathway metabolites tertiles in **Table 2.4**. No differences are seen for any outcome among L-Arginine or L-Arginine/ADMA tertiles. Mean PI mean, pathological Doppler of the uterine arteries and pregnancy-induced hypertension prevalence did not differ among ADMA, SDMA or ADMA/SDMA Ratio tertiles.

Table 2.4: Mean pulsatility index, pathological Doppler of the uterine arteries and pregnancy-induced hypertension according to maternal first trimester plasma L-Arginine pathway metabolites tertiles.

L-Arginine	Mean PI[1]	Pathological Doppler[2]	Pregnancy-induced hypertension[2]
Lowest tertile	1.00 (0.90, 1.09) [48]	17.4 (9.1, 30.7) [8/46]	10.8 (4.3, 24.7) [4/37]
Mid tertile	0.99 (0.88, 1.10) [48]	13.6 (7.4, 30.4) [6/44]	8.3 (2.9, 21.8) [3/36]
Highest tertile (ref)	0.99 (0.89, 1.09) [51]	22.0 (12.8, 35.2) [11/50]	17.1 (8.5, 31.3) [7/41]
ADMA			
Lowest tertile (ref)	1.07 (0.96, 1.19) [49]	24.4 (14.2, 38.7) [11/45]	7.0 (2.4, 18.6) [3/43]
Mid tertile	0.93 (0.84, 1.02) [48]	15.2 (7.6, 28.2) [7/46]	15.2 (6.7, 30.9) [5/33]
Highest tertile	0.97 (0.88, 1.06) [50]	14.3 (7.1, 26.7) [7/49]	15.8 (7.4, 30.4) [6/38]
SDMA			
Lowest tertile (ref)	1.05 (0.92, 1.17) [44]	26.2 (15.3, 41.1) [11/42]	10.3 (4.1, 23.6) [4/39]
Mid tertile	0.99 (0.91, 1.08) [51]	12.5 (5.9, 24.7) [6/48]	12.5 (5.0, 28.1) [4/32]
Highest tertile	0.96 (0.87, 1.05) [51]	16.3 (8.5, 29.0) [8/49]	14.3 (6.7, 27.8) [6/42]
L-Arg/ADMA Ratio			
Lowest tertile	0.98 (0.88, 1.08) [46]	14.0 (6.6, 27.3) [6/43]	8.6 (3.0, 22.4) [3/35]
Mid tertile	1.04 (0.94, 1.15) [50]	22.9 (13.3, 36.5) [11/48]	7.5 (2.6, 19.9) [3/40]
Highest tertile (ref)	0.95 (0.86, 1.05) [51]	16.3 (8.5, 29.0) [8/49]	20.5 (10.8, 35.5) [8/39]
ADMA/SDMA Ratio			
Lowest tertile (ref)	1.00 (0.91, 1.09) [51]	14.6 (7.3, 27.2) [7/48]	10.0 (4.0, 23.1) [4/40]
Mid tertile	1.03 (0.91, 1.14) [51]	26.5 (16.2, 40.3) [13/49]	13.5 (5.9, 28.0) [5/37]
Highest tertile	0.95 (0.86, 1.05) [44]	11.9 (5.2, 25.0) [5/42]	13.9 (6.1, 28.7) [5/36]

ADMA, Asymmetric Dimethylarginine; SDMA, Symmetric Dimethylarginine. [1] Mean, 95% confidence interval. [2] Percentage, 95% confidence interval. Comparisons between tertiles were by ANOVA for continuous variables and χ^2 for categorical variables. First trimester L-Arginine pathway metabolites tertiles were adjusted for GW. L-Arginine tertiles cut-offs: highest tertile ≥ 50.72 µmol/L, mid tertile < 50.72 µmol/L and ≥41.79 µmol/L and lowest tertile <41.79 µmol/L. ADMA tertiles cut-offs: lowest tertile ≤ 0.39 µmol/L, mid tertile >0.39 µmol/L and ≤ 0.45 µmol/L and highest tertile > 0.45 µmol/L. SDMA tertiles cut-offs: lowest tertile ≤ 0.35 µmol/L, mid tertile >0.35 µmol/L and ≤ 0.41 µmol/L and highest tertile > 0.41 µmol/L. L-Arginine/ADMA Ratio tertiles cut-offs: highest tertile ≥ 119.84, mid tertile ≥ 100.64 and < 119.84 and lowest tertile < 100.64 µmol/L. ADMA/SDMA Ratio tertiles cut-offs: lowest tertile ≤ 1.01, mid tertile >1.01 and ≤ 1.19 and highest tertile > 1.19 µmol/L. *p <0.05 compared with reference tertile. Statistical differences are adjusted by the Bonferroni tests for multiple comparison corrections.

Figure 2.4 and **Figure 2.5** show plasma L-Arginine pathway metabolite concentrations throughout pregnancy according to the presence of pathological Doppler of the uterine arteries and pregnancy-induced hypertension, respectively. The pattern of changes in the L-Arginine pathway metabolites did not differ in mothers with pathological Doppler of the uterine arteries compared with controls (**Figure 2.4**).

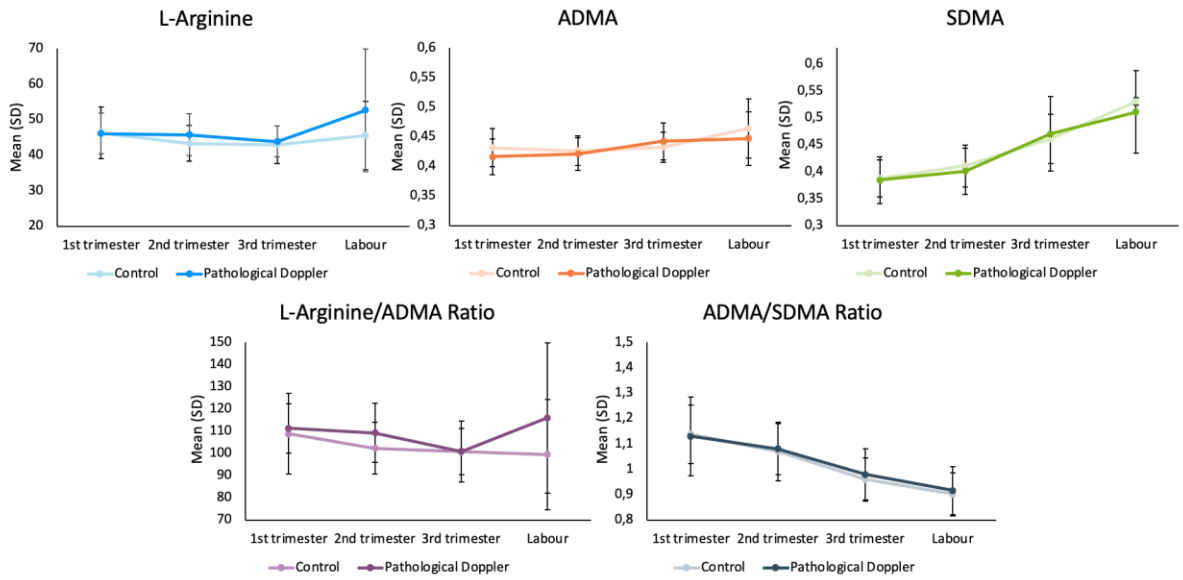


Figure 2.4: Mean plasma L-Arginine pathway metabolite concentrations throughout pregnancy according to pathological Doppler of the uterine arteries. ADMA, Asymmetric Dimethylarginine; SDMA, Symmetric Dimethylarginine. Comparisons between controls and pathological Doppler of the uterine arteries were by ANOVA and ANCOVA analysis (adjusted for maternal age, BMI, smoking habits (never, first trimester only and throughout pregnancy), low versus mid-high socio-economic status, previous pregnancies >20 GW and plasma creatinine in each trimester. $^{*}p$ <0.05 compared with controls. Controls: first trimester n=114, second trimester n=93, third trimester n=88, Birth n=105. Pathological Doppler: first trimester n=25, second trimester n=25, third trimester n=18, Labour n=21.

Figure 2.5 illustrates changes in plasma concentrations of L-arginine pathway metabolites in mothers with pregnancy-induced hypertension and control mothers. Mothers with pregnancy-induced hypertension had higher ADMA and a lower L-Arginine/ADMA Ratio throughout pregnancy compared with controls. However, these differences were no longer statistically significant after adjusting for maternal factors. Mothers with pregnancy-induced hypertension had a higher first trimester ADMA/SDMA Ratio compared to normotensive mothers.

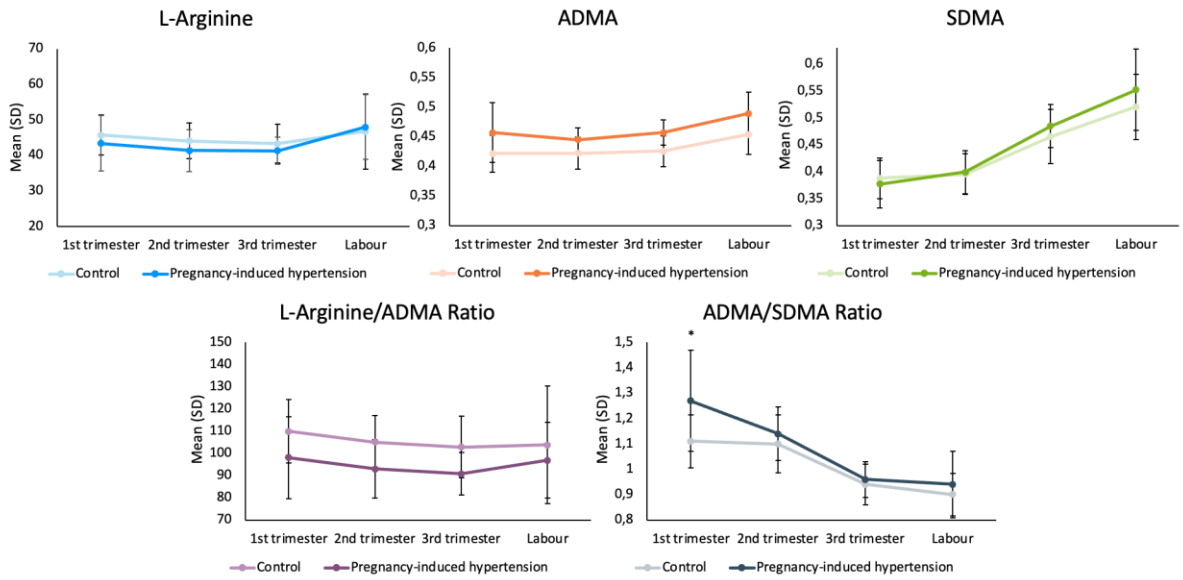


Figure 2.5: Maternal plasma L-Arginine pathway metabolite concentrations throughout pregnancy according to pregnancy-induced hypertension. ADMA, Asymmetric Dimethylarginine; SDMA, Symmetric Dimethylarginine. Comparisons between controls and pregnancy-induced hypertension were by ANOVA and ANCOVA analysis (adjusted for maternal age, BMI, smoking habits (never, first trimester only and throughout pregnancy), low versus mid-high socio-economic status, previous pregnancies >20 GW and plasma creatinine in the moment of each trimester. *p <0.05 compared with controls. Controls: first trimester n=100, second trimester n=86, third trimester n=76, Labour n=92. Pregnancy-induced hypertension: first trimester n=14, second trimester n=10, third trimester n=13, Labour n=13.

None of the L-Arginine pathway metabolites were associated with mean pulsatility index of the uterine arteries at 20 GW (data not shown). In **Table 2.5** the associations between the first trimester plasma concentrations of the L-Arginine pathway metabolites and pathological Doppler of the uterine arteries and with pregnancy-induced hypertension are shown. In the case of pathological Doppler of the uterine arteries, as an outcome, none of the multiple logistic regression models were statistically significant. The same occurs with pregnancy-induced hypertension as an outcome, except in the case of the ADMA/SDMA Ratio. An increase of 0.1 in this Ratio at <12 GW was associated with a 40% increased risk of pregnancy-induced hypertension (OR [95% CI] (1.4 [1.1, 1.9]) after adjusting for several maternal characteristics.

Table 2.5: Multiple logistic regression analysis testing the association between plasma L-Arginine pathway metabolites and pathological Doppler of the uterine arteries and pregnancy-induced hypertension.

		Pathological Doppler of uterine arteries			Pregnancy-induced hypertension		
	Model	n	R^2	OR (95%CI)	n	R^2	OR (95%CI)
L-Arginine	Unadjusted	138	0.000	1.0 (1.0, 1.0)	110	0.001	1.0 (1.0, 1.0)
	Adjusted		0.108	1.0 (1.0, 1.0)		0.168	1.0 (1.0, 1.0)
ADMA	Unadjusted	138	0.013	0.7 (0.3, 1.4)	110	0.016	1.6 (0.6, 3.9)
	Adjusted		0.116	0.7 (0.3, 1.6)		0.165	1.2 (0.4, 3.4)
SDMA	Unadjusted	137	0.001	0.9 (0.5, 1.7)	109	0.006	0.8 (0.4, 1.8)
	Adjusted		0.114	0.8 (0.4, 1.6)		0.201	0.5 (0.2, 1.4)
L-Arginine/ADMA Ratio	Unadjusted	138	0.000	1.0 (1.0, 1.0)	110	0.013	1.0 (1.0, 1.0)
	Adjusted		0.114	1.0 (1.0, 1.0)		0.173	1.0 (1.0, 1.0)
ADMA/SDMA Ratio	Unadjusted	137	0.000	1.0 (0.8, 1.2)	109	0.050	1.2 (1.0, 1.5)
	Adjusted		0.113	1.1 (0.9, 1.3)		0.243*	1.4 (1.1, 1.9)

ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine. Multiple logistic regression analysis was used. Unadjusted model: first trimester maternal plasma L-Arginine pathway metabolites. Metabolite concentrations were multiplied by 10 to facilitate results interpretation. Adjusted model: same variables as unadjusted model as well as maternal age, BMI, smoking habits (never, first trimester only and throughout pregnancy), low versus mid-high socio-economic status and previous pregnancies >20 GW. When pregnancy-induced hypertension was the dependent outcome, the model was also adjusted for first trimester plasma creatinine. Nagelkerke R^2. *p <0.05; **p <0.01; ***p <0.001.

4.4 Discussion

Major findings

Elevated first trimester maternal tHcy and the *MTHFR* C677T polymorphism were associated with first trimester L-Arginine pathway metabolites. These associations were not maintained for L-Arginine pathway metabolites determined at later time in pregnancy. Maternal first trimester L-Arginine pathway metabolites were not associated with increased pulsatility index nor pathological Doppler of the uterine arteries. In contrast, maternal first trimester ADMA/SDMA Ratio was associated with increased risk of pregnancy-induced hypertension.

One-Carbon metabolism and L-Arginine pathway in pregnancy

Maternal tHcy status decreases from the first trimester and reaches its lowest at the second trimester, returning to a status similar to at baseline value in the third trimester and exceeding them at birth. Murphy et al. reported this pattern in normal pregnancies and that it occurs independently of whether the mothers take folic acid supplements [190, 268]. This same pattern has been seen by Walker et al. (1999) in normal pregnancies [269]. There are many factors that can affect tHcy concentrations throughout pregnancy, including folic acid supplementation [270], haemodilution and increased demands of methionine by the foetus [191], pregnancy hormones [271], among others. In our population, from 15 GW, after the first trimester folic acid supplement use has stopped, plasma folate concentration starts to decrease and continues to do so until the end of pregnancy. However, this reduction does not occur in red blood cell folate concentration until the third trimester, the same trimester as homocysteine increases. Plasma folate concentration is indicative of recent folate

intake (either from folic acid supplementation or diet), whereas red blood cell folate is indicative of storages in the liver over a longer period of time [272]. Folic acid supplementation prevalence was reduced from 73.2% to 36.2% after the first trimester, which would partly explain the decrease in plasma folate, combined with haemodilution and provision of the nutrient to the foetus. However, red blood cell folate does not decline until the third trimester despite the reduction in folic acid intake and the decline in plasma folate status. In the last trimester rapid foetal growth occurs, so the increased demand for one-carbon units from the foetus would decrease RBCF reserves [184].

Maternal plasma B12 concentrations decreased as the pregnancy progressed in our study. This is consistent with other studies that observed a decrease in plasma cobalamin in normal pregnancies [273, 274]. Methionine synthase catalyses the transfer of a methyl group from bound methylcobalamin in order to remethylate homocysteine into methionine [21]. Optimal B12 status during pregnancy is crucial as homocysteine remethylation demands from the foetus increase [191].

In our population, maternal L-Arginine concentrations did not change throughout pregnancy as seen in a Japanese cohort of 221 pregnant women. At midgestation (25.4 ± 1.3 weeks) L-Arginine values were decreased compared with early gestation (11.3 ± 1.3 weeks) in uncomplicated pregnancies. This decrease did not persist until the end of pregnancy (37.4 ± 0.4 weeks) [254]. In contrast, other authors did not observe a difference in plasma L-Arginine between the different trimesters [276, 277]. Holden et al. (1998) [200] observed in a population of 20 non-pregnant and 145 pregnant women, that maternal first trimester plasma ADMA means were lower (0.4 ± 0.15 µmol/L) than in non-pregnant women (0.82 ± 0.31). In addition, plasma and serum ADMA concentrations have been found to increase as pregnancy progresses [200,

222, 276]. We observed that this increase was no longer significant after correcting for the Bonferroni test. Of the aforementioned studies that have observed an increase in ADMA throughout pregnancy, only one has performed a Bonferroni post-hoc adjustment [222]. It should be noted that this study only included 41 with uncomplicated pregnancies.

We observed that plasma SDMA concentrations significantly increased as the pregnancy progressed, and the significance was sustained after applying post hoc Bonferroni correction. Few studies have investigated this metabolite throughout pregnancy. In a study of malaria during pregnancy, plasma SDMA concentrations were assessed in 94 out of the 384 women that enrolled for the study. After adjusting for gestational age, SDMA plasma concentrations increased over the course of pregnancy. They also saw, like us, that at enrolment (20.9 GW, SD, 3.4) ADMA concentrations were higher than SDMA, but by the third trimester, SDMA exceeded ADMA concentrations [276]. In this same study, L-Arginine/ADMA Ratio did not differ in the progress of pregnancy, the same results are seen in our study. Berlinguer et al. (2019) studied the evolution of both L-Arginine/ADMA and ADMA/SDMA ratios in sheep, controls and those on feed-restricted diets. Even though the L-Arginine/ADMA Ratio decreased during the 140 days of pregnancy, it did not differ between the two groups. However, ADMA/SDMA Ratio was significantly higher in controls compared to feed-restricted ewes at day 50 and day 80 of pregnancy, which indicates a decrease activity in the ADMA degrading enzyme, DDAH [278]. Valtonen et al. (2009), compared the ADMA/SDMA Ratio among non-pregnant women (n=61) and women that were in the first (n=13; ≤14 GW), second (n=2; 15-27 GW) and third trimester (n=23; ≥28 GW). Second trimester ADMA/SDMA Ratio was significantly higher compared to the third trimester [279]. In our population, the ADMA/SDMA Ratio was

significantly higher in the second trimester (mean [95% CI] (1.10 [1.06, 1.14]) than in the third trimester (mean [95% CI] (0.97 [0.93, 1.00]) after Bonferroni corrections. An increased ADMA/SDMA Ratio suggests an impaired DDAH activity [116].

Several authors have investigated 1CM or the L-Arginine pathway, but few of them have studied the association between them during pregnancy. It is hypothesised that high tHcy concentrations decrease NO synthesis by increasing ADMA production and therefore NOS would be inhibited [125]. In our population we observed that the first trimester plasma ADMA concentrations of those mothers with first trimester tHcy in the mid tertile are higher than those in the lowest tertile. In the lowest tertile, but not in the mid or highest tertile, plasma ADMA concentrations are in the second and third trimester compared to the first trimester. In the mid and highest tHcy tertiles, there are no changes in plasma ADMA concentrations throughout pregnancy. These differences may simply be due to the fact that baseline plasma ADMA concentrations are lower in the lowest tHcy tertile. Also, the mean maximum plasma ADMA concentrations in the third trimester and at labour are at the highest tertile of tHcy in the first trimester.

High concentrations of plasma ADMA may be conditioned by an increase in its synthesis (85% synthesised by type I PRMT [280]) or a decrease in its degradation. PRMT enzymes have been found to be involved in a number of functions such as gene expression regulation, mRNA splicing, protein localization, signal transduction, development and even cancer. However, its role in foetal programming remains understood [281]. Ito et al. (2009) saw that DDAH activity of the myometrium of rats was up-regulated in mid-gestation accompanied by a reduction in plasma ADMA and other vasoconstrictors. This may reduce the contractility of the myometrium for uterine quiescence during gestation. Nonetheless, at term gestation, DDAH was decreased, again accompanied by plasma ADMA increase, which may suggest that increased

myometrial vasoconstrictors may increase contractions and tone for the labour onset [282]. Stühlinger et al. (2001) saw that in bovine aortic endothelial cells cultured in DL-homocysteine (10 µmol/L to 1 mmol/L), ADMA concentrations in cells cultured with 10 µmol/L of homocysteine were significantly higher (0.54 ± 0.04 µmol/L) compared to those cells cultured without homocysteine (0.33 ± 0.02 µmol/L). Plasma ADMA, but not SDMA, concentrations parallelly increased with homocysteine concentrations. In addition, DDAH activity was reduced by 19% when cells were cultured in 10 µmol/L of homocysteine [161]. In addition, Ayar et al. (2003) observed an increase in spontaneous contractions in the myometrium from pregnant women after homocysteine was applied to the organ compared to before and after the application [283].

In our study, only 1% of the women had tHcy concentrations above 10 µmol/L at the beginning of the pregnancy. This percentage increased in the second trimester (4.5%) but only reached 5% by the end of pregnancy. This may explain the lack of association between tHcy concentrations in the first trimester and plasma ADMA throughout pregnancy. The tHcy values would not be high enough to inhibit the DDAH enzyme and consequently decrease the degradation of ADMA. However, a model of mice with endothelial dysfunction studied by Dayal et al. (2007) concluded that the decreased expression of DDAH enzyme due to hyperhomocysteinemia did not alter plasma ADMA concentrations [284]. Nevertheless, we need to consider that tHcy is subject to intense physiological and hormonal effects that change its concentrations, independently of its status within 1CM. This may complicate the investigation of its interaction with other pathways. Furthermore, homocysteine is central in a metabolic network that is upregulated to meet pregnancy demands and foetal developmental

requirements during pregnancy so pregnancy related changes may prevail over interactions observed outside of pregnancy.

SDMA is not a substrate for DDAH enzyme and increases throughout pregnancy in our study, independently of first trimester tHcy status. The literature indicates that during pregnancy there is an increase in glomerular filtration rate and haemodilution, which contradicts the increase in SDMA (which is mainly degraded by urine) during pregnancy. It has been postulated that CAT enzymes, responsible for methylarginines transports in and out of the cell, may be enhanced at the end of the pregnancy [285].

As mentioned in chapter 1, the association between the *MTHFR* C677T polymorphism and the L-Arginine pathway has not been studied in depth and to our knowledge, no study has been conducted to investigate their association in a longitudinal cohort during pregnancy.

In two studies conducted by Dimitroulas et al. (2016) and Fernández-Macías et al. (2022) of rheumatoid arthritis patients and healthy women, respectively, serum ADMA concentrations were higher in those participants with the TT genotype of the *MTHFR* C677T polymorphism compared to those with the wild-type genotype [157, 286]. In addition, Sgarra et al. (2020), in patients with a history of stroke, observed lower L-Arginine/ADMA Ratio in both TT and CT genotypes compared to CC [287]. In a study on epilepsy, no association was found between plasma ADMA concentrations and the different genotypes of this polymorphism [158]. In contrast, we observed a lower first-trimester plasma ADMA concentration in women with the CT or TT genotypes of the *MTHFR* C677T polymorphism when compared to those with the CC genotype. These differences are not observed in the rest of the pregnancy. As mentioned in the previous chapter, the TT genotype could reduce SAM (methyl group donor) synthesis by limiting the availability of folate, and reducing methylation [159]. Compared to the first

trimester, RBCF concentrations are always higher at the different points of pregnancy in our population. This could indicate that folate status would be sufficient that, even if its availability is limited by a decreased activity of the *MTHFR* enzyme due to the mutated genotype, SAM synthesis would not be reduced. This could explain why we only observed differences in ADMA concentrations in the first trimester, when RBCF status is lower and represents long-term folate intake (only 36% of women supplemented before pregnancy). We also must consider that the time of pregnancy when tHcy is least affected by physiological changes of pregnancy is during the first trimester. These changes may mask possible association in mid and late pregnancy.

It has been postulated that in healthy early pregnancies, the upregulation of the L-Arginine-NO system and plasma ADMA decrease may be a hemodynamic adaptation for uterine relaxation and organ perfusion demands. The increase in ADMA as the pregnancy progresses may prepare uterine muscle fibres for delivery as contractile activity increases. However, if this ADMA increase occurs at early stages of pregnancy, relaxation-contraction disequilibrium may lead to a poor vascular adaptation that could trigger pregnancy complications such as pregnancy-induced hypertension or pre-eclampsia [200].

L-Arginine pathway and adverse pregnancy outcomes

L-Arginine pathway metabolites and Doppler ultrasounds parameters

We did not observe any association between L-Arginine pathway metabolites and Doppler ultrasound parameters such as mean pulsatility index of the uterine arteries at 20 GW or the presence of bilateral notches in the uterine artery waveforms. Doppler ultrasound is of great importance in predicting pregnancy complications and gives information regarding utero-placental blood flow [288]. If

the uterine arteries do not decrease their resistance and increase blood supply due to poor trophoblast invasion, this will be reflected in the Doppler ultrasound parameters. Savvidou et al. (2003) did see higher plasma ADMA concentrations in those mothers with the presence of bilateral notches (median, 2.4; IQR, 1.97-3.14) compared to those without notches (median, 0.81; IQR, 0.49-1.08) [221]. Our median plasma ADMA concentrations at 24-27 GW are 0.41 µmol/L (IQR, 0.39-0.46) for mothers with mean pulsatility index ≥ percentile 95 or presence of bilateral notch and 0.42 µmol/L (IQR, 0.39-0.46) for mothers with neither of these features. The Savvidou study's plasma ADMA concentrations compared with ours are much higher, which could explain the lack of association between plasma ADMA and the presence of bilateral notch in uterine arteries of our population. In addition, tHcy was not determined in that study and it was not specified whether the participants took folic acid supplements, which could modulate tHcy status.

The conformational change that the uterine arteries must undergo from high to low resistance and increased blood flow, allows optimal interchange and transport of nutrients and oxygen from the mother to the foetus [207]. If this conformational change does not occur optimally, the effects on placental morphology and function are drastic, leading to poor/reduced placental perfusion [289]. In these conditions, several factors affecting blood flow and pressure are released that trigger a cascade leading to maternal endothelial dysfunction. Among others, reduced NO synthesis has been associated with pre-eclampsia [290].

L-Arginine pathway metabolites and pregnancy-induced hypertension

Plasma ADMA concentrations were higher in pregnancies with pre-eclampsia than in the control group, and higher concentrations have been observed in early pre-eclampsia [199, 291].

It has been proposed that plasma ADMA may have a negative effect on the placentation process [292]. In a study of 35 pregnant women, prone to recurrent hypertension, plasma ADMA, SDMA and ADMA/SDMA Ratio concentrations at 12, 16 and 20 GW did not differ between normotensive and hypertensive mothers [277]. The L-Arginine/ADMA Ratio was lower at 20 GW in hypertensive, but not in normotensive mothers, compared to 16 GW. Our study shows that, even though plasma ADMA and L-Arginine/ADMA Ratio in women with pregnancy-induced hypertension were higher and lower (respectively) throughout pregnancy compared to normotensive mothers, the differences did not reach statistical significance. However, the first trimester ADMA/SDMA Ratio was higher in women with hypertension compared to those with normal blood pressure. In addition, an increase of 0.1 unit in first trimester ADMA/SDMA Ratio was associated with an increased risk of pregnancy-induced hypertension (OR [95% CI] (1.4 [1.1, 1.9]), after adjusting for several maternal cofactors. Increased ADMA/SDMA Ratio may suggest 1) changes in the two classes of PRMT enzyme, class 1 and class 2, that methylate ADMA and SDMA or 2) impaired DDAH activity [116]. LDL cholesterol increases the expression of the PRMT enzyme [177]. This variable was not available in our study, however, first trimester BMI of mothers with pregnancy-induced hypertension was higher (26.5 ± 5.5 kg/m^2) compared to normotensive mothers (23.9 ± 4.9 kg/m^2). In addition, in this same population, in the association of first trimester maternal tHcy ≥ 90th percentile and pregnancy-induced hypertension, BMI, but not tHcy, was associated with a higher risk

of this adverse outcome (OR=1.1, 95%CI=1.05-1.15) [293]. LDL cholesterol and obesity have been associated in a population of 4260 participants [294].

4.5 Strengths and limitations

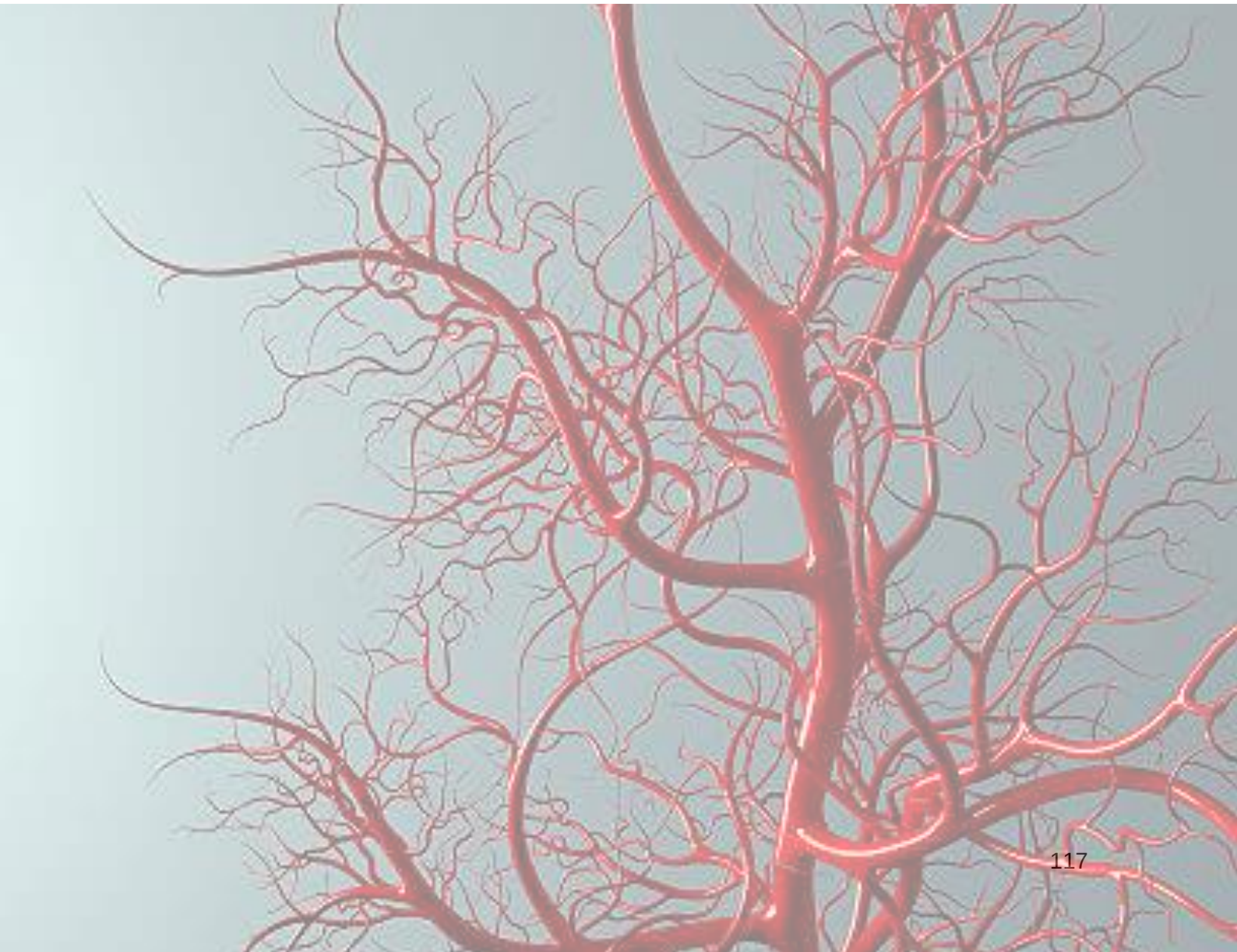
A strength of this study is its early starting point and its longitudinal design. Maternal blood samples were obtained from before the end of the first trimester (12 GW) until birth. Usually, the earliest blood samples available in prospective pregnancy studies are from the second trimester. Thus, we were able to examine the metabolites of our target pathways in early stages of pregnancies, where haemodilution and other physiological changes in pregnancy are still not completely established. Previously 1CM metabolism and the L-Arginine pathway have been studied in pregnancy separately. In our study, both pathways' metabolites were determined, which allowed us to study the interaction between these two pathways during pregnancy. The observational study did not set out to investigate causality, the primary aim of the study, on which this thesis is based, was to identify reliable early pregnancy biomarkers of pregnancy complications. The study included several covariables to control for confounding, a limitation of observational studies. We used plasma cotinine measurements to corroborate self-reported smoking data by the participants and used flash cards to identify precisely folic acid or other nutritional supplements that they took.

Even though 810 mothers were recruited for the study, the L-Arginine pathway was not a target pathway at the beginning of the study, and we only have information for approximately 20% of participants, which reduced statistical power.

Future perspectives

Due to ethical reasons, no study could explore the evolution of One-Carbon metabolites like tHcy or folate in the absence of the effect of folic acid supplementation during pregnancy, as pregnant women are recommended to take it at periconception. However, the potential mediation effect of L-Arginine pathway in the association of tHcy and pregnancy complications, a population of mothers with high tHcy status might be considered to investigate.

Chapter 3
The Reus-Tarragona Birth Cohort Fathers



5. CHAPTER 3: THE REUS-TARRAGONA BIRTH COHORT FATHERS

5.1 Introduction

Although the mother plays a crucial role in pregnancy, the paternal influence on optimal embryological development is often overlooked. While different paternal factors such as age, ethnicity or genetic factors have been associated with different pregnancy conditions such as low birth weight [295], preterm birth [296] or gestational diabetes [297], we will focus on conditions where pregnancy-induced hypertension occurs.

Since half of the foetal genetic material is of paternal origin, the foetus is considered a semi-allograft to the maternal host, so that immune tolerance occurs in pregnancy to prevent rejection [298]. At the maternal-foetal interface we find maternal immune cells such as regulatory T cells of human lymphocyte antigens (HLAs) or natural killer (NK) cells. Maternal immune system tolerance is induced by regulatory T-cells, and these cells have been shown to be decreased in preeclampsia [299]. Uterine NKs influence trophoblast invasion and vascular spiral artery remodelling through angiogenesis, and express inhibitory and activatory killer-cell immunoglobulin-like receptors (KIR) capable of recognising HLA-class I molecules [300].

Trophoblasts express a unique combination of HLA, HLA-C, -E and -G [301]. HLA-G is a tolerogenic molecule of the major histocompatibility complex and induces proliferation of regulatory T cells and inhibits NK activity, among other functions [302]. In addition, the two HLA-C groups interact with different NK receptors. Therefore, each pregnancy will have different combinations of paternal-foetal HLA-C and maternal KIR [303]. More schematic information is shown in **Figure 3.1**.

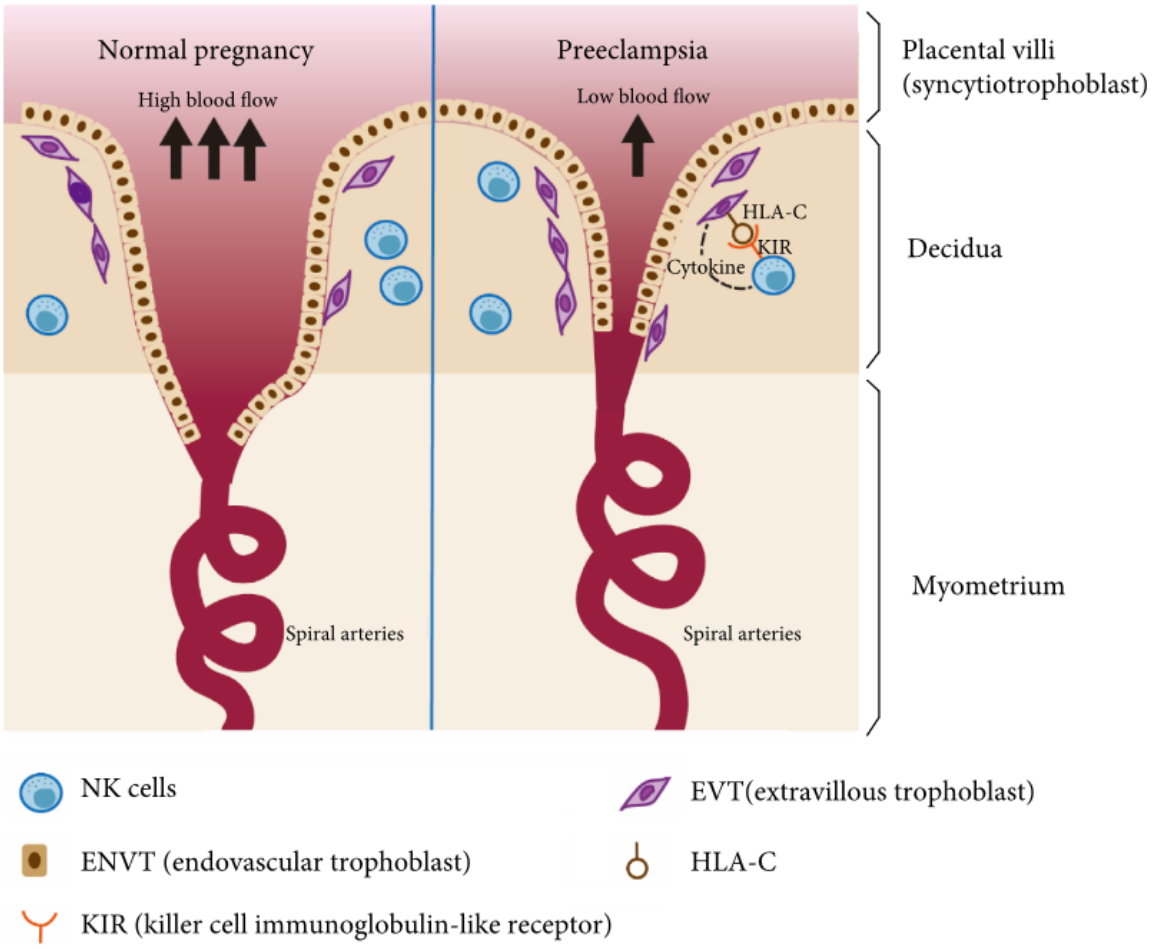


Figure 3.1: Poor placentation in early pregnancy. From Yang et al. (2020) [304].

5.1.1 Paternal risk factors and its influence in pregnancy complications

Non-modifiable paternal factors such as age and ethnicity have been associated with various pregnancy-induced hypertension complications [303].

Maternal **age** is a well-established risk factor for several pregnancy complications. Being older than 45 years in both the mother and the father has been associated with an increased risk of gestational hypertension (OR [95% CI] (1.73 [1.48, 2.02]), with this risk being higher in multiparous women (OR [95% CI] (2.07 [1.73, 2.49]). However, after stratifying by maternal age, the significance was lost [305]. In addition, in a cohort study of 81,213 couples, paternal age above 45 years increased the risk of pre-

eclampsia by 80 % (OR [95% CI] (1.8 [1.4, 2.3]) after adjusting for maternal age [306]. While the mechanism by which paternal age affects these gestational diseases is not yet known, it is hypothesised that increased DNA damage due to age or altered exposure to paternal sperm may be involved in the aetiology [303].

When parents are of different **ethnicities**, the risk of different gestational diseases such as prematurity [307] or low birth weight babies [295] is higher than when the parents are of the same ethnicity. In two studies in the United States, ethnic discordance between the two parents was found to be associated with an increased risk of pre-eclampsia by 13 and 90% compared with white couples, after adjusting for confounding variables [308, 309]. In addition, Asian fathers were associated with the lowest rate of pre-eclampsia [308]. One of the possible mechanisms has a genetic basis. The killer immunoglobulin receptors (KIR) are expressed by the NK cells and a genotype KIR AA generally has no activating receptors. The HLA-C molecules (ligands of KIR) are also separated in HLA-C1 or HLA-C2. The mother could possess KIR genes for paternal HLA-C allotypes that she herself does not have or the foetus could lack HLA-C KIR ligands that are present in the mother [310]. Hiby et al. (2004) observed that the combination of maternal KIR AA genotype with a foetal HLA-C2 is associated with an increased risk of pre-eclampsia. They exposed that the poor placentation observed in pre-eclampsia might be due to an increased inhibition of the NK cells, as the HLA-C2 will only engage a inhibitory specific KIR receptor [310]. Different human populations have a reciprocal relationship between KIR AA frequency and HLA-C2 frequency which may cause immunological tolerance problems in discordance ethnicities [303].

Having a second pregnancy with a **different father** has also been related with the risk of pre-eclampsia. When a normotensive pregnancy has occurred, a change of father in a subsequent pregnancy has been associated with an increased risk of pre-eclampsia [311]. However, studies that took into account interpregnancy intervals, observed that the risk of pre-eclampsia increased with increasing time between pregnancies in women without previous pre-eclampsia, regardless of whether it was the same or a different father [312, 313]. This may be explained for maternal sensitization to paternal or foetal antigens due to cohabitation time and sperm exposure and inadequate tolerance induction via regulatory T cells [314].

Paternal **history of pre-eclampsia** has also been associated with increased risk of pre-eclampsia. Lie et al. (1998) used familial patterns of recurrence of pre-eclampsia in order to investigate the contribution of paternal or maternal risk to develop pre-eclampsia. If a woman becomes pregnant by a man who previously fathered a pre-eclamptic pregnancy, the risk of developing pre-eclampsia increases (OR [95% CI] (1.8 [1.2, 2.6]) [315]. In addition, if the male partner was born of a pregnancy complicated by pre-eclampsia, the risk of his partner developing pre-eclampsia is more than double [316].

This may have a **genetic** component, the so-called “dangerous father”. Many authors have investigated different genes that may be involved in the aetiology of pre-eclampsia and have focused on the different biological alterations that occur in this disease. Among them are genes associated with oxidative stress (such as SOD2, GSTP1 or LPL) or blood pressure (genes for endothelin 1 or angiotensin II). It has been observed that different SNPs for these genes are associated with an increased risk of pre-eclampsia [317–320].

5.1.2 Paternal 1CM and L-Arginine pathway and its association with pregnancy complications.

Even if we focus on pregnancy complications, the paternal effect may start earlier, during spermatogenesis, and different compounds from both the 1CM network and the L-Arginine pathway have been found to be associated with this process.

DNA methylation is crucial for gametogenesis, implantation and embryo development [321] and both 1CM and L-Arginine play an important role in methylation as we have discussed before. In a study of fertile and subfertile men, plasma homocysteine was positively associated with seminal homocysteine. In addition, in those fertile men, lower folate concentrations were found to be associated with increased DNA damage (β, -0.36; $p \leq 0.05$) [322]. In a study of 156 couples undergoing assisted reproduction techniques, it was observed 1 μmol/L decrease of homocysteine concentrations in the ejaculate was associated with a 1.2-fold higher chance of achieving a reasonable/high quality embryo [323]. Furthermore, in a double-blind, randomised, placebo-controlled trial, treating subfertile men with 5 mg of folic acid and 66 mg of zinc sulphate, increased the total sperm count by 74% [324]. And, in another case-control study of Indian men, lower status of plasma folate and cobalamin and higher tHcy were seen in infertile men compared with fertile ones. In addition, low folate status (<5.7 ng/ml) was associated with an increased risk of infertility (OR [95% CI] (2.5 [1.4, 4.6]) [325]. Although a study with *Mthfr* -/- mice demonstrated the detrimental effects of this polymorphism on spermatogenesis and subsequent subfertility [326] and TC or TT versus CC genotype had an increased risk of infertility in men [327], other authors suggested that folate, rather than the polymorphism may have a stronger influence in spermatogenesis [328].

Saygin et al. (2021), observed in a case-control study that participant with oligozoospermia (low spermatozoa count) had higher serum ADMA concentrations (mean, 0.38; ± SD, 0.07) compared with men with normozoospermia (mean, 0.35; ± SD, 0.05; p, 0.046) [329]. Furthermore, treatment with a drug that stimulates eNOS activity in endothelial cells, combined with L-Arginine supplementation, significantly increased semen volume, spermatozoa concentration, percentage of motile spermatozoa and percentage of spermatozoa with normal morphology compared with the placebo group [330].

Regarding pregnancy outcomes, in a mouse model exposed to low dietary folate, paternal (and not just maternal) folate status was associated with differential nDNA methylation in genes implicated in development and chronic disease [331].

Few epidemiological studies have looked at the possible association of paternal 1CM and pregnancy complications. In two Australian populations, both paternal hyperhomocysteinemia and the *MTHFR* C677T polymorphism were associated with pregnancy loss [332, 333]. In addition, paternal tHcy was positively associated with NTD risk (OR [95% CI] (26.5 [2.6, 262.4]) [334].

Another prospective cohort study conducted in three clinics of Oceania recruited 3,196 women and their partners, to investigate the association between 1CM and different pregnancy complications including pre-eclampsia, gestational diabetes, gestational hypertension, small for gestational age and preterm birth. Paternal CT versus CC genotype of the *MTHFR* C677T polymorphism was associated with an increased risk of gestational hypertension (OR [95% CI] (1.6 [1.1, 2.4]). The same trend was observed for the TT variant (OR, 1.8), although not statistically significant (95%CI; 0.97, 3.33) [335].

Our group recently observed in the Reus Tarragona Birth Cohort study, an increased risk of pathological Doopler when the father had the CT (OR [95% CI] (4.0 [1.3, 12.6]) or TT (OR [95% CI] (7.1 [1.6, 32.8]) genotypes of the *MTHFR* C677T polymorphism compared to the wild genotype. This risk of pathological Doppler was also more than doubled in women whose partners had tHcy was ≥ 90th percentile (OR [95% CI] (2.7 [1.2, 6.0]) [293].

As far as we know, no epidemiological studies have investigated the association of paternal metabolites of the L-Arginine pathway and pregnancy outcomes. Pappa et al. (2011), in their study on pre-eclampsia risk saw that the frequency of the T allele of the *eNOS* G894T polymorphism was higher in men fathering a normal pregnancy (34.4%) than a pre-eclamptic pregnancy but as they recognized, further studies are required to establish whether the eNOS polymorphism is associated with pre-eclampsia [318].

Here we explore the association of impaired paternal One-carbon metabolism with L-Arginine pathway components in the fathers of the Reus-Tarragona Birth Cohort and study the involvement of paternal L-Arginine pathway metabolites with adverse pregnancy outcomes.

5.2 Material and methods

The participating mothers in the Reus Tarragona Birth Cohort study were asked if they were willing to provide us with the contact information of the baby's fathers so that we could invite them to participate in the study. Those that agreed provided the father’s contact details and those fathers were contacted to explain the nature of the study and

what father participation consisted of. Fathers that agreed to participate provided their signed informed consent. We tried to programme the fathers' blood samples as close as possible to conception, but the timing depended on how soon the mother provided the contact, when contact was established with the fathers and their availability to attend an appointment at the hospital. These factors led to temporal differences in the timing of the paternal blood samples extractions. 416 fathers participated in the study between 2003 and 2020 (**Figure 3.2**).

As with the pregnant women, fathers provided a fasting blood sample in the participating University Hospitals and completed two questionnaires regarding food frequency and lifestyle around the time of conception.

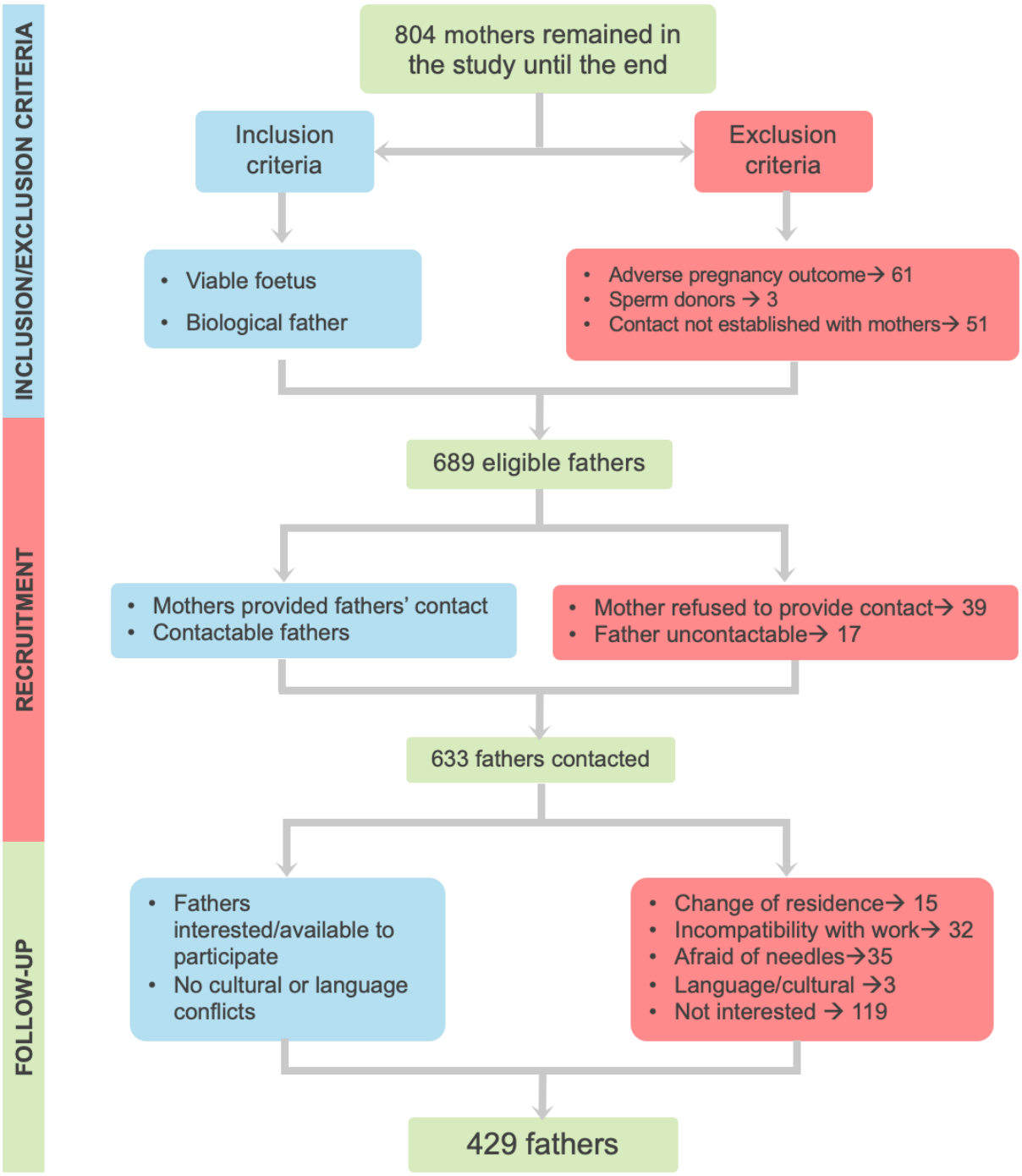


Figure 3.2: Flow charts of the fathers study.

In the habits and lifestyle questionnaire, information was collected on paternal date of birth, weight and height in the six months prior to pregnancy, in order to calculate age and BMI (kg/m^2). In addition, medical information such as medical history or blood type was collected. Vitamin supplement use, brand and frequency were collected in order to identify intake of B vitamins or folic acid, in addition to dietary intake. The use of toxic substances such as tobacco, alcohol or illegal substances was recorded. In the case of smoking, information was collected on the frequency (cigarettes per day) between the previous 5 years and during pregnancy. Whether this habit had been maintained, reduced or ceased during pregnancy was also collected. In addition to the self-reported questions, this information was confirmed by blood cotinine concentrations (>10 ng/ml was classified as smoker). For alcohol and illegal substances, duration, frequency (drinks per week) and maintenance or cessation during pregnancy were also collected by questionnaire.

The blood samples were processed in the Physiology laboratory of the Faculty of Medicine and Health Sciences of the Universitat Rovira i Virgili. The processing and storage of blood samples, the biochemical determinations of metabolites and the polymorphisms determinations were carried out following the same protocols as with maternal and cord blood samples.

Statistical analysis

Paternal characteristics are reported as percentages and 95% CI for categorical variables. and as arithmetic means and 95% CI. Normality of the distributions of paternal plasma metabolites were tested. Normal distributed variables are reported as arithmetic means and 95% CI. These latter variables were ln-transformed for the application of parametric statistical tests.

Mean plasma paternal L-Arginine pathway components were described according to paternal tHcy tertiles and *MTHFR* C667T polymorphism genotypes. Metabolite concentrations of mid and highest tHcy tertiles were compared with those in the lowest tHcy tertile. In addition, L-Arginine pathway metabolite status among *MTHFR* 667 CT and TT genotypes were compared with CCs. The comparisons were performed using ANCOVA analysis, adjusting for paternal age, first trimester BMI, smoking habits (smokers/non-smokers) and low versus mid-high socio-economic status. For the genotype comparisons, paternal plasma cobalamin and red blood cell folate were also included as covariable.

Multiple linear regression analysis was used for the association of paternal tHcy tertiles and other predictors with L-Arginine pathway metabolites. All models were adjusted for paternal age, first trimester BMI, smoking habits (smokers/non-smokers) and low versus mid-high socio-economic status.

According to paternal L-Arginine pathway metabolites tertiles, maternal mean PI mean, pathological Doppler of the uterine arteries and pregnancy-induced hypertension prevalence was described. As in the previous chapter, ADMA, SDMA and ADMA/SDMA Ratio reference tertile was the lowest tertile, while for L-Arginine and the L-Arginine/ADMA Ratio, it was the highest one. Difference between mean pulsatility index among tertiles was tested by ANOVA comparisons and by chi-square for pathological Doppler of the uterine arteries and pregnancy-induced hypertension.

The status of paternal metabolites of the L-Arginine pathway were also described according to whether or not the mother suffered any adverse pregnancy outcome (pathological Doppler of the uterine arteries or pregnancy-induced hypertension). The ANCOVA compared cases and controls adjusted for paternal age, BMI, smoking

habits (smokers/non-smokers), plasma creatinine and maternal and paternal low versus mid-high socio-economic status.

To study whether impaired concentrations of paternal metabolites of the L-Arginine pathway were associated with an increased risk of pregnancy-induced hypertension in the mothers, multiple logistic regression analyses were performed. In the first model, only paternal metabolites were included. These continuous variables, as in the previous chapter, were multiplied by 10 for the interpretation of the results. Model 2 included the same variables as model 1 as well as paternal age, BMI, smoking habits (smokers/non-smokers), plasma creatinine and maternal and paternal low versus mid-high socio-economic status. Maternal characteristics such as age, BMI, smoking habits (never [reference category], first trimester only and throughout pregnancy) and previous pregnancies >20 GW were also included in model 3. In the fully adjusted model, maternal first trimester plasma L-Arginine pathway metabolites were added to those included in model 3.

Paternal *NOS* G894T polymorphism genotypes frequencies were explored according to whether the pregnancy was affected by pathological Doppler or pregnancy-induced hypertension. χ^2 comparing between cases and controls was performed. In addition, a multiple logistic regression analysis of the association between paternal T allele of the *NOS* G894T polymorphism and pregnancy-induced hypertension was carried out. In the non-adjusted model GT+TT genotypes versus GG were tested as an independent variable. Model 2 was also adjusted for paternal characteristics such as, paternal age, BMI, smoking habits (smokers/non-smokers), plasma creatinine, plasma ADMA and maternal and paternal low versus mid-high socio-economic status. The most adjusted model (Model 3), was also adjusted for maternal age, BMI, smoking habits (never [reference category], first trimester only and throughout pregnancy) and

previous pregnancies >20 GW. Again, plasma metabolites were multiplied by 10 for the interpretation of the results. Maternal *NOS* G894T polymorphism could not be included as a covariable in the most adjusted model as this information was only available for <10% of mothers of the population. The same regression was performed substituting paternal ADMA for SDMA.

P-values were corrected for multiple comparisons using the post-hoc Bonferroni test. Statistical significance was accepted at $p < 0.05$. The software SPSS (version 28.0) was used for data analysis.

5.3 Results

Paternal characteristics and lifestyle habits during the first trimester of pregnancy are reported in **Table 3.1**. Regarding lifestyle habits, 17.1% of the fathers smoked more than 10 cigarettes per day before and during pregnancy and 1.5% had heavy alcohol intake. Before pregnancy, 6.8% of fathers took supplements that included folic acid. In the paternal population, 14.1% and 15.5% had the homozygote variant genotype of *MTHFR* C677T and *NOS* G894T polymorphisms, respectively.

Table 3.1: Paternal characteristics during the first trimester of pregnancy.

	n	Mean/Frequencies
Age (years)[1]	415	34.6 (34.1, 35.1)
BMI (kg/m^2)[1]	414	26.2 (25.9, 26.6)
Smokers[2]	415	36.6 (32.1, 41.4)
Nº of cigarettes before and during pregnancy[2]		
None	255	63.3 (58.5, 67.8)
1-5 cig/day	40	9.9 (7.4, 13.2)
6-10 cig/day	39	9.7 (7.2, 13.0)
>10 cig/day	69	17.1 (13.8, 21.1)
Alcohol consumption[2]		
None	196	48.3 (43.5, 53.1)
Low intake	119	29.3 (25.1, 33.9)
Moderate intake	85	20.9 (17.3, 25.2)
High intake	6	1.5 (0.7, 3.2)
Illegal drug use[2]	397	16.4 (13.1, 20.3)
Folic acid use[2]	414	6.8 (4.7, 9.6)
***MTHFR* C677T polymorphism[2]**		
CC	142	36.3 (31.7, 41.2)
CT	194	49.6 (44.7, 54.6)
TT	55	14.1 (11.0, 17.9)
***NOS* G894T polymorphism[2]**		
GG	157	40.5 (35.7, 45.4)
GT	171	44.1 (39.2, 49.1)
TT	60	15.5 (12.2, 19.4)

MTHFR, methylenetetrahydrofolate reductase. [1]Mean, 95% confidence interval. [2]Percentage, 95% confidence interval. *MTHFR* C677T and *NOS* G894T polymorphisms were in Hardy-Weinberg equilibrium.

Table 3.2 shows plasma 1CM and L-Arginine pathway nutrients and metabolites in the fathers that participated in the study. 64.7% of fathers had plasma folate deficiency defined as <10 nmol/L by the WHO [12] and mean plasma folate was 8.5 nmol/L in the fathers. 30.5% of the fathers had RBCF deficiency (<340 nmol/L). Mean plasma

L-Arginine was higher in the fathers (89.6 ± 18.8 µmol/L) compared to the mothers (first trimester, 47.0 ± 13.8 µmol/L, p <0.001) and men of the population study (66.9 ± 18.5 µmol/L, p <0.001). The L-Arginine/ADMA Ratio was 176.1 (SD, 37.6). ADMA 0.51 µmol/L (SD, 0.08), SDMA 0.54 µmol/L (SD, 0.14) and ADMA/SDMA Ratio 0.98 (SD, 0.21).

Table 3.2: Paternal 1CM and L-Arginine pathway metabolites status.

	n	Means (95% CI)
One-carbon metabolism		
tHcy (µmol/L)[1]	360	10.0 (9.7, 10.3)
Plasma B12 (pmol/L)[1]	360	386.5 (375.0, 398.4)
RBCF (nmol/L)[1]	387	427.8 (406.5, 450.3)
Plasma folate (nmol/L)[1]	360	8.5 (8.1, 9.0)
L-Arginine pathway		
L-Arginine (µmol/L)[2]	360	89.6 (87.7, 91.6)
ADMA (µmol/L)[2]	360	0.51 (0.50, 0.52)
SDMA (µmol/L)[2]	360	0.54 (0.52, 0.55)
L-Arginine/ADMA Ratio[2]	360	176.1 (172.2, 180.0)
ADMA/SDMA Ratio[2]	360	0.98 (0.96, 1.00)

tHcy, total plasma homocysteine; RBCF, Red blood cell folate; ADMA, Asymmetric Dimethylarginine; SDMA, Symmetric Dimethylarginine. [1] Geometric mean (95% confidence interval). [2] Arithmetic mean (95% confidence interval).

Paternal One-carbon metabolism and L-Arginine pathway association

Plasma L-Arginine pathway metabolite means according to tHcy tertiles are represented in **Figure 3.3**. Fathers with tHcy in the highest tertile had higher plasma L-Arginine (92.0 ± 19.7; p, 0.005), ADMA (0.52 ± 0.07; p, 0.052) and SDMA (0.57 ± 0.2; p, 0.005) concentrations compared to the lowest tertile (L-Arginine, 85.8 ± 15.9; ADMA, 0.50 ± 0.07; SDMA, 0.52 ± 0.1). None of the studied Ratio values differed among the tHcy tertiles.

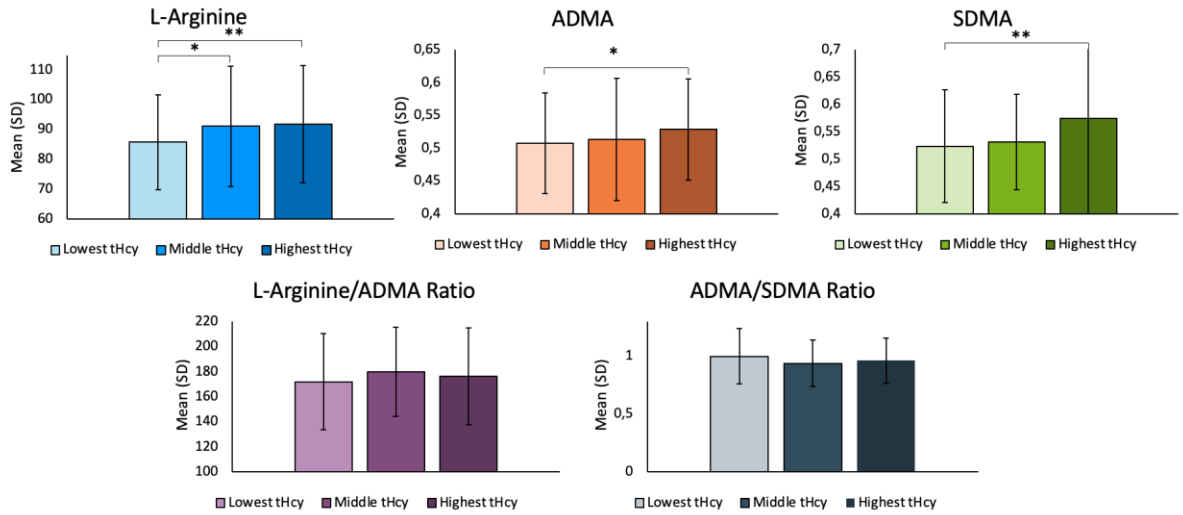


Figure 3.3: Paternal L-Arginine pathway metabolite means according to tHcy tertiles. tHcy, total plasma homocysteine; ADMA, Asymmetric Dimethylarginine; SDMA, Symmetric Dimethylarginine. tHcy cut offs: lowest tertile ≤8.78 µmol/L, mid tertile >8.78 µmol/L and ≤10.54 µmol/L, highest tertile >10.54 µmol/L. Lowest tHcy tertiles n=114; mid tHcy tertile n=125; highest tHcy tertile n=111. ANCOVA (mid versus lowest tertile & highest versus lowest tertile) adjusted for paternal age, first trimester BMI, smoking habits (smokers/non-smokers) and low versus mid-high socio-economic status. $^{*}p < 0.05$; $^{**}p < 0.01$

No significant differences were observed in L-Arginine pathway metabolites among the different *MTHFR* C677T genotypes (**Figure 3.4)**.

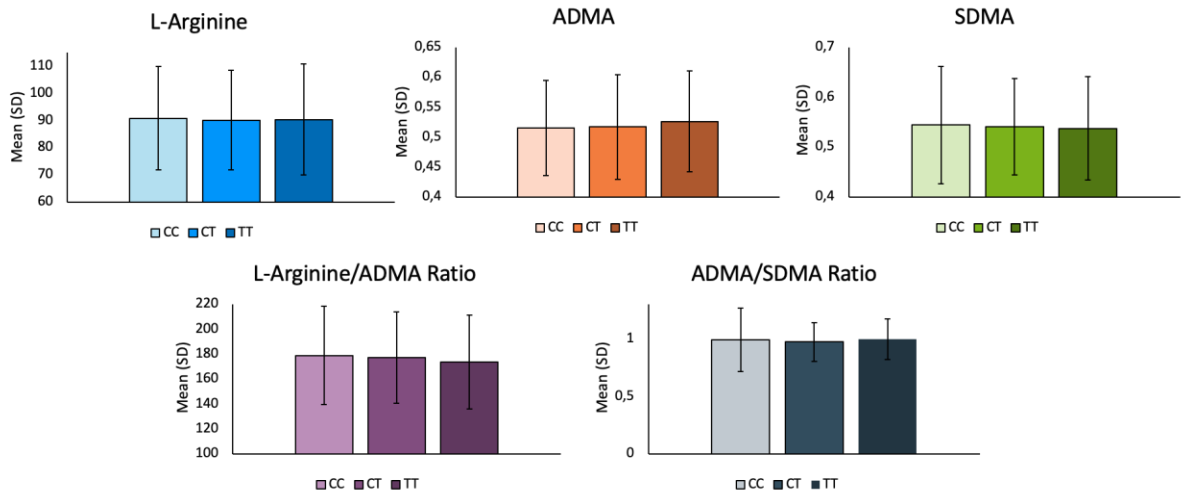


Figure 3.4: Paternal L-Arginine pathway metabolite means according to *MTHFR* C677T genotype. MTHFR, methylenetetrahydrofolate reductase; ADMA, Asymmetric Dimethylarginine; SDMA, Symmetric Dimethylarginine. CC n=120; CT n=168; TT n=48. ANCOVA (CT versus CC & TT versus CC) adjusted for age, first trimester BMI, smoking habits (smokers/non-smokers), low versus mid-high socio-economic status, red blood cell folate (nmol/L) and plasma cobalamin (pmol/L). $^{*}p < 0.05$ $^{**}p < 0.01$.

The associations between tHcy status (tertile) and plasma L-Arginine metabolites are shown in **Table 3.3**. Having tHcy in the mid or in the highest compared with the lowest tertile was positively associated with plasma L-Arginine but only the association in the highest tHcy tertile remained significant after Bonferroni correction for multiple comparisons (p, 0.012). THcy was also positively associated with plasma ADMA and SDMA, but the models were only significant for SDMA after Bonferroni correction. THcy was not associated with either the L-Arginine/ADMA or ADMA/SDMA Ratios.

Table 3.3: Multiple linear regression analyses of the association between tHcy and plasma L-Arginine pathway metabolites in the fathers.

	R^2	B-coefficient[1]	SE	p
L-Arginine				
tHcy mid versus lowest tertile	0.028*	5.55	2.41	0.063
tHcy highest versus lowest tertile		6.96	2.44	0.012
ADMA				
tHcy mid versus lowest tertile	0.004	0.005	0.011	1.926
tHcy highest versus lowest tertile		0.022	0.011	0.153
SDMA				
tHcy mid versus lowest tertile	0.029*	0.008	0.014	1.719
tHcy highest versus lowest tertile		0.038	0.014	0.018
L-Arginine/ADMA Ratio				
tHcy mid versus lowest tertile	0.00	8.38	4.9	0.273
tHcy highest versus lowest tertile		5.46	4.95	0.813
ADMA/SDMA Ratio				
tHcy mid versus lowest tertile	0.024*	-0.022	0.028	1.293
tHcy highest versus lowest tertile		-0.033	0.028	0.753

tHcy, total plasma homocysteine; ADMA, Asymmetric Dimethylarginine; SDMA, Symmetric Dimethylarginine. tHcy cut-offs: lowest tertile ≤8.78 µmol/L, mid tertile >8.78 µmol/L and ≤10.54 µmol/L, highest tertile >10.54 µmol/L. Models adjusted for mid and highest tHcy tertile versus lowest tHcy tertile, paternal age, first trimester BMI, smoking (smokers/non-smokers) and low versus mid-high socio-economic status. Nagelkerke R^2. [1]Unstandardized B-coefficients and standard error. N for all regressions= 349. *p <0.05 **p <0.01. Reported statistical significance were adjusted by the Bonferroni tests for multiple comparison corrections.

In the association of *MTHFR* 677TT genotype and L-Arginine pathway metabolites, neither CT or TT genotypes were significantly associated with any L-Arginine metabolites compared with the *MTHFR* 677CC genotype (data not shown).

Paternal L-Arginine pathway and pregnancy adverse outcomes

In **Table 3.4**, adverse pregnancy outcomes are represented according to paternal plasma L-Arginine pathway metabolite status (tertiles). Regarding maternal mean pulsatility index, when fathers had L-Arginine/ADMA Ratio in the mid tertile, mothers had lower mean pulsatility index (mean [95% CI] (0.92 [0.86, 0.98]), compared to the pulsatility index of mothers (mean [95% CI] (1.02 [0.95, 1.08]) whose partner had L-Arginine/ADMA Ratio in the highest tertile. Pathological Doppler of the uterine arteries prevalence in mothers did not differ among any paternal plasma L-Arginine pathway metabolite tertiles. In addition, the prevalence of pregnancy-induced hypertension was lower when fathers had plasma L-Arginine in the mid tertile compared to the highest one. However, the frequency of mothers that suffered pregnancy-induced hypertension was more than double (% [95% CI] (20.5 [13.2, 30.4]) when paternal ADMA concentrations were in the highest tertile compared to the frequency of pregnancy-induced hypertension (% [95% CI] (7.9 [3.9, 15.4]) when fathers had plasma ADMA concentrations in the lowest tertile.

Table 3.4: Mean pulsatility index, pathological Doppler of the uterine arteries and pregnancy-induced hypertension characteristics according to paternal L-Arginine pathway metabolites tertiles.

L-Arginine	Mean PI[1]	Pathological Doppler[2]	Pregnancy-induced hypertension[2]
Lowest tertile	0.97 (0.90, 1.04) [112]	15.1 (2.6, 6.7) [16/106]	20.0 (12.8, 30.0) [16/80]
Mid tertile	0.98 (0.92, 1.04) [115]	15.9 (10.3, 23.8) [18/113]	6.0 (2.6, 13.3) [5/83]**
Highest tertile (ref)	0.96 (0.90, 1.02) [106]	20.6 (13.9, 29.4) [21/102]	12.4 (7.2 , 20.4) [12/97]
ADMA			
Lowest tertile (ref)	0.97 (0.91, 1.04) [111]	18.3 (12.2, 26.7) [20/109]	7.9 (3.9, 15.4) [7/89]
Mid tertile	0.97 (0.91, 1.03) [112]	14.8 (9.3, 22.7) [16/108]	10.2 (4.4, 18.3) [9/88]
Highest tertile	0.97 (0.91, 1.03) [110]	18.3 (12.0, 26.8) [19/104]	20.5 (13.2, 30.4) [17/83]*
SDMA			
Lowest tertile (ref)	0.98 (0.92, 1.04) [110]	19.0 (12.7, 27.6) [20/105]	10.2 (5.5, 18.3) [9/88]
Mid tertile	0.95 (0.90, 1.0) [110]	13.0 (7.9, 20.6) [14/108]	12.2 (7.0, 20.6) [11/90]
Highest tertile	0.98 (0.90, 1.05) [113]	19.4 (13.1, 27.9) [21/108]	15.9 (9.5, 25.3) [12/82]
L-Arg/ADMA Ratio			
Lowest tertile	1.02 (0.95, 1.08) [112]	13.9 (8.6, 21.7) [15/108]	13.6 (7.8, 22.7) [11/81]
Mid tertile	0.92 (0.86, 0.98) [111]*	16.5 (10.7, 24.6) [18/109]	11.6 (6.44, 20.10) [10/86]
Highest tertile (ref)	0.98 (0.91, 1.04) [110]	21.2 (14.4, 30.0) [22/104]	12.9 (7.5, 21.2) [12/93]
ADMA/SDMA Ratio			
Lowest tertile (ref)	0.94 (0.88, 1.00) [111]	14.7 (9.2, 22.5) [16/109]	12.5 (7.1, 21.0) [11/88]
Mid tertile	0.98 (0.91, 1.05) [110]	18.3 (12.0, 26.8) [19/104]	7.1 (3.3, 14.6) [6/85]
Highest tertile	0.99 (0.93, 1.05) [112]	18.5 (12.3, 26.9) [20/108]	18.4 (11.7, 27.8) [16/87]

ADMA, Asymmetric Dimethylarginine; SDMA, Symmetric Dimethylarginine. [1] Mean, 95% CI, [n]. [2] Percentage, 95% CI [n/N]. Comparisons between tertiles were by ANOVA for continuous variables and χ^2 for categorical variables. L-Arginine tertiles cut-offs: highest tertile ≥ 96.13 µmol/L, mid tertile < 96.13 µmol/L and ≥80.80 µmol/L and lowest tertile <80.80 µmol/L. ADMA tertiles cut-offs: lowest tertile ≤ 0.47 µmol/L, mid tertile >0.47 µmol/L and ≤ 0.55 µmol/L and highest tertile > 0.55 µmol/L. SDMA tertiles cut-offs: lowest tertile ≤ 0.50 µmol/L, mid tertile >0.50 µmol/L and ≤ 0.57 µmol/L and highest tertile > 0.57 µmol/L. L-Arginine/ADMA Ratio tertiles cut-offs: highest tertile ≥ 188.79, mid tertile ≥ 160.56 and < 188.79 and lowest tertile < 160.56 µmol/L. ADMA/SDMA Ratio tertiles cut-offs: lowest tertile ≤ 0.89, mid tertile >0.89 and ≤ 1.05 and highest tertile > 1.05 µmol/L. *p <0.05; **p <0.01; ***p <0.001 compared with reference tertile.

Plasma L-Arginine pathway metabolite concentrations in non-complicated pregnancies and those with adverse outcomes (pathological Doppler of the uterine arteries and pregnancy-induced hypertension) are shown in **Figure 3.5**. Plasma L-Arginine, ADMA and SDMA concentrations were higher in fathers of pregnancies affected by pregnancy-induced hypertension compared to normal pregnancies. Paternal L-Arginine pathway metabolites did not differ between pregnancies with pathological Doppler of the uterine arteries compared to controls.

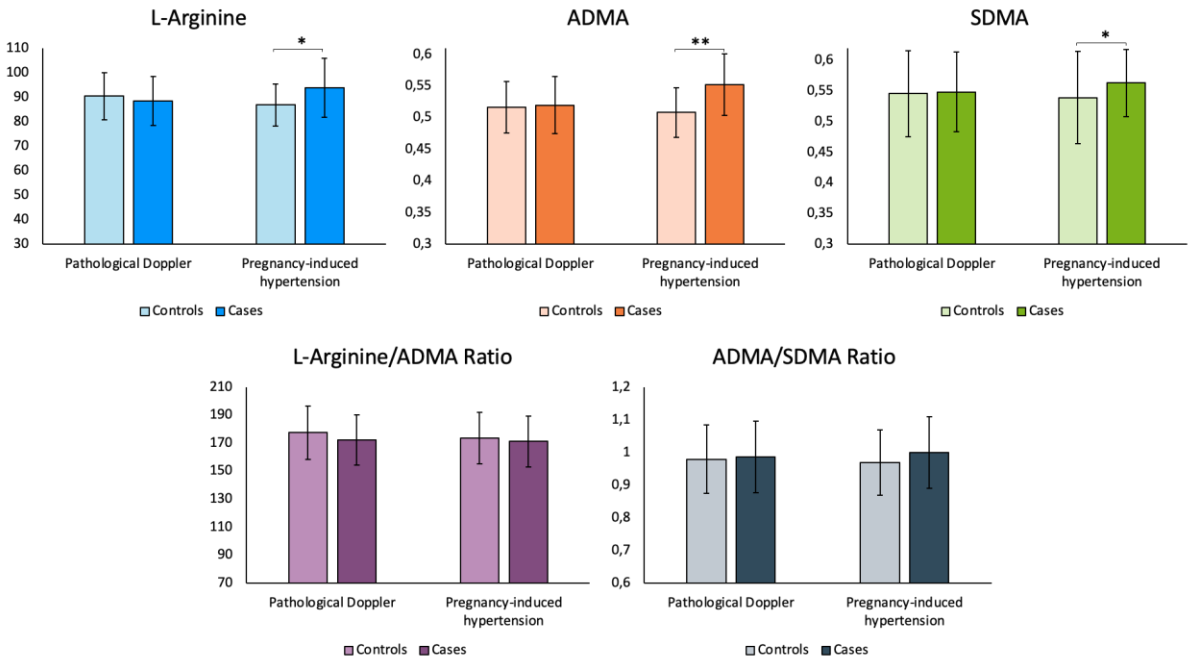


Figure 3.5: Paternal plasma L-Arginine pathway metabolite means according to cases and controls of pathological Doppler of the uterine arteries and pregnancy-induced hypertension. ADMA, Asymmetric Dimethylarginine; SDMA, Symmetric Dimethylarginine. Pathological Doppler: Controls n=266 and Cases=55. Pregnancy-induced hypertension: Controls n=227 and Cases n=33. ANCOVA comparing cases and controls adjusted for paternal age, BMI, smoking habits (smokers/non-smokers), plasma creatinine and maternal and paternal low versus mid-high socio-economic status. $^{*}p < 0.05$, $^{**}p < 0.01$.

The results of the multiple logistic regression analysis of the association of paternal L-Arginine pathway metabolites with pregnancy-induced hypertension is shown in **Table 3.5**. An increase of 0.1 µmol/L of paternal plasma ADMA was associated with a twofold increase in the risk of pregnancy-induced hypertension that was maintained after adjusting for both maternal and paternal characteristics (OR [95% CI] (2.0 [1.2, 3.3]). However, when including first trimester maternal plasma ADMA, the model (not significant) was reduced from 241 to 67 participants and it was maternal ADMA that increased the risk of pregnancy-induced hypertension (OR [95% CI] (3.3 [0.7, 16.5]) rather than paternal (OR [95% CI] (0.3 [0.1, 1.5]). In contrast, paternal plasma SDMA was associated with a 60% increased risk of pregnancy-induced hypertension when its concentration rose 0.1 µmol/L after adjusting for maternal and paternal variables (OR [95% CI] (1.6 [1.1, 2.4]). This risk doubled when including first trimester maternal

SDMA as a covariable, however, the model was reduced again to 67 participants and was no longer significant.

Table 3.5: Multiple logistic regression in the association between paternal and maternal L-Arginine pathway metabolites with pregnancy-induced hypertension.

				Paternal L-Arginine pathway metabolite	Maternal L-Arginine pathway metabolite
	Model	n	R^2	OR (95%CI)	OR (95%CI)
L-Arginine	1	260	0.029*	1.0 (1.0, 1.0)	-
	2	251	0.062	1.0 (1.0, 1.0)	-
	3	241	0.161*	1.0 (1.0, 1.0)	-
	4	67	0.332	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)
ADMA	1	260	0.056**	1.9 (1.2, 2.9)	-
	2	251	0.079*	1.9 (1.2, 2.9)	-
	3	241	0.171*	2.0 (1.2, 3.3)	-
	4	67	0.439	0.3 (0.1, 1.5)	3.3 (0.7, 16.5)
SDMA	1	260	0.004	1.1 (0.9, 1.3)	-
	2	251	0.050	1.4 (1.0, 2.0)	-
	3	241	0.155*	1.6 (1.1, 2.4)	-
	4	67	0.495	2.0 (0.6, 6.7)	0.1 (0.0, 0.8)
L-Arginine/ADMA Ratio	1	260	0.001	1.0 (1.0, 1.0)	-
	2	251	0.022	1.0 (1.0, 1.0)	-
	3	241	0.110	1.0 (1.0, 1.0)	-
	4	67	0.421	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)
ADMA/SDMA Ratio	1	260	0.006	1.1 (0.9, 1.3)	-
	2	251	0.024	1.1 (0.9, 1.3)	-
	3	241	0.110	1.0 (0.8, 1.2)	-
	4	67	0.876***	0.1 (0.0, 4.8)	207.8 (0.0, $1.2 \cdot 10^{12}$)

ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine. Multiple logistic regression analysis was used. Model 1: Paternal L-Arginine pathway metabolites. Metabolite concentrations were multiplied by 10 for results interpretation. Model 2: same variables as model 1 as well as paternal age, BMI, smoking habits (smokers/non-smokers), plasma creatinine and maternal and paternal low versus mid-high socio-economic status. Model 3: same variables as model 2 as well as maternal age, BMI, smoking habits (never, first trimester only and throughout pregnancy) and previous pregnancies >20 GW. Model 4: same variables as model 3 as well as maternal first trimester plasma L-Arginine pathway metabolites. Metabolite concentrations were multiplied by 10 for results interpretation. Nagelkerke R^2. *p <0.05; **p <0.01; ***p <0.001.

Figure 3.6 shows the paternal *NOS* G894T genotypes (GT+TT versus GG) according to case and control pregnancies with pathological Doppler of the uterine arteries or pregnancy-induced hypertension. No differences were seen between cases and controls of pathological Doppler of the uterine arteries among *NOS* G894T genotypes.

However, a tendency (p, 0.13) for lower frequency of the paternal T allele for the *NOS* G894T polymorphism was seen in pregnancies with pregnancy-induced hypertension compared to normotensive pregnancies.

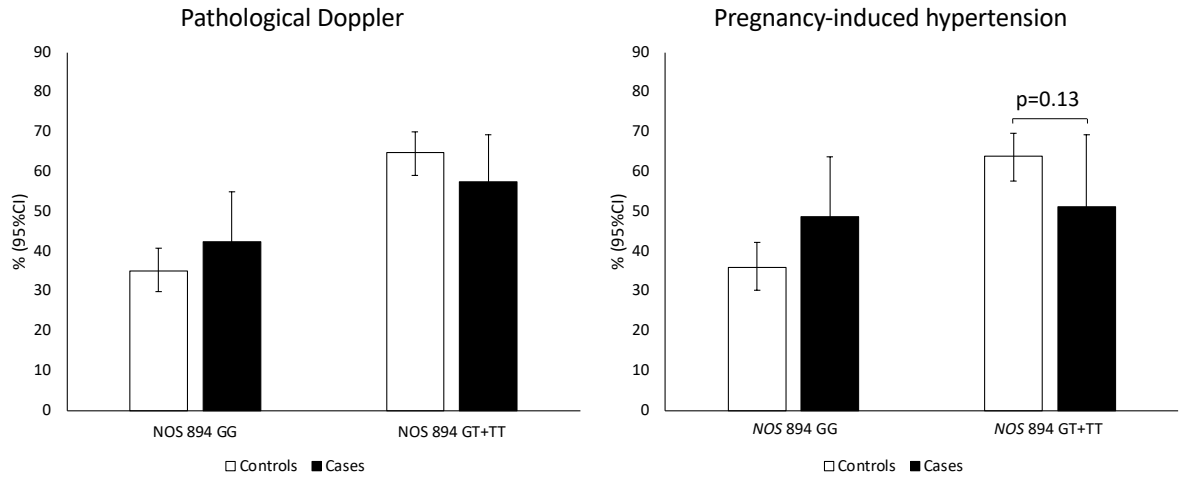


Figure 3.6: Paternal *NOS* G894T GT+TT versus GG genotype frequency according to cases and controls for pregnancies affected by pathological Doppler of the uterine arteries and pregnancy-induced hypertension. Pathological Doppler: Controls n= 290 and Cases= 59. Pregnancy-induced hypertension: Controls n= 241 and Cases n= 39. χ^2 comparing GT+TT genotypes with CC.

Multiple logistic regression testing the association between paternal *NOS* 894 GT+TT versus GG and pregnancy-induced hypertension is shown in **Table 3.5**. The T allele of the *NOS* G894T polymorphism reduced the risk of pregnancy-induced hypertension by approximately 50%, but this association was not significant. Paternal NOS inhibitors (ADMA and SDMA) independently increased the risk of pregnancy-induced hypertension, more than double (OR [95% CI] (2.2 [1.3, 4.0]) and by 60% (OR [95% CI] (1.6 [1.1, 2.3]), respectively, and this was maintained after adjusting for both paternal and maternal characteristics.

Table 3.5: Multiple logistic regression analysis of the association between paternal *NOS* G894T GT+TT versus GG and risk of pregnancy-induced hypertension.

			Paternal *NOS* G894T (GT+TT versus GG)	Paternal plasma ADMA	Paternal plasma SDMA
Model	n	R^2	OR (95%CI)	OR (95%CI)	OR (95%CI)
1	280	0.014	0.6 (0.3, 1.2)	-	-
2	237	0.109*	0.5 (0.2, 1.1)	2.0 (1.3, 3.2)	-
3	227	0.205**	0.5 (0.2, 1.1)	2.2 (1.3, 4.0)	-
1	280	0.014	0.6 (0.3, 1.2)	-	-
2	237	0.067	0.6 (0.3, 1.3)	-	1.4 (1.0, 2.0)
3	227	0.172*	0.6 (0.3, 1.4)	-	1.6 (1.1, 2.3)

ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine. Multiple logistic regression analysis was used. Model 1: Paternal *NOS* G894T GT+TT versus GG. Model 2: same variables as model 1 as well as paternal age, BMI, smoking habits (smokers/non-smokers), plasma creatinine, paternal plasma ADMA or SDMA and maternal and paternal low versus mid-high socio-economic status. Model 3: same variables as model 2 as well as maternal age, BMI, smoking habits (never, first trimester only and throughout pregnancy) and previous pregnancies >20 GW. Metabolite concentrations were multiplied by 10 for results interpretation. Nagelkerke R^2. *p <0.05; **p <0.01; ***p <0.001.

5.4 Discussion

Major findings

Paternal tHcy in the highest tertile was associated with increased plasma L-Arginine and SDMA. No association between the *MTHFR* C677T polymorphism and plasma L-Arginine pathway metabolites was observed in fathers. Paternal plasma ADMA and SDMA, but not the T allele of the *NOS* G894T polymorphism, were associated with increased risk of pregnancy-induced hypertension.

One-Carbon metabolism and L-Arginine pathway association

The positive association between tHcy and plasma SDMA, in the fathers, is in agreement with the results found in the male population in chapter 1 and with other studies that have observed an association between these two metabolites in both men and women [153, 155]. As mentioned in the first chapter, the higher concentrations of

tHcy in men [162] and the deleterious effect of testosterone on the kidney [164] (the main degradation system of SDMA) may explain the positive association of these two metabolites.

However, it does not explain the lack of association between tHcy and plasma ADMA in the fathers. Like men in the population study (chapter 1), 40% of fathers have tHcy above 10 µmol/L, the concentration at which the DDAH enzyme activity was reduced *in vitro* [161]. Our results show that plasma ADMA as tHcy tertiles increase, although the multiple linear regression testing the association of tHcy tertiles and plasma ADMA model did not confirm this association after adjusting for several paternal cofounders. The same results are seen in men of our population study of chapter 1. This may indicate that the influence of age on the association of tHcy with ADMA may be greater than sex differences.

Little is known about the association between the *MTHFR* C677T polymorphism and L-Arginine pathway metabolites and the results are inconsistent. Sniezawska et al. (2011) reported higher plasma ADMA concentrations in epileptic patients with the CT variant of this polymorphism compared to the wild genotype [158]. This was also seen by Dimitroulas et al. (2016) in the TT genotypes compared to the CC or CT genotypes in rheumatoid arthritis patients. However, when including tHcy in the multiple linear regression, it was an independent variable predicting plasma ADMA concentrations and no significant differences in serum ADMA concentrations were seen among *MTHFR* C677T polymorphism genotypes [157]. We also observed in the men from the population study of chapter 1, that the TT versus CC genotype of the *MTHFR* C677T polymorphism was not associated with plasma ADMA, there was an indirect association via tHcy.

L-Arginine pathway and adverse pregnancy outcomes

In this paternal population, we did not observe an association between increased concentrations of L-Arginine pathway metabolites with higher mean pulsatility index or increased risk of pathological Doppler. However, a 0.1 µmol/L increase in paternal plasma ADMA or SDMA increased the risk of pregnancy-induced hypertension about twofold, after adjusting for characteristics such as maternal age or smoking. However, when these models were adjusted for maternal metabolite concentrations in the first trimester, the models went from having an n of 241 to 67, as the available number of L-Arginine pathway metabolite concentrations for mothers was lower. Thus, in the totally adjusted models for maternal and paternal metabolite concentrations and other maternal and paternal characteristics, significance was not reached. Although not significant, the increased risk of pregnancy-induced hypertension was maintained for an increase of 0.1 µmol/L of paternal plasma SDMA. As seen in chapter 2, mothers with a high ADMA/SDMA Ratio do not have higher mean pulsatility index or an increased risk of pathological Doppler of the uterine arteries, but they do have an increased risk of pregnancy-induced hypertension.

Our group recently reported that maternal first trimester tHcy ≥ 90th percentile was not associated with either higher mean pulsatility index nor pathological Doppler of the uterine arteries [293]. This could be explained by the influence of folic acid supplementation on maternal tHcy reduction, which would indicate that the tHcy risk factor would be reduced. In fathers, of which only 7% took folic acid supplements, tHcy ≥ 90th percentile was associated with higher mean pulsatility index and an increased risk of pathological Doppler, but not with an increased risk of pregnancy-induced hypertension [293].

The role of the father on adverse pregnancy outcomes is usually overlooked, and the main factors studied are non-modifiable such as age, ethnicity or genetic factors. The placenta is formed from the foetus [336], which has half the genetic heritage of the father, and, as seen by others, paternal genetics may have an influence on adverse pregnancy outcomes of placental origin [335]. In addition, Zhou et al. (2013) observed a higher risk of bilateral notching in uterine arteries when fathers had the mutant allele for two different polymorphisms of the receptor of angiotensin type II, exposing that paternal genetics may have a role in placenta function [320].

Nevertheless, our group also took into consideration paternal plasma concentrations of different metabolites of the 1CM network and L-Arginine pathway on the influence of the father on adverse pregnancy outcomes. Elevated paternal elevated tHcy has been shown to be associated with sperm and embryo quality [324, 337], and impaired placentation [293]. Even though low serum low ADMA concentrations and L-Arginine supplementation have been associated with a higher sperm quality [329, 330], to the best of our knowledge, the association between paternal plasma/serum L-Arginine pathway metabolite concentrations on embryo development and placentation has not been studied yet.

Like us, Pappa et al. (2011) saw that the presence of the paternal T allele of the *NOS* G894T polymorphism was higher in normal pregnancies (n=108) than in pre-eclamptic pregnancies (n=51) [318]. However, we observed that increased paternal plasma ADMA or SDMA were associated with an increased risk of pregnancy-induced hypertension beyond the protective effect of the T allele of *NOS* G894T polymorphism. This may confirm Pappa et al's statement that the polymorphism by itself may not explain the aetiology of pre-eclampsia.

NO and NOS play an important role in sperm motility, capacitation and acrosome reaction [338]. The acrosome reaction of sperm cells occurs in the oviducts, as other physiological processes such as fecundation and early embryonic development [339]. Higher plasma ADMA concentrations have been observed in men with fertility disease, such as varicocele (an enlargement of the veins within scrotum) compared to controls or post surgery [340]. In human extravillous trophoblast cells, the invasion in fibrin gels and the motility of the cells were inhibited by L-NMMA (another NOS inhibitor), demonstrating the importance of NO synthesis on trophoblast cell function [341]. Looking at all of this evidence so far, it is possible that paternal plasma ADMA may also influence a poor trophoblast invasion, leading to pregnancy-induced hypertension when trophoblast is impaired.

5.5 Strengths and limitations

The role of the father in the development of pregnancy complications is usually overlooked so this is a strength of this study. Sixty five percent of eligible fathers participated. Some adverse pregnancy outcomes cannot be explained by maternal factors, which gives the father an important role. In addition, in Spain there is no mandatory folic acid fortification and few fathers took supplements, which allow us to examine one-carbon metabolism components, such as *MTHFR* C677T genotype or tHcy without the influence of folic acid.

Paternal blood samples were taken as near as possible to conception, but precise preconception information is relevant to collect. Our questionnaires were specifically designed to interrogate fathers about factors occurring around the time of conception. They were asked whether they changed their habits or diet or health status between conception and the time of blood sampling. If not, we would expect tHcy and B vitamin

status to be similar. Furthermore, the genetic determinations are not affected by timing of the sample. A possible bias is that fathers that accepted to participate may have been more health conscious than those that did not. Regarding paternity, we could not test this for ethical reasons, but we made it clear to the mothers before obtaining the fathers' contacts that their participation in the study would require investigating their genetic contribution to pregnancy outcome. The mothers provided the paternal contacts knowing this. Also, we carried out internal controls on a battery of 15 SNPs in the mother-father-cord triads to ensure compatibility in this regard.

Future perspectives

To further investigate how paternal plasma L-Arginine pathway metabolites may be associated with pregnancy outcomes, it would be interesting to investigate their association in sperm quality, genetic or methylation. We are currently collecting blood and semen samples from young men in the LED-Fertyl study of male fertility in the Tarragona province region. We will test associations between 1CM parameters, the L-Arginine pathway and a battery of indicators of male fertility.

6. GENERAL DISCUSSION

Concern about cardiovascular diseases has increased in recent decades, as the prevalence has risen [342], actually they are the leading cause of death in the world. High blood pressure, a major risk factor for CVDs, is present in 1.13 billion people worldwide [343]. Hypertension can also develop during pregnancy and complicates 5–10% of all pregnancies and is an important cause of maternal morbidity and mortality worldwide [227]. The WHO states that prevention, early diagnosis and appropriate treatment to lower blood pressure is essential to improve blood pressure [344].

Although risk factors for hypertension have been established, many cases develop in the absence of known risk factors. The L-arginine pathway and 1CM have been associated with different hypertensive diseases, but few studies investigate both pathways simultaneously. The L-arginine pathway has been proposed as a linking mechanism to explain the association between the 1C metabolic network and the circulatory system [252] both outside pregnancy and in pregnant women. Furthermore, usually only maternal factors are taken into account in the management of gestational hypertension, pre-eclampsia or pregnancy complications of placental origin. Considering the role of paternal factors in placental development, considering the father's contribution to pregnancy complications is justified, but has been mostly overlooked. This is especially the case for pregnancy complications that apparently cannot be explained by maternal factors alone.

In this thesis we show evidence that the L-Arginine pathway may be a potential mechanism in the association between poor 1CM and arterial and gestational hypertension. In the first study included in this thesis, a population-based study of adult

men and women unexposed to folic acid fortification and B vitamin supplement use, we confirmed the association between these two pathways and each of their associations with hypertension. However, we add novel evidence of interaction between 1CM and the L-Arginine pathway in the presence of hypertension in adults over 50 years of age. We showed this with statistical mediation analysis in which the previously reported association between tHcy and hypertension is mediated by plasma ADMA concentrations.

In the Reus-Tarragona Birth Cohort, we observe an increase in SDMA and a decrease in the ADMA/SDMA Ratio as pregnancy progresses. We did not confirm the association between 1CM and the L-Arginine pathway in the first trimester of pregnancy. Unlike our population-based study, tHcy is influenced by perinatal folic acid supplementation in keeping with protocol to prevent neural tube defects. However, we observed that an increased ADMA/SDMA Ratio in early pregnancy was associated with the risk of pregnancy-induced hypertension. Consider ADMA and SDMA alone as predictors of pregnancy-induced hypertension might not be sufficient. ADMA/SDMA Ratio, in addition to taking into account both L-Arginine analogues, provides information regarding other parts of the pathway, such as the activity of the DDAH enzyme, since it catabolises ADMA but not SDMA.

In the case of the fathers from this same cohort, uninfluenced by folic acid supplementation, we observed the association between the two pathways studied. In addition, we propose potential new paternal risk factors, such as elevated plasma concentrations of ADMA and SDMA that were associated with an increased risk of pregnancy-induced hypertension in the mothers. This supports the argument that the

role of fathers in the development of pregnancy complications should be further studied.

It has already been observed in a randomised, double-blind, placebo-controlled study in healthy subjects, that following a methionine load, plasma tHcy and ADMA but not SDMA were increased. Furthermore, endothelial function was also reduced[252]. The cytotoxic effect of homocysteine on the endothelium has been observed by many authors, however, in this clinical trial, the association between plasma ADMA and endothelial dysfunction was stronger than between tHcy and endothelial dysfunction. We consider that the L-Arginine pathway should be taken into account when exploring the implications of impaired 1CM and high blood pressure.

Trophoblast invasion and placental development are completed by 20-22 weeks' gestation [205]. It is important to highlight the fact that in this thesis, unlike most studies, maternal plasma metabolites were measured before 12 weeks of gestation. Early screening for both 1CM and L-Arginine pathway biomarkers, before the end of the first trimester and fully established maternal-foetal unit and strong influence of physiological effects of pregnancy, should be considered for the prevention and early diagnosis of hypertensive disorders of pregnancy.

Age, ethnicity and paternal genetic factors have been found to be risk factors for hypertensive disorders of pregnancy [345]. In mice, folate deficient diet decreases sperm methylation and these changes were associated with genes implicated in offspring development [331]. However, few studies have investigated the role of impaired paternal 1CM in pregnancy outcomes and, to the best of our knowledge, this is the first study to investigate the paternal plasma L-Arginine pathway and pregnancy complications.

In Spain there is no folic acid fortification, and as we have seen, not even 7% of fathers take any kind of supplement. In the same way that supplementation is recommended for mothers before pregnancy, this thesis suggests that future studies should test vitamin supplementation with folic acid and L-Arginine for fathers.

7. CONCLUSIONS

Chapter 1

GENERAL AIMS

To explore whether there is evidence for the involvement of the L-Arginine pathway in the 1CM-hypertension link, in a representative sample of an adult population.

SPECIFIC AIMS

To investigate the association of high tHcy and the *MTHFR* 677TT genotype with L-Arginine pathway metabolites according to age group and sex differences.

THcy was positively associated with plasma ADMA concentration in participants not on medication. tHcy was positively associated with plasma SDMA concentrations in participants not on medication in the entire population, in those ≤ 50 years and in men.

To explore the association of impaired L-Arginine pathway metabolites and the *NOS* G894T polymorphism on the increased risk of hypertension in adult men and women.

Plasma ADMA concentration, the L-Arginine/ADMA Ratio, and the *NOS* G894T polymorphism were associated with increased risk of hypertension in adults over 50 years of age.

To study whether the L-Arginine pathway plays a mediating role in the association between elevated tHcy, the *MTHFR 677*TT genotype and hypertension risk.

Our data support the L-Arginine pathway as a potential mechanistic link, more specifically via ADMA, between moderately elevated tHcy and hypertension. However, the association between the *MTHFR* 677TT genotype and hypertension was not mediated via the L-Arginine pathway.

Chapter 2

GENERAL AIMS

To explore the association between impaired One-Carbon metabolism and the L-Arginine pathway and to investigate the association of the L-Arginine pathway with adverse outcomes of pregnancy.

SPECIFIC AIMS

To describe plasma L-Arginine pathway metabolite fluctuations throughout the three trimesters of pregnancy, at labour and in cord.

Maternal plasma SDMA concentrations increase and ADMA/SDMA Ratio decreases, as pregnancy progresses, peaking at labour and in the cord, regardless of tHcy concentrations and *MTHFR* C677T genotype. Plasma L-Arginine, ADMA and L-Arginine/ADMA Ratio concentrations do not change throughout pregnancy.

To assess the association between elevated first trimester tHcy and *MTHFR* C677T genotype with L-Arginine pathway metabolites during pregnancy.

Mothers with first trimester higher tHcy concentrations showed higher plasma ADMA and SDMA concentrations. Mothers with the *MTHFR* 677TT genotype showed lower plasma ADMA and SDMA concentrations.

To determine the association between impaired first trimester L-Arginine pathway metabolites and risks of impaired placentation (diagnosed by

pathological Doppler of the uterine arteries at 20 GW) and pregnancy-induced hypertension.

High maternal first trimester plasma ADMA/SDMA Ratio was associated with increased risk of pregnancy-induced hypertension but not with impaired placentation.

Chapter 3

GENERAL AIMS

To explore the involvement of paternal L-Arginine pathway metabolites with adverse pregnancy outcomes in fathers.

SPECIFIC AIMS

To investigate the association of impaired paternal One-carbon metabolism with L-Arginine pathway.

The association of tHcy, but not the *MTHFR* C677T polymorphism, and L-Arginine pathway was confirmed in fathers.

To study the associations between impaired paternal L-Arginine pathway metabolites, the *NOS* G894T polymorphism and poor placentation and pregnancy-induced hypertension.

Paternal plasma ADMA and SDMA concentrations were associated with increased risk of maternal pregnancy-induced hypertension but not with impaired placentation. The *NOS* G894T polymorphism in the fathers was not associated with protection against pregnancy-induced hypertension.

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SCIENTIFIC AND ACADEMIC CONTRIBUTIONS AND OTHER MERITS

Articles

Rojas-Gómez, A., Solé-Navais, P., Cavallé-Busquets, P., Ornosa-Martin, G., Grifoll, C., Ramos-Rodriguez, C., ... & Murphy, M. M. (2022). Pregnancy homocysteine and cobalamin status predict childhood metabolic health in the offspring. *Pediatric Research*, 1-10. PMID: 35641553

Cavallé-Busquets, P., Inglès-Puig, M., Fernandez-Ballart, J. D., Haro-Barceló, J., Rojas-Gómez, A., Ramos-Rodriguez, C., ... & Murphy, M. M. (2020). Moderately elevated first trimester fasting plasma total homocysteine is associated with increased probability of miscarriage. The Reus-Tarragona Birth Cohort Study. *Biochimie*, *173*, 62-67. PMID: 31962182

The association between the MTHFR C677T polymorphism and fasting total plasma homocysteine with hypertension via the L-Arginine pathway: a cross-sectional study. Submitted to BMC Medicine. Carla Ramos-Rodríguez, Alejandra Rojas-Gomez, Santiago Ceruelo, Lídia Ríos, Per M Ueland, Joan D Fernandez- Ballart and Michelle M Murphy.

Conference Contribution

13th International Conference One-Carbon Metabolism, B Vitamins and Homocysteine. Poznan (Poland), 2021

- Title: One-carbon metabolism and L-Arginine pathway interaction is associated with increased risk of hypertension
- Authors: Carla Ramos-Rodríguez, Alejandra Rojas-Gomez, Santiago Ceruelo, Lídia Ríos, Per M Ueland, Joan D Fernandez-Ballart and Michelle M Murphy.
- Format: Oral communication.

The Epigenome in Human Health and Diseases Conference. FASEB (Virtual), 2021

- Title: Differences in One-carbon metabolism and L-Arginine pathway metabolites and polymorphisms according to sex in adults.
- Authors: Carla Ramos-Rodríguez, Alejandra Rojas-Gomez, Santiago Ceruelo, Lídia Ríos, Per M Ueland, Joan D Fernandez-Ballart and Michelle M Murphy.
- Format: Poster.

Folate, Vitamin B12, and One-Carbon Metabolism Conference. FASEB (North Carolina), 2022

- Title: Maternal and paternal One-Carbon metabolism, L-Arginine analogues and pregnancy-induced hypertension
- Authors: Carla Ramos-Rodríguez, Luis Adolfo Santos-Calderón, Pere Cavallé-Busquets, Julia Haro Barceló, Alejandra Rojas-Gómez, Per M Ueland, Joan D Fernandez-Ballart and Michelle M Murphy.
- Format: Oral communication.

Teaching and academic activities

60 hours per year teaching the subject of “Research and development foundations” for Medicine degree
30 hours per year teaching “General epidemiology” for Medicine degree

FASEB conference (Asheville, NC, USA) The folate, Vitamin B12 and One-Carbon Metabolism Conference. <u>ORAL</u> <u>PRESENTATION</u> and <u>POSTER</u>

Summary Agenda

The Folate, Vitamin B12, and One-Carbon Metabolism Conference

Sunday, August 14, 2022 - Friday, August 19, 2022

Renaissance Asheville Hotel

Asheville, North Carolina, USA

Register Now

Already registered?

AGENDA

Here's what's scheduled for the event.

Viewing in Eastern Time

August 18, 2022

Breakfast

7:30 AM-9:00 AM ET

General Session 7 -1-carbon nutrients in maternal and offspring health: molecules to populations

9:00 AM-12:00 PM ET

Chair: Julia Finkelstein, Cornell U, USA

9:00am - 9:30am *miRNAs, 1-carbon nutrients and neural tube closure* - Ron Parchem, Baylor College of Medicine

9:30am - 10:00am *Glycine cleavage system in neural & brain dev* - Kit-Yi Leung, UC-London, UK

10:15am - 10:45am *Clinical supplementation trials in pregnant Indian women* - Julia Finkelstein, Cornell U.

10:45am - 11:15am *Is riboflavin a determinant of hypertension & anemia in pregnancy?* - Bethany Duffy, Ulster U, N. Ireland

11:15am - 11:30am **Short talk 13** *Transgenerational Transmission of Behavioral Deficits in Rats Exposed to Folate Receptor Alpha Antibody In Utero,* Natasha Bobrowski Khoury

11:30am - 11:45am **Short talk 14** *Influence of methionine synthase on proliferation and differentiation of neural stem cells & postnatal neurogenesis,* RosaMaria Gueant-Rodriguez

11:45am - 12:00pm **Short talk 15** *Maternal & paternal 1-Carbon metabolism, L-Arginine analogues & hypertensive pregnancies,* Carla Ramos

Maternal and paternal One-Carbon metabolism, L-Arginine analogues and hypertensive pregnancies

Carla Ramos-Rodríguez[1,2], Luis Adolfo Santos-Calderón[1,2], Pere Cavallé-Busquets[3,4], Julia Haro Barceló[1,2], Alejandra Rojas-Gómez[1,2], Per M Ueland[5], Joan D Fernandez-Ballart[1,2,4] and Michelle M Murphy[1,2,4].
[1]Unitat de Medicina Preventiva, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Spain (FMCS URV); [2]IISPV Spain; [3]Area of Obstetrics and Gynecology, Hospital Sant Joan de Reus Spain; [4]CIBERobn ISCIII, Spain; [5]Bevital AS, Norway.

Background: In the formation of homocysteine from methionine, S-adenosylmethionine provides methyl groups for the methylation of L-Arginine. The L-Arginine analogues, Asymmetric dimethylarginine (ADMA) and Symmetric dimethylarginine (SDMA) can inhibit the synthesis of nitric oxide by endothelial nitric oxide synthase from L-Arginine, as they compete with L-Arginine. Fasting plasma total homocysteine (tHcy) has been positively associated with ADMA and SDMA. First trimester maternal tHcy, ADMA and MTHFR 677TT genotype (rs180113) have been associated with hypertensive pregnancies. We have observed that paternal impaired 1CM has been associated with impaired placentation. The influence of paternal plasma ADMA and SDMA concentrations on pregnancy outcomes has not been studied.

Aims: To investigate the association between parental tHcy and *MTHFR* C677T polymorphism and ADMA and SDMA with hypertensive pregnancies.

Methods: 810 pregnant mothers and 416 fathers participated in the longitudinal study. Parental lifestyle data and fasting blood samples were collected for biomarker and genotype determinations. Pregnancy induced hypertension (PIH) and preeclamptic pregnancies were classified together as PIH cases and studied in pregnancies with available ADMA and SDMA data for both mothers and fathers. Plasma ADMA and SDMA concentrations were compared between tHcy terciles and *MTHFR* C677T genotype by ANOVA. Parental predictors of probability of PIH were identified using multiple logistic regression analysis.

Results: 75 PIH cases (including 14 cases of preeclampsia) and 463 controls were studied. Maternal first trimester ADMA and SDMA concentrations were higher in mid (mean±SD; ADMA=0.43±0.07) and high (SDMA=0.39±0.08) versus low (ADMA=0.40±0.05; SDMA=.0.36±0.07; p <0.05) first trimester tHcy tertiles. Maternal first trimester ADMA (0.40±0.05) and SDMA (0.35±0.06) concentrations were lower in participants with the *MTHFR* TT versus CC genotype (ADMA=0.44±0.06; SDMA=0.40±0.08; p=0.02). Fathers with tHcy in the high tertile had higher ADMA (0.52±0.07; p=0.052) and SDMA (0.57±0.2; p=0.005) concentrations versus low tercile (ADMA 0.50±0.07; SDMA=0.52±0.1). Paternal ADMA and SDMA concentrations did not differ between *MTHFR* C677T genotypes. An increase of 0.1 in maternal first trimester ADMA/SDMA Ratio was associated with a 40% increased risk of hypertensive pregnancies (OR [95%CI] 1.4 [1.1-1.9]). An average increase of 0.1 µmol/L in paternal plasma of ADMA or SDMA was associated with increased risk of hypertensive pregnancies (2.0 [1.2-3.3]) and (1.6 [1.1-2.4]), respectively. This increased risk

was lost after adding maternal first trimester ADMA or SDMA concentrations to the fully-adjusted models.

Conclusion: Parental impaired 1CM may influence ADMA and SDMA concentrations and these are associated with increased risk of hypertensive pregnancies.

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Maternal and paternal One-Carbon metabolism, L-Arginine analogues and pregnancy-induced hypertension

Carla Ramos-Rodríguez[1,2], Luis Adolfo Santos-Calderón[1,2], Pere Cavallé-Busquets[3,4], Julia Haro Barceló[1,2], Alejandra Rojas-Gómez[1,2], Per M Ueland[5], Joan D Fernandez-Ballart[1,2,4] and Michelle M Murphy[1,2,4].
[1]Unitat de Medicina Preventiva, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Spain (FMCS URV); [2]IISPV Spain; [3]Area of Obstetrics and Gynecology, Hospital Sant Joan de Reus Spain; [4]CIBERobn ISCIII, Spain; [5]Bevital AS, Norway.

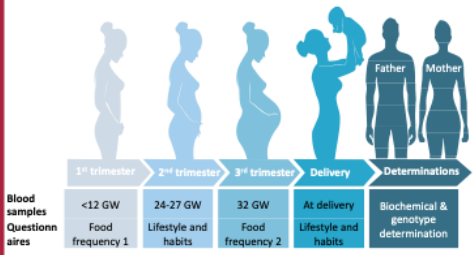
BACKGROUND

In the formation of homocysteine from methionine, S-adenosylmethionine provides methyl groups for the methylation of L-Arginine. The L-Arginine analogues, Asymmetric dimethylarginine (ADMA) and Symmetric dimethylarginine (SDMA) compete with L-Arginine and can inhibit the synthesis of nitric oxide by endothelial nitric oxide synthase from L-Arginine. Fasting plasma total homocysteine (tHcy) has been positively associated with ADMA and SDMA. First trimester maternal tHcy, ADMA and *MTHFR* 677TT genotype (rs180113) have been associated with pregnancy induced hypertension (PIH).

We observed that impaired paternal 1CM is associated with impaired placentation. The influence of paternal plasma ADMA and SDMA concentrations on pregnancy outcomes has not been studied.

AIM To investigate the association between parental tHcy, *MTHFR* C677T genotype and ADMA and SDMA with PIH.

METHODS



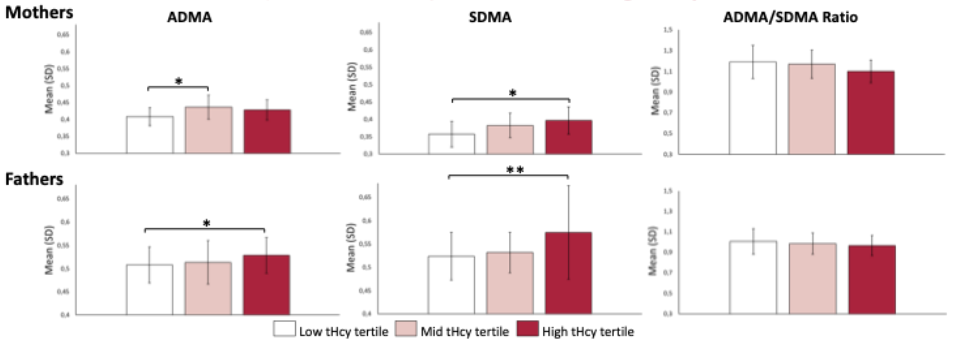
810 mothers and **416** fathers participated in the study. Parental lifestyle data and fasting blood samples were collected for biomarker and genotype determinations. Pregnancies with available ADMA and SDMA data for both mothers and fathers were studied. PIH and preeclampsia were classified together as cases. Parental plasma ADMA and SDMA concentrations were compared between tHcy terciles (at 1st trimester for mothers) and *MTHFR* C677T genotypes by ANOVA. Parental predictors of probability of PIH were identified using multiple logistic regression analysis.

RESULTS

	n	Mothers	n	Fathers
Age, years[1]	801	32.3 (32.0-32.7)	415	34.6 (34.1-35.1)
BMI, (kg/m^2)[1]	736	24.3 (24.0-24.7)	414	26.2 (25.9-26.6)
Smoking[2]	766		415	
Never		74.3 (71.1-77.3)		63.4 (58.6-67.9)
First trimester trimester		9.4 (7.5-11.7)		36.6 (32.1-41.4)
Throughout pregnancy		16.3 (13.9-19.1)		
Previous pregnancies[2]	793	54.1 (20.6-57.5)		-
Periconception Folic Acid supplementation[2]	259	85.3 (80.5-89.1)	414	6.8 (4.7-9.6)
Low socioeconomic status[2]	764	12.2 (10.0-14.7)*		
Pregnancy induced hypertension[2]	538	13.9 (11.3-17.1)		-
Plasma folate, (nmol/l)[3]	707	34.6 (32.4-36.7)	360	9.6 (9.1-10.1)
Plasma cobalamin, (pmol/l)[3]	707	381.6 (368.3-397.8)	360	404.7 (390.1-419.2)
tHcy, (µmol/l)[3]	707	5.4 (5.3-5.5)	360	10.5 (10.1-11.0)
ADMA, (µmol/l)[1]	157	0.42 (0.42-0.43)	360	0.52 (0.51-0.53)
SDMA, (µmol/l)[1]	156	0.38 (0.37-0.39)	360	0.54 (0.53-0.56)
ADMA/SDMA Ratio[1]	156	1.15 (1.11-1.19)	360	0.98 (0.96-1.00)
MTHFR 677CC[2]	739	33.7 (30.4-37.2)	391	36.3 (31.7-41.2)
MTHFR 677CT[2]		49.3 (45.7-52.9)		49.6 (44.7-54.6)
MTHFR 677TT[2]		17.1 (14.5-19.9)		14.1 (11.0-17.9)

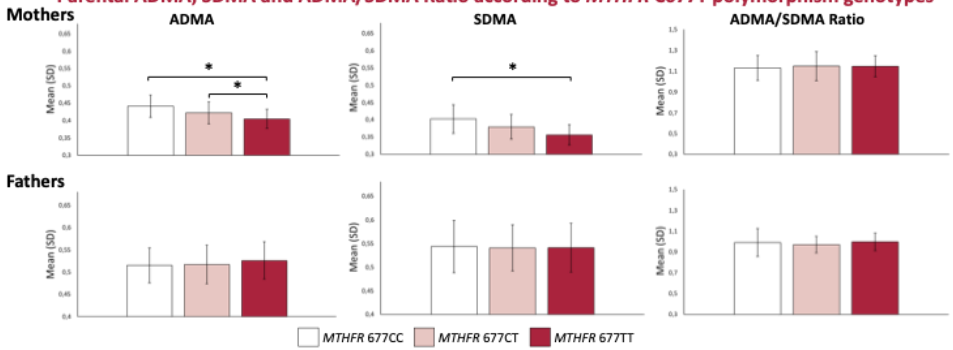
Maternal and paternal characteristics during first trimester. [1]Arithmetic mean, 95% confidence Interval. [2] Percentage, 95% confidence interval. [3]Geometric mean, 95% confidence interval. *MTHFR C677T* polymorphism was in Hardy-Weinberg equilibrium. *Combined socioeconomic status was based on household income and maternal and paternal educational level and occupation.

Parental ADMA, SDMA and ADMA/SDMA Ratio according to tHcy terciles



Maternal plasma ADMA, SDMA, ADMA/SDMA Rato and tHcy terciles were analysis at 1st trimester. Maternal first trimester tHcy terciles were adjusted by gestational weeks. *p-value<0.05 compared with low tHcy terciles. Reported statistical differences are adjusted by the Bonferroni tests for multiple comparison corrections. Maternal tHcy terciles cut-offs: Low tercile ≤ 4.80 µmol/L, mid tercile >4.80 µmol/L and ≤ 5.73 µmol/L and high tercile > 5.73 µmol/L. Low tHcy tercile: n=42, Mid tHcy tercile: n=56, High tHcy tercile: n=59. Paternal tHcy cut-offs: Low tercile ≤8.78 µmol/L, mid tercile >8.78 µmol/L and ≤10.54 µmol/L, high tercile >10.54 µmol/L. Low tHcy terciles n=114; mid tHcy tercile n=125; high tHcy tercile n=111.

Parental ADMA, SDMA and ADMA/SDMA Ratio according to *MTHFR* C677T polymorphism genotypes



Maternal plasma ADMA, SDMA and ADMA/SDMA Rato were analysis at 1st trimester. *p-value<0.05 compared with *MTHFR* 677 CC genotype. Reported statistical differences are adjusted by the Bonferroni tests for multiple comparison corrections. *MTHFR* 677CC: n=49, *MTHFR* 677CT: n=83, *MTHFR* 677TT: n=24.

Multiple logistic regression in the association of first trimester maternal, paternal or maternal+paternal plasma ADMA, SDMA and ADMA/SDMA Ratio with PIH

		Mother or Father Model			Mother+Father Model	
ADMA	n	R2	OR (95% CI)	n	R2	OR (95% CI)
Maternal ADMA	110	0.165	1.2 (0.4-2.4)	67	0.439	3.3 (0.7-16.5)
Paternal ADMA	251	0.079*	1.9 (1.2-2.9)			0.3 (0.1-1.5)
SDMA						
Maternal SDMA	109	0.201	0.5 (0.2-1.3	67	0.495	0.1 (0.0-0.8)
Paternal SDMA	251	0.050	1.4 (1.0-2.0)			2.0 (0.6-6.7)
ADMA/SDMA Ratio						
Maternal ADMA/SDMA	109	0.243*	1.4 (1.1-1.9)	67	0.876***	207.8 (0.0-1.2E+12)
Paternal ADMA/SDMA	251	0.024	1.0 (0.9-1.3)			0.1 (0.0-4.8)

Mothers model: first trimester maternal plasma ADMA, SDMA or ADMA/SDMA Ratio, maternal age, BMI, smoking habits (never, first trimester and throughout pregnancy), low v. mid-high socio-economic status, previous pregnancies >20 GW first trimester plasma creatinine. Maternal+Paternal model: Paternal ADMA, SDMA or ADMA/SDMA Ratio, paternal age, BMI, smoking habits (smokers/non-smokers), plasma creatinine, maternal and paternal low v. mid-high socio-economic status, maternal age, BMI, smoking habits (never, first trimester and throughout pregnancy) and previous pregnancies >20 GW and maternal first trimester plasma ADMA, SDMA or ADMA/SDMA Ratio. Metabolites concentrations were multiplied by 10 for results interpretation. NagelkerkeR^2. * p-value <0.05; ** p-value <0.01; *** p-value <0.001.

CONCLUSIONS Impaired parental 1CM may influence ADMA and SDMA concentrations and these are associated with increased risk of hypertensive pregnancies.

FASEC conference (Virtual). The epigenome in Human Health and Diseases Conference. POSTER.

Differences in One-carbon metabolism and L-Arginine pathway metabolites and polymorphisms, and associations with hypertension, according to sex in adults.

Carla Ramos-Rodríguez[1,2], Alejandra Rojas-Gomez[1,2], Santiago Ceruelo[3], Lídia Ríos[4], Per M Ueland[5], Joan D Fernandez-Ballart[1,2,6] and Michelle M Murphy [1,2,6].

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Background: One carbon metabolism (1CM) and the L-Arginine pathway interact when S-adenosylmethionine provides methyl groups to form Asymmetric dimethylarginine (ADMA) from L-Arginine, after homocysteine remethylation. ADMA competes with L-Arginine for eNOS, responsible for nitric oxide synthesis. *MTHFR*677TT genotype, elevated fasting total plasma homocysteine (tHcy), ADMA and variant T allele of the *eNOS*G894T polymorphism have been associated with hypertension.
Aim: to study whether L-Arginine-NO pathway mediates the association between 1CM and hypertension risk according to sex in an adult population.
Methods: 788 adults, aged 18-75 years, unexposed to mandatory folic acid fortification and B vitamin supplement use, stratified by sex and age group, participated in the cross-sectional study. At the check-up lifestyle data and blood samples were collected for biomarker and genotype determinations. Predictors of ADMA and hypertension probability were identified by lineal and logistic regressions, respectively. Mediation by ADMA in the 1CM-hypertension association was assessed by mediation analysis.
Results: tHcy was associated with ADMA in women (β=0.118; P=0.02) and men (β=0.112; P=0.02). ADMA did not differ between *MTHFR* genotypes in either sex. In women, high versus mid-low ADMA tertile increased hypertension risk (OR [95% CI] 2.7 [1.1-6.2]). Overall, mid-high tertile ADMA and the *eNOS* 894T allele increased the risk of hypertension (2.1 [1.1-4.0]) but this was only in women (2.9 [1.0-8.2]). Mediation analysis confirmed tHcy as a mediator in the *MTHFR* 677TT-hypertension link but not ADMA.
Conclusions: stratifying by sex, 1CM and L-Arginine pathway are associated with each other and independently with hypertension risk but the 1CM-hypertension link was not shown to be via the L-Arginine pathway.
Funding: Instituto de Salud Carlos III (ISCIII) FIS (PI00/0954; PI03/0870), AGAUR Generalitat de Catalunya (SGR:2009-1237, 2014-332); CIBERobn; C. Ramos (Martí-Franques, URV) and A. Rojas (EU Horizon 2020 research and innovation programme (Marie Skłodowska-Curie grant agreement No. 713679)/URV) are PhD fellows.

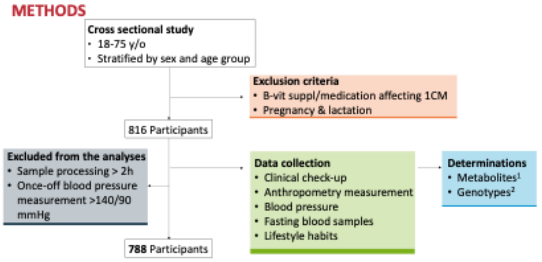
Differences in One-carbon metabolism and L-Arginine pathway metabolites and polymorphisms according to sex in adults.

Carla Ramos-Rodríguez[1,2], Alejandra Rojas-Gomez[1,2], Santiago Ceruelo[3], Lidia Ríos[4], Per M Ueland[5], Joan D Fernandez-Ballart[1,2,6] and Michelle M Murphy[1,2,6],
[1]Unitat de Medicina Preventiva, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain (FMCS URV). [2]IISPV, Àrea of Family and Community Medicine, [3]Centre d'Atenció Primària, El Morell and [4]Hospital Lleuger Antoni de Gimbernat de Cambrils, Spain; [5]Bevital A/S, Bergen, Norway; [6]CIBERobn ISCIII, Spain.

BACKGROUND The one carbon metabolic network (1CM) and L-Arginine pathway interact when S-adenosylmethionine provides methyl groups to form Asymmetric dimethylarginine (ADMA) from L-Arginine in the remethylation of homocysteine. ADMA competes with L-Arginine for *eNOS* binding, the enzyme responsible for nitric oxide synthesis. *MTHFR* 677TT genotype (rs180113), elevated fasting total plasma homocysteine (tHcy), ADMA and the T allele of the *eNOS* G894T polymorphism (rs1799983) have been associated with hypertension.

AIM to study the potential mediation by the L-Arginine-NO pathway in the association between 1CM and hypertension risk according to sex in an adult population.

METHODS



Predictors of ADMA and hypertension probability were identified by lineal and logistic regressions, respectively. The potential mediation by ADMA in the association between 1CM and hypertension was assessed by mediation analysis. [1] Plasma folate, (nmol/l), Plasma cobalamin, (pmol/l), EGRAC, tHcy, (µmol/l), ADMA, (µmol/l), Plasma total cholesterol, (mmol/l). [2] *MTHFR* C677T and *NOS* G894T.

RESULTS

Characteristics of the study population stratified by sex

	Women (n=407)	Men (n=381)	P
Age, years	43.0 (41.5-44.5)	43.0 (41.5-44.5)	0.994
BMI, (kg/m^2)	26.67 (26.11-27.23)	27.46 (27.01-27.9)	0.033
Smokers %	30.4 (26.2-35.1)	36.4 (31.8-41.4)	0.074
‡Alcohol consumption, %			
No intake	80.3 (76.2-83.9)	37.0 (32.3-41.9)	
Moderate intake	14.7 (11.6-18.5)	35.7 (31.0-40.6)	<0.001
High intake	4.9 (3.2-7.4)	27.3 (23.0-31.9)	
Diagnosed hypertension, %	15.2 (12.0-19.0)	12.8 (9.8-16.6)	0.339
Hypertensive medication/ Medication affecting L-Arginine pathway, %	13.5 (10.5-17.1)	11.5 (8.7-15.1)	0.066
Low socioeconomic status, %	47.4 (42.6-52.2)	27.0 (22.8-31.7)	<0.001
Plasma folate, (nmol/l)	12.25 (11.60-12.93)	10.71 (10.15-11.29)	<0.001
Plasma cobalamin, (pmol/l)	349.74 (336.66-363.36)	343.40 (331.65-355.56)	0.247
EGRAC	1.36 (1.33-1.38)	1.35 (1.33-1.37)	0.481
Plasma total cholesterol, (mmol/l)	5.24 (5.14-5.34)	5.32 (5.22-5.43)	0.236
tHcy, (µmol/l)	8.79 (8.56-9.03)	10.51 (10.23-10.81)	<0.001
ADMA, (µmol/l)	0.54 (0.53-0.54)	0.53 (0.52-0.54)	0.226
MTHFR C677T			
CC	35.7 (31.2-40.5)	35.7 (31.05-40.6)	
CT	47.7 (42.9-52.6)	44.9 (40.0-50.0)	0.577
TT	16.5 (13.1-20.4)	19.3 (15.6-23.5)	
NOS G894T			
GG	37.0 (32.3-41.9)	35.4 (30.7-40.4)	
GT	44.5 (39.6-49.55)	48.2 (43.1-53.3)	0.935
TT	18.3 (14.8-22.5)	16.3 (12.9-20.4)	

BMI, body mass index; tHcy, total plasma homocysteine; ADMA, Asymmetric Dimethylarginine; EGRAC, erythrocyte glutathione reductase activation coefficient; MTHFR, methylenetetrahydrofolate reductase 677C>T polymorphism; NOS, Nitric oxide synthase 894 G>T polymorphism. ‡: Twenty-four participants were excluded after the medical check-up due to declared B vitamin supplement use. A further fifty-one participants were excluded from all analyses involving tHcy because their blood samples were not processed within 2 h of collection and five participants because they had suspected altered renal function (plasma creatinine >97 mmol/l for women and >124 mmol/l for men). ‡ Category of habitual alcohol intake: moderate (<16 g/d in women and <24 g/d in men) and high (≥16 g/d in women and ≥24 g/d in men. Descriptive data are reported as means and 95 % confidence interval (CI) for normally distributed variables and as geometric means and 95 % CI when variables with skewed distributions were ln-transformed for the application of parametric statistical tests. Categorical variables are reported as percentages and 95 % CI.

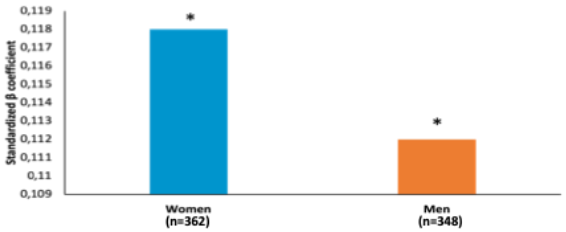
FACTORS PREDICTING ADMA

Association between *MTHFR* C677T genotype and ADMA, stratified by sex

		Women (n=355)			Men (n=340)		
		R^2	β[1]	P	R^2	β	P
Model 1 (*MTHFR* C677T)	CT v. CC	0.001	-0.085	0.150	-0.004	-0.051	0.402
	TT v. CC		-0.066	0.266		-0.007	0.907
Model 2 (model 1 + non-modifiable factors)	CT v. CC	0.108***	-0.061	0.273	0.096***	-0.045	0.438
	TT v. CC		-0.053	0.345		-0.032	0.574
	Age		0.333	<0.001		0.325	<0.001
Model 3 (model 2 + modifiable lifestyle factors)	CT v. CC	0.129***	-0.071	0.207	0.117***	-0.073	0.210
	TT v. CC		-0.064	0.256		-0.036	0.526
	Age		0.281	<0.001		0.186	0.007
	BMI		0.166	0.007		0.167	0.004
Model 4 (model 3 + 1CM nutrient status)	CT v. CC	0.123***	-0.071	0.210	0.112***	-0.070	0.230
	TT v. CC		-0.066	0.254		-0.029	0.620
	Age		0.279	<0.001		0.054	0.312
	BMI		0.164	0.007		0.167	0.021
	Plasma cobalamin		0.015	0.773		0.170	0.003
	Plasma folate		-0.019	0.725		0.046	0.437
	EGRAC		-0.025	0.640		-0.013	0.816

Multiple linear regression analysis. [1]Standardised β coefficient. Model 1: (basic model) MTHFR 677 polymorphism CT v. CC and TT v. CC. Model 2: included the same variables as model 1 as well as age and plasma creatinine. Model 3: included the same variables as model 2 as well as, smoking (cigarettes/day), category of habitual alcohol intake (moderate (<16 g/d in women and <24 g/d in men) v. none; high v. none (≥16 g/d in women and ≥24 g/d in men)), low v. mid-high socio-economic status, BMI and category of medication use. Model 4: included the same variables as model 3 as well as plasma cobalamin (µmol/l), plasma folate (nmol/l) and EGRAC. MTHFR, methylenetetrahydrofolate reductase; 1CM, One-carbon metabolism; EGRAC, erythrocyte glutathione reductase activation assay; BMI, body mass index; ADMA, Asymmetric dimethylarginine. Adjusted R^2 values and β-coefficients. * P<0.05; **P<0.01*** P<0.001

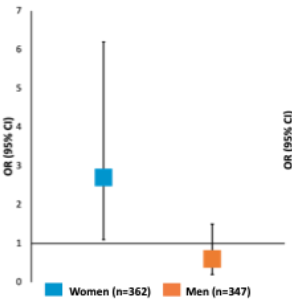
Association between tHcy and ADMA, stratified by sex



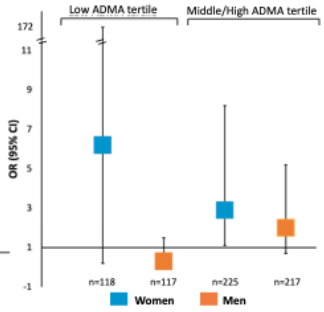
Multiple linear regression analysis. Showing change in ADMA for unit increase in tHcy. Model adjusted for age, smoking (cigarettes/day), category of habitual alcohol intake (moderate (<16 g/d in women and <24 g/d in men) v. none; high v. none (≥16 g/d in women and ≥24 g/d in men)), low v. mid-high socio-economic status and BMI. * P<0.05

FACTORS PREDICTING HYPERTENSION RISK

Association between high ADMA tertiles and diagnosed hypertension stratified by sex

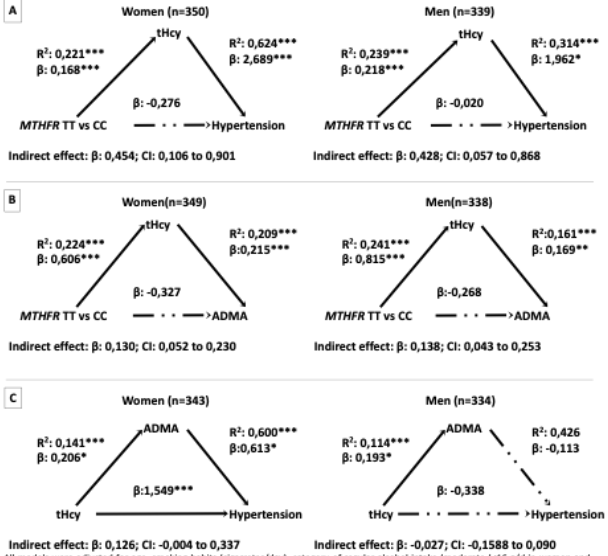


Association between *NOS* G894T and diagnosed hypertension stratified by sex, according to ADMA tertiles



Multiple logistic regressions. Cut-offs for high ADMA tertiles were ≥0.573 µmol/l in women ≤50 years, ≥0.574 µmol/l in women >50, ≥0.566 µmol/l in men ≤50 years, ≥0.570 µmol/l in men >50. Model adjusted by age low v. mid-high socio-economic status, smoking habits (cigarettes/day), category of regular alcohol intake (moderate (<16 g/d in women and <24 g/d in men) v. none; high v. none (≥16 g/d in women and ≥24 g/d in men)), BMI, total cholesterol and plasma creatinine.

MEDIATION ANALYSIS to test whether the association of 1CM-hypertension was via ADMA.



All models were adjusted for age, smoking habits (cigarettes/day), category of regular alcohol intake (moderate (<16 g/d in women and <24 g/d in men) v. none; high v. none (≥16 g/d in women and ≥24 g/d in men)), low v. mid-high socio-economic status and BMI. Models including *MTHFR* 677 TT vs CC polymorphism were also adjusted for plasma cobalamin (µmol/l), plasma folate (nmol/l) and EGRAC. Models including diagnosed hypertension were also adjusted for total cholesterol and NOS GT+TT vs GG* P<0.05; **P<0.01*** P<0.001.

CONCLUSION when stratifying by sex, 1CM and L-Arginine pathway are associated to each other and independently to hypertension, however, the mediation effect in the 1CM-hypertension link was not seen to be via L-Arginine pathway in men or women.

Funding Instituto de Salud Carlos III (ISCIII) FIS (PI00/0954 and PI03/0870), AGAUR Generalitat de Catalunya (SGR:2009-1237, 2014-332); CIBERobn ISCIII; C. Ramos and A. Rojas are PhD fellows from the Martí-Franques, URV and EU Horizon 2020 research and innovation programme (Marie Skłodowska-Curie grant agreement No. 713679)/URV programmes, respectively.

13th international conference. One-Carbon metabolism, B Vitamins and Homocysteine. (Poznan, Poland). ORAL PRESENTATION

One-carbon metabolism and L-Arginine pathway interaction is associated with increased risk of hypertension.

Carla Ramos-Rodríguez[1,2], Alejandra Rojas-Gomez[1,2], Santiago Ceruelo[3], Lídia Ríos[4], Per M Ueland[5], Joan D Fernandez-Ballart[1,2,6] and Michelle M Murphy [1,2,6].

[1]Unitat de Medicina Preventiva, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain (FMCS URV); [2]IISPV; Areas of Family and Community Medicine, [3]Centre d'Atenció Primària, El Morell and [4]Hospital Lleuger Antoni de Gimbernat de Cambrils, Spain; [5]Bevital A/S, Bergen, Norway; [6]CIBERobn ISCIII, Spain.

Background: Elevated fasting total plasma homocysteine (tHcy) and the *MTHFR* 677TT genotype (rs180113) are associated with increased risk of hypertension. Asymmetric dimethylarginine (ADMA) inhibits nitric oxide (NO) production from L-arginine via eNOS. The variant allele for *eNOS* G894T (rs1799983) has been associated with hypertension. The 1C metabolic network and L-Arginine-NO pathway interact when S-adenosylmethionine (SAM) provides methyl groups to form ADMA from L-Arginine and homocysteine inhibits dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that degrades ADMA into L-

Citrulline.
Aim: to investigate whether the association between impaired 1C metabolism and hypertension risk is mediated via the L-Arginine-NO pathway.
Methods: A representative, age and sex-stratified sample of 788 adults (18-75 years) from towns in Tarragona province (Spain) participated in the cross-sectional study. B vitamin supplement users were excluded and mandatory fortification with folic acid is absent in Spain. Medical and lifestyle data were recorded during a check-up and a fasting blood sample collected. Plasma folate, cobalamin, creatinine, homocysteine, ADMA, total cholesterol and erythrocyte folate and glutathionine reductase activation coefficient (riboflavin status), as well as the *MTHFR* C677T and *NOS* G894T genotypes were determined. Linear regressions were performed to identify predictors of ADMA and logistic regressions to investigate the associations between ADMA and eNOS with probability of hypertension. We investigated whether associations between the *MTHFR* 677TT genotype, elevated tHcy and hypertension were mediated via the L-Arginine pathway, using mediation analysis.
Results: tHcy and ADMA were positively associated in the medication-free population (β=0.096; P=0.026). The *MTHFR* 677TT genotype was not associated with ADMA. In participants over 50 years of age, highest tertile ADMA concentrations in women and men, respectively (≥0.573 and 0.566 μmol/L, ≤50y; ≥0.574 and 0.570 μmol/L, >50y), were associated with increased risk of hypertension (OR [95% CI] 2.0 [1.0-3.7]). The *NOS* 894T variant genotypes were associated with increased risk of hypertension compared to the GG genotype, only when ADMA was in the middle or highest tertiles (2.4 [1.1-5.2]). The mediation models indicate that the association between tHcy and hypertension was mediated by ADMA levels. Furthermore, the association between the *MTHFR* 677TT genotype and ADMA and hypertension risk was mediated by tHcy levels.
Conclusions: The associations between the *MTHFR* 677TT genotype and tHcy on hypertension might be mediated by the L-Arginine pathway.
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APPENDICES

Supplementary table: Participants medication use code, active principle and category

Participants	Medication code (CI-9)	Active principle	Treatment for	Medication category
1	A02	-	Antiacid	No medication use/Sporadic medication
2	A02	Aluminium hydroxide	Antiacid	No medication use/Sporadic medication
3	A02	Aluminium hydroxide	Antiacid	No medication use/Sporadic medication
4	A02	Aluminium hydroxide	Antiacid	No medication use/Sporadic medication
5	A02	Aluminium hydroxide	Antiacid	No medication use/Sporadic medication
6	A02 + M01 + N02B	-	Antiacid + Pain, inflammation & fever	No medication use/Sporadic medication
7	A02A	-	Antiacid	No medication use/Sporadic medication
8	A02B	-	Antiacid	No medication use/Sporadic medication
9	A02B	-	Antiacid	No medication use/Sporadic medication
10	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
11	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
12	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
13	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
14	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
15	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
16	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
17	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
18	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
19	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
20	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
21	M01	Clonixin	Pain, inflammation and fever	No medication use/Sporadic medication
22	M01	Ibuprofen	Pain, inflammation and fever	No medication use/Sporadic medication
23	M01A	Naproxen	Pain, inflammation and fever	No medication use/Sporadic medication
24	N02	-	Pain, inflammation and fever	No medication use/Sporadic medication
25	N02	-	Pain, inflammation and fever	No medication use/Sporadic medication
26	N02	Acetaminophen	Pain, inflammation and fever	No medication use/Sporadic medication
27	N02 + N02B	-	Pain, inflammation and fever	No medication use/Sporadic medication
28	N02B	-	Pain, inflammation and fever	No medication use/Sporadic medication
29	N02B	Metamizole	Pain, inflammation and fever	No medication use/Sporadic medication
30	N02B	Metamizole	Pain, inflammation and fever	No medication use/Sporadic medication
31	N02B	Acetaminophen	Pain, inflammation and fever	No medication use/Sporadic medication
32	N02B	Acetaminophen	Pain, inflammation and fever	No medication use/Sporadic medication
33	N02B	Acetaminophen	Pain, inflammation and fever	No medication use/Sporadic medication
34	N02B	Acetaminophen	Pain, inflammation and fever	No medication use/Sporadic medication
35	N02B	Acetaminophen	Pain, inflammation and fever	No medication use/Sporadic medication
36	N02B	Tramadol	Pain, inflammation and fever	No medication use/Sporadic medication
37	N02B	Acetaminophen	Pain, inflammation and fever	No medication use/Sporadic medication
38	R03	Fluticasone	Nasal congestion	No medication use/Sporadic medication
39	R06	-	Allergies	No medication use/Sporadic medication

40	R06	-	Allergies	No medication use/Sporadic medication
41	R06 + A03F	Cinitapride	Allergies + Reflux	No medication use/Sporadic medication
42	R06 + A07E	Budesonide	Allergies	No medication use/Sporadic medication
43	R06A	Ebastine	Allergies	No medication use/Sporadic medication
44	R06A	Loratadine	Allergies	No medication use/Sporadic medication
45	R06A	Mizolastine	Allergies	No medication use/Sporadic medication
46	A02 + A02B	Aluminium hydroxide + Omeprazole	Antiacid	Medication not affecting L-Arginine pathway
47	A02 + N06A	Aluminium hydroxide + Sertraline	Antiacid + Anxiety	Medication not affecting L-Arginine pathway
48	A02A	Ranitidine	Ulcer	Medication not affecting L-Arginine pathway
49	A02B	Omeprazole	Antiacid	Medication not affecting L-Arginine pathway
50	A02B	Omeprazole	Antiacid	Medication not affecting L-Arginine pathway
51	A02B	Omeprazole	Antiacid	Medication not affecting L-Arginine pathway
52	A02B	Omeprazole	Antiacid	Medication not affecting L-Arginine pathway
53	A02B	Omeprazole	Antiacid	Medication not affecting L-Arginine pathway
54	A02B + C07	Omeprazole + Atenolol	Antiacid + Hypertension	Medication not affecting L-Arginine pathway
55	A10B	Gliclazide	Diabetes	Medication not affecting L-Arginine pathway
56	A10B + A10A	Glyburide + Insulin human	Diabetes	Medication not affecting L-Arginine pathway
57	A10B + H04	Glyburide + Insulin human	Diabetes	Medication not affecting L-Arginine pathway
58	A12A	Ossein-hydroxyapatite + Raloxifene + Fluoxetine	Osteoposthorosis	Medication not affecting L-Arginine pathway
59	A12A + A02B	Ossein-hydroxyapatite + Famotidine	Osteoposthorosis + Ulcer	Medication not affecting L-Arginine pathway
60	B01A	Acenocoumarol	Stroke	Medication not affecting L-Arginine pathway
61	B04	Gemfibrozil	Hypertriglyceridemia	Medication not affecting L-Arginine pathway
62	B04	Gemfibrozil	Hypertriglyceridemia	Medication not affecting L-Arginine pathway
63	B04A	Lovastatin	Hypercholesterolemia	Medication not affecting L-Arginine pathway
64	C02	Nifedipine	Hypertension	Medication not affecting L-Arginine pathway
65	C07	Atenolol	Hypertension	Medication not affecting L-Arginine pathway
66	C07A	Bisoprolol	Hypertension	Medication not affecting L-Arginine pathway
67	C10A	Fenofibrate	Dyslipemia	Medication not affecting L-Arginine pathway
68	C10A	Bezafibrate	Hypercholesterolemia	Medication not affecting L-Arginine pathway
69	C10A	Simvastatin	Hypercholesterolemia	Medication not affecting L-Arginine pathway
70	C10A + C07	Lovastatin + Atenolol	Hypercholesterolemia + Hypertension	Medication not affecting L-Arginine pathway
71	G03	-	Contraceptive	Medication not affecting L-Arginine pathway
72	G03 + A03	Ethinylestradiol + Otilonium	Contraceptive + Spasms	Medication not affecting L-Arginine pathway
73	G03G	Raloxifene	Osteoposthorosis	Medication not affecting L-Arginine pathway
74	H03B	Carbimazole	Thyroid disorder	Medication not affecting L-Arginine pathway
75	H03B	Methimazole	Thyroid disorder	Medication not affecting L-Arginine pathway
76	H04	Insulin human	Diabetes	Medication not affecting L-Arginine pathway
77	H04	Insulin human	Diabetes	Medication not affecting L-Arginine pathway
78	H04	Insulin human	Diabetes	Medication not affecting L-Arginine pathway
79	H04 + G04C	Insulin human + Tamsulosin	Diabetes + Benign hyperplasia	Medication not affecting L-Arginine pathway
80	L01G	Tamoxifen	Cancer	Medication not affecting L-Arginine pathway
81	M01 + A02B	Omeprazole	Pain, inflammation and fever + Ulcer	Medication not affecting L-Arginine pathway
82	M01 + B04	Pravastatin	Pain, inflammation and fever + Hypercholesterolemia	Medication not affecting L-Arginine pathway
83	M01 + N05B + N06A	Alprazolam + Sertraline	Pain, inflammation and fever + Anxiety + Depression	Medication not affecting L-Arginine pathway
84	M01 + N06	-	Pain, inflammation and fever + Depression	Medication not affecting L-Arginine pathway
85	M03	Tetrazepam	Rehabilitation or functional re-education	Medication not affecting L-Arginine pathway

86	M03	Tetrazepam	Rehabilitation or functional re-education	Medication not affecting L-Arginine pathway
87	M03 + N06A	Tetrazepam + Paroxetine	Rehabilitation or functional re-education + Depression	Medication not affecting L-Arginine pathway
88	M03 + N06A + H04	Tetrazepam + Celecoxib + Venlafaxine + Calcitonin	Pain, inflammation and fever + Depression + Osteoposthorosis	Medication not affecting L-Arginine pathway
89	M05	Alendronic acid	Osteoposthorosis	Medication not affecting L-Arginine pathway
90	M05	Alendronic acid	Osteoposthorosis	Medication not affecting L-Arginine pathway
91	M05 + G02C	Alendronic acid+ Raloxifene	Osteoposthorosis	Medication not affecting L-Arginine pathway
92	N02 + G03H	Flutamide	Pain, inflammation and fever + Cancer	Medication not affecting L-Arginine pathway
93	N02B + C02E	Bisoprolol	Pain, inflammation and fever + Hypertension	Medication not affecting L-Arginine pathway
94	N02B + G03C	Acetaminophen + Tibolone	Pain, inflammation and fever + Menopause	Medication not affecting L-Arginine pathway
95	N02B + M01A	Acetaminophen + Diclofenac	Pain, inflammation and fever + Arthrosis	Medication not affecting L-Arginine pathway
96	N02B + N05B + M05	Bromazepam + Calcitonin	Pain, inflammation and fever + Depression + Osteoposthorosis	Medication not affecting L-Arginine pathway
97	N02B + N06	Metamizole + Reboxetine	Pain, inflammation and fever + Depression	Medication not affecting L-Arginine pathway
98	N02B + R03A	Acetaminophen + Salbutamol	Pain, inflammation and fever + Asthma	Medication not affecting L-Arginine pathway
99	N02C	Sumatriptan	Migraine	Medication not affecting L-Arginine pathway
100	N02C	Sumatriptan	Migraine	Medication not affecting L-Arginine pathway
101	N02C	Sumatriptan	Migraine	Medication not affecting L-Arginine pathway
102	N02C	Sumatriptan	Migraine	Medication not affecting L-Arginine pathway
103	N02C	Dihydroergotamine	Migraines and vertigo	Medication not affecting L-Arginine pathway
104	N02C	Dihydroergotamine	Migraines and vertigo	Medication not affecting L-Arginine pathway
105	N03	Phenytoin	Epilepsy	Medication not affecting L-Arginine pathway
106	N03A	Carbamazepine	epilepsies	Medication not affecting L-Arginine pathway
107	N05B	Alprazolam	Anxiety	Medication not affecting L-Arginine pathway
108	N05B	Alprazolam	Anxiety	Medication not affecting L-Arginine pathway
109	N05B	Alprazolam	Anxiety	Medication not affecting L-Arginine pathway
110	N05B	Alprazolam	Anxiety	Medication not affecting L-Arginine pathway
111	N05B	Alprazolam	Anxiety	Medication not affecting L-Arginine pathway
112	N05B	Lorazepam	Anxiety	Medication not affecting L-Arginine pathway
113	N05B	Bromazepam	Depression	Medication not affecting L-Arginine pathway
114	N05B	Citalopram	Depression	Medication not affecting L-Arginine pathway
115	N05B	Diazepam	Depression	Medication not affecting L-Arginine pathway
116	N05B	Diazepam	Depression	Medication not affecting L-Arginine pathway
117	N05B	Lormetazepam	Insomnia	Medication not affecting L-Arginine pathway
118	N05B + A10B	Alprazolam + Miglitol	Anxiety + Diabetes	Medication not affecting L-Arginine pathway
119	N05B + C07	Diazepam + Atenolol	Anxiety + Hypertension	Medication not affecting L-Arginine pathway
120	N05B + C10A	Alprazolam + Lansoprazole	Anxiety + Ulcer	Medication not affecting L-Arginine pathway
121	N05B + G03A	Hydroxyzine + Ethinylestradiol + Gestodene	Anxiety + Contraceptive	Medication not affecting L-Arginine pathway
122	N05B + M03 + C07	Hydroxyzine + Tetrazepam + Atenolol	Anxiety + Rehabilitation or functional re-education + Hypertension	Medication not affecting L-Arginine pathway
123	N05B + N02C	Alprazolam + Dihydroergotamine	Anxiety + Migraine	Medication not affecting L-Arginine pathway
124	N05B + N02C	Diazepam + Sumatriptan	Depression + Migraine	Medication not affecting L-Arginine pathway
125	N05B + N07 + S01	Alprazolam + Insulin human	Anxiety + Diabetes	Medication not affecting L-Arginine pathway
126	N05C + C10A	Clomethiazole + Pravastatin	Anxiety + Hypercholesterolemia	Medication not affecting L-Arginine pathway
127	N06	-	Depression	Medication not affecting L-Arginine pathway
128	N06A	Paroxetine	Anxiety	Medication not affecting L-Arginine pathway
129	N06A	Paroxetine	Depression	Medication not affecting L-Arginine pathway
130	N06A	Paroxetine	Depression	Medication not affecting L-Arginine pathway

131	N06A	Paroxetine	Depression	Medication not affecting L-Arginine pathway
132	N06A	Sertraline	Depression	Medication not affecting L-Arginine pathway
133	N06A	Venlafaxine	Depression	Medication not affecting L-Arginine pathway
134	N06A + B04	Paroxetine + Simvastatin	Depression + Hypercholesterolemia	Medication not affecting L-Arginine pathway
135	N06A + N02C	Venlafaxine + Dihydroergotamine	Depression + Migraine	Medication not affecting L-Arginine pathway
136	N07C	Flunarizine	Migraines and vertigo	Medication not affecting L-Arginine pathway
137	R03A	Salbutamol	Asthma	Medication not affecting L-Arginine pathway
138	R03A	Salbutamol	Asthma	Medication not affecting L-Arginine pathway
139	R03A	Salbutamol	Asthma	Medication not affecting L-Arginine pathway
140	R03A	Salbutamol	Asthma	Medication not affecting L-Arginine pathway
141	R03A	Salbutamol	Asthma	Medication not affecting L-Arginine pathway
142	R03A	Salbutamol	Asthma	Medication not affecting L-Arginine pathway
143	R03A	Salbutamol	Asthma	Medication not affecting L-Arginine pathway
144	R03A	Terbutaline	Asthma	Medication not affecting L-Arginine pathway
145	R03A	-	Bronchi	Medication not affecting L-Arginine pathway
146	R03A	-	Bronchi	Medication not affecting L-Arginine pathway
147	R03A + H04	Terbutaline + Insulin human	Asthma + Diabetes	Medication not affecting L-Arginine pathway
148	R03B	-	Asthma	Medication not affecting L-Arginine pathway
149	R06 + R03A	-	Allergies + Bronchi	Medication not affecting L-Arginine pathway
150	A02 + C02 + H03A	Omeprazole + Quinapril + Levothyroxine	Ulcer + Hypertension + Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
151	A02 + N02 + N06A	Aluminium hydroxide + Acetaminophen + Fluoxetine	Antiacid + Pain, inflammation and fever + Bulimia	Hypertensive medication/ Medication affecting L-Arginine pathway
152	A02A + C03	Ranitidine + Chlorthalidone	Ulcer + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
153	A02A + M05	Glucosamine	Vomiting + Arthrosis	Hypertensive medication/ Medication affecting L-Arginine pathway
154	A02B + C02 + M04	Omeprazole + Indapamide + Allopurinol	Antiacid + Hypertension + Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
155	A10 + C01D + C02	Metformin + Repaglinide + Acetylsalicylic acid + Telmisartan	Diabetes + Atherosclerosis + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
156	A10A + C02B4	Insulin human + Irbesartan	Diabetes + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
157	A10B	Glimepiride	Diabetes	Hypertensive medication/ Medication affecting L-Arginine pathway
158	A10B	Glimepiride	Diabetes	Hypertensive medication/ Medication affecting L-Arginine pathway
159	A10B + B04A	Acarbose + Glimepiride + Atorvastatin	Diabetes + Hypercholesterolemia	Hypertensive medication/ Medication affecting L-Arginine pathway
160	A10B + C02	-	Diabetes + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
161	A10B + C02	Gliclazide + Candesartan	Diabetes + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
162	A10B + C02	Acarbose + Indapamide	Diabetes + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
163	A10B + C02 + R03A	Gliclazide + Enalapril	Diabetes + Hypertension + Bronchi	Hypertensive medication/ Medication affecting L-Arginine pathway
164	A10B + C05A	Gliclazide + Nicardipine	Diabetes + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
165	A10B + C09C	Glimepiride + Losartan	Diabetes + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
166	A10B + C09C	Losartan	Diabetes + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
167	A10B + S01	Glyburide + Acarbose + Metformin + Latanoprost	Diabetes + Ocular hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
168	A12A + G02C +N06A	Ossein-hydroxyapatite + Raloxifene + Fluoxetine	Osteoposthorosis + Bulimia	Hypertensive medication/ Medication affecting L-Arginine pathway
169	B04 + C02	Gemfibrozil+ Indapamide	Hypertriglyceridemia + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
170	B04 + C02B4	Atorvastatin + Enalapril	Hypercholesterolemia + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway

171	B04HF	Atorvastatin	Hypercholesterolemia	Hypertensive medication/ Medication affecting L-Arginine pathway
172	B04H	Atorvastatin	Hypercholesterolemia	Hypertensive medication/ Medication affecting L-Arginine pathway
173	C02	Amlodipine	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
174	C02	Amlodipine	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
175	C02	Amlodipine	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
176	C02	Enalapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
177	C02	Indapamide	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
178	C02	Indapamide	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
179	C02	Lacidipine	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
180	C02	Quinapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
181	C02	Quinapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
182	C02	Quinapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
183	C02 + C02B4	Doxazosin + Enalapril + Indapamide	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
184	C02 + C02B4	Indapamide + Irbesartan	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
185	C02B + C02B4	Diltiazem + Irbesartan	Hypertension + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
186	C02B4	Captopril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
187	C02B4	Captopril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
188	C02B4	Enalapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
189	C02B4	Enalapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
190	C02B4	Enalapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
191	C02B4	Irbesartan	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
192	C02B4 + M04	Captopril + Allopurinol	Hypertension + Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
193	C02B4 + M04A	Enalapril + Allopurinol	Hypertension + Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
194	C03A	Midamor	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
195	C03A	Midamor	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
196	C03A	Midamor	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
197	C03A	Enalapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
198	C03A	Enalapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
199	C03A	Enalapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
200	C03A	Valsartan	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
201	C03A + M04A	Enalapril + Allopurinol	Hypertension + Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
202	C03B	Furosemide	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
203	C03B + H03	Furosemide + Levothyroxine	Hypertension + Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
204	C04A + B04A + C02	Hidrosmin + Atorvastatin + Lisinopril	Edema + Hypercholesterolemia + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
205	C05C	Ruscogenin	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
206	C05C + C02	Troxerutin + Doxazosin	Atherosclerosis + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
207	C07A	Propranolol	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
208	C09A + M04A	Lisinopril + Allopurinol	Hypertension + Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
209	C09C	Losartan	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
210	C10A + C07A + C09A	Pravastatin + Sotalol + Lisinopril	Hypercholesterolemia + Tachyarrhythmias + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
211	G04 + B04A	Alfuzosin + Atorvastatin	Prostate + Hypercholesterolemia	Hypertensive medication/ Medication affecting L-Arginine pathway
212	H02A	Deflazacort	Arthrosis	Hypertensive medication/ Medication affecting L-Arginine pathway
213	H03	Levothyroxine	Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
214	H03A	Levothyroxine	Atherosclerosis	Hypertensive medication/ Medication affecting L-Arginine pathway
215	H03A	Levothyroxine	Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway

216	H03A	Levothyroxine	Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
217	H03A	Levothyroxine	Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
218	H03A	Levothyroxine	Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
219	H04 + B04 + C02	Insulin human + Gemfibrozil + Quinapril	Diabetes + Hypertriglyceridemia + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
220	J04A + M04	Rifampicin + Allopurinol	Tuberculosis + Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
221	M04	Allopurinol	Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
222	M04A	Allopurinol	Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
223	M05	Glucosamine	Arthrosis	Hypertensive medication/ Medication affecting L-Arginine pathway
224	M05 + B04A + C03A	Elcatonin + Atorvastatin + Midamor	Osteoposthorosis + Hypercholesterolemia + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
225	M05 + C05A	Glucosamine + Escin	Arthrosis + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
226	N02	Troxerutin	Atherosclerosis	Hypertensive medication/ Medication affecting L-Arginine pathway
227	N02 + C02 + H03	Enalapril + Levothyroxine	Pain, inflammation and fever + Hypertension + Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
228	N02 + C05A	-	Pain, inflammation and fever + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
229	N02B + C02	Acetaminophen + Indapamide	Pain, inflammation and fever + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
230	N02B + C02 + M04	Doxazosin + Allopurinol	Pain, inflammation and fever + Hypertension + Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
231	N02B + C02B + M04Z	Acetaminophen + Amlodipine + Allopurinol	Pain, inflammation and fever + Hypertension + Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
232	N02B + C02B4	Acetaminophen + Cilazapril + Hydrochlorothiazide	Pain, inflammation and fever + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
233	N02B + D05 + C07A	Acetaminophen + Tacalcitol + Propranolol	Pain, inflammation and fever + Psoriasis + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
234	N02B + M05	Acetaminophen + Glucosamine	Pain, inflammation and fever + Arthrosis	Hypertensive medication/ Medication affecting L-Arginine pathway
235	N02B + N05B + C02B4	Acetaminophen + Diazepam + Valsartan	Pain, inflammation and fever + Depression + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
236	N02B + N05B + H03A	Lorazepam + Levothyroxine	Pain, inflammation and fever + Anxiety + Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
237	N03A + B01B	Phenytoin + Ticlopidine	Epilepsy + Atherosclerosis	Hypertensive medication/ Medication affecting L-Arginine pathway
238	N05	Amitriptyline	Depression	Hypertensive medication/ Medication affecting L-Arginine pathway
239	N05 + C02	Loprazolam + Quinapril	Insomnia + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
240	A16 + C03B	Atorvastatin + Furosemide	Hypercholesterolemia + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
241	N05B + C02	Bromazepam + Indapamide	Anxiety + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
242	N05B + N06A	Zolpidem + Fluoxetine	Insomnia + Bulimia	Hypertensive medication/ Medication affecting L-Arginine pathway
243	N06A	Amitriptyline	Depression	Hypertensive medication/ Medication affecting L-Arginine pathway
244	N06A	Fluoxetine	Bulimia	Hypertensive medication/ Medication affecting L-Arginine pathway
245	N06A	Fluoxetine	Bulimia	Hypertensive medication/ Medication affecting L-Arginine pathway
246	R03A + C02	Formoterol + Budesonide +Theophylline	Asthma + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
247	R05D + A02 + B01B	Dextromethorphan + Pantoprazole + Clopidogrel	Coughing + Esophageal problems + Heart attack	Hypertensive medication/ Medication affecting L-Arginine pathway
248	R06 + M05 + C02	Cetirizine + Glucosamine + Amlodipine	Allergies + Arthrosis + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway

12 semanas

Estudio NUTCIR 1

Nombre..... *Fecha ....*

CUESTIONARIO DE FRECUENCIA DE CONSUMO ALIMENTARIO 1

INSTRUCCIONES PARA CONTESTAR

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

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

La frecuencia de consumo se tiene que especificar en los recuadros de la derecha del listado de alimentos de este cuestionario. Para cada alimento del listado debe apuntar el **número de veces** que lo consume.

- Si lo consume **todos los días de la semana**, escriba un 7 en la columna **A LA SEMANA.**
- Si el consume **alguna vez a la semana,** escriba las veces: 1-2-3-4-5 o 6 en la columna **A LA SEMANA.**

  Piense siempre en sumar el consumo de todas las comidas del día (desayuno, almuerzo, merienda, cena, otros,…). Por ejemplo, si toma todos los días leche para desayunar y alguna vez a la semana para cenar: 7 + 4 = 11 veces a la semana.
- Si consume el alimento **alguna vez al mes**, escriba las veces: 1-2-3 etc… en la columna: **AL MES**
- Si no lo consume **nunca** o casi nunca, deje la casilla en blanco, sin escribir nada.

Ejemplo: Una mujer desayuna habitualmente un vaso de leche (7 veces) con magdalenas (7 veces), y para cenar a veces toma leche (4 veces) y a veces toma yogur (3 veces) de postres. Además, toma pescado algunas veces a la semana para almorzar (2 veces) y otras veces para cenar (4 veces). De legumbres consume alguna vez al mes (aproximadamente 4 veces). Si no consume nunca un alimento deje la casilla en blanco, sin contestar nada.
Este consumo lo apuntaría de la siguiente manera:

LISTADO DE ALIMENTOS	**¿CUÁNTAS VECES COME…?**	
	A LA SEMANA	**AL MES**
Leche	*11*	
Yogur	*3*	
Bizcocho, magdalenas, …	*7*	
…		
Pescado	*6*	
…		
Legumbres		*4*
…		
Queso de régimen		

CUESTIONARIO DE FRECUENCIA DE CONSUMO ALIMENTARIO

LISTADO DE ALIMENTOS	¿CUÁNTAS VECES COME...?	
	A LA SEMANA	AL MES
Leche		
Yogur		
Chocolate: tableta, bombones, "Kit-Kat", "Mars"…		
Cereales de desayuno ("Corn-Flakes" "Kellog's")		
Galletas tipo "maría"		
Galletas con chocolate, crema...		
Magdalenas, bizcocho ...		
Ensaimada, Donut, croissant...		

	A LA SEMANA	AL MES
Ensalada: lechuga, tomate, escarola…		
Judías verdes, acelgas, o espinacas		
Verduras de guarnición: berenjena, calabacín, champiñones…		
Patatas al horno, fritas o hervidas		
Legumbres: lentejas, garbanzos, judías blancas…		
Arroz blanco, paella		
Pasta: fideos, macarrones, espaguetis …		
Sopas y cremas		

	A LA SEMANA	AL MES
Huevos		
Pollo o pavo		
Ternera, cerdo, cordero (bistec, empanada…)		
Carne picada: longaniza, hamburguesa …		
Pescado blanco: merluza, mero…		
Pescado azul: sardinas, atún, salmón …		
Marisco: mejillones, gambas, langostinos, pulpo, calamares …		
Croquetas, empanadillas, pizza		
Pan (en bocadillos, en las comidas)		

	QUANTES VEGADES MENJA...?	
	A LA SEMANA	AL MES
Jamón, jamón dulce, embutidos		
Queso fresco (Burgos…) o bajo en calorías		
Quesos curados o semicurados, cremosos		

CUESTIONARIO DE PREFERENCIAS Y HÁBITOS ALIMENTARIOS

	A LA SEMANA	AL MES
Frutas cítricas: naranja, mandarina		
Otras frutas: manzana, pera, melocotón, albaricoque, plátano		
Frutas en conserva (en almíbar...)		
Zumos de fruta natural		
Zumos de fruta comercial		
Frutos secos: cacahuetes, avellanas, almendras		
Postres lácteos: natillas, flan, requesón		
Pasteles de crema o chocolate		
Bolsas de aperitivo ("chips", "cheetos", "fritos")		
Golosinas: gominolas, caramelos,…		
Helados		

	A LA SEMANA	AL MES
Bebidas azucaradas ("coca-cola", "Fanta")		
Bebidas bajas en calorías (coca-cola light…)		
Vino, sangría		
Cerveza		
Cerveza sin alcohol		
Bebidas destiladas (Whisky, ginebra, coñac…)		

Indique con una X la respuesta que usted desee señalar:

1.- ¿En la mesa, se añade sal a las comidas?

Nunca_ / Alguna vez__ / Frecuentemente_/ Casi siempre_

2.- ¿Cómo definiría su apetito? Mucho__ Bastante_ Normal__ Poco__ Ninguno_

3.- ¿Qué tipo de leche toma habitualmente?: Entera_ Semidesnatada_ Desnatada_

4.- ¿Qué tipo de yogur toma habitualmente?

a) Natural ___ b) Natural desnatado ___
c) De sabores ___ d) De sabores desnatado ___
e) Con trozos de fruta ___ f) Con trozos de frutas desnatado ___

5.- ¿Qué tipo de pan toma habitualmente?: Blanco Integral _

6.- ¿Unta el pan con tomate y aceite en los bocadillos?:

Siempre_ / Habitualmente_/ Alguna vez__/ Casi nunca_

20 semanas

Nom: Data:

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

ANOTE LAS RESPUESTAS EN LOS ESPACIOS CORRESPONDIENTES A CADA PREGUNTA.
Estos datos servirán a la Universitat Rovira i Virgili para realizar un estudio comparativo entre diferentes poblaciones. En los resultados nunca aparecerá su nombre.

USO DE SUPLEMENTOS DE VITAMINAS / MINERALES

Por diferentes motivos, los suplementos de vitaminas y minerales recomendados no se toman siempre: por olvido, por sentimiento de que no son necesarios, por no encontrarse bien, porque dan molestias, etc. Por favor, conteste sinceramente estas preguntas para ayudarnos a valorar la realidad del uso de los suplementos.

- ¿Ha tomado por iniciativa propia o recetado por un médico algún tipo de suplemento vitamínico / mineral?
  Nunca he tomado ☐ **Si he tomado ☐**

En el caso que si, escriba el nombre del preparado e indique las veces a la semana que lo ha tomado marcando el cuadrado. Marque el cuadrado correspondiente a los meses que lo ha tomado.

Ejemplo, una mujer que ha tomado cada día FOLIDOCE durante los primeros 3 meses, escribiría:

Nombre del preparado	¿Cuántas veces a la semana?	Meses del embarazo 1	2	3	4	5
ÁCIDO FÓLICO	■ Cada día	■	■	■	☐	☐
	☐ La mayoría de los días (4-6 veces)	☐	☐	☐	☐	☐
¿Cuál? : FOLIDOCE	☐ Algunos días (1-3 veces)	☐	☐	☐	☐	☐

Nombre del preparado	¿Cuántas veces a la semana?	Meses del embarazo 1	2	3	4	5
ÁCIDO FÓLICO	☐ Cada día	☐	☐	☐	☐	☐
	☐ La mayoría de los días (4-6 veces)	☐	☐	☐	☐	☐
¿Cuál?:_____	☐ Algunos días (1-3 veces)	☐	☐	☐	☐	☐
	☐ Cada día	☐	☐	☐	☐	☐
HIERRO	☐ La mayoría de los días (4-6 veces)	☐	☐	☐	☐	☐
¿Cuál?:_____	☐ Algunos días (1-3 veces)	☐	☐	☐	☐	☐
MULTI-VITAMINAS	☐ Cada día	☐	☐	☐	☐	☐
	☐ La mayoría de los días (4-6 veces)	☐	☐	☐	☐	☐
¿Cuál?:_____	☐ Algunos días (1-3 veces)	☐	☐	☐	☐	☐

- ¿Tomó ácido fólico en los 3 meses antes de quedarse embarazada? **Sí ☐** **No ☐**
- ¿Tomó hierro en los 3 meses antes de quedarse embarazada? **Sí ☐** **No ☐**

DESAYUNO (durante el embarazo)

	Sí	No
¿Tiene la costumbre de desayunar?	☐	☐
¿Desayuna cereales inflados habitualmente (p.ej. tipo Kelloggs / Nestlé etc) ?	☐	☐
¿Toma café con cafeína?	☐	☐

Nom: Data:

TABACO

- ¿Es fumadora pasiva (expuesta al humo habitualmente en casa o en el trabajo) ? **Sí** ☐ **No** ☐
- ¿Es fumadora activa? **Sí** ☐ **No** ☐

Sólo para fumadoras en los últimos 5 años

	0 cigs/día	1-5 cigs/día	6-10 cigs/día	>10 cigs/día
Actualmente fuma	☐	☐	☐	☐
Fumaba durante los 12 meses antes del embarazo	☐	☐	☐	☐

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

ALCOHOL

	Nunca / Ocasionalmente	<3 copas / semana	Cada día como aperitivo y/o con las comidas	>7 copas / semana
Actualmente bebe alcohol	☐	☐	☐	☐
En los 12 meses antes del embarazo	☐	☐	☐	☐

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

PESO, ALTURA, EDAD, ORIGEN Y PARTICIPACIÓN EN ESTUDIOS

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

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

Origen padres:
Origen abuelos:

Nom: Data:

SUSTANCIAS TÓXICAS

- ¿Ha tomado algún otro tipo de sustancia tóxica (p.ej. marihuana, cocaína, heroína, etc...) en los últimos 5 años?

Sí ☐ **No** ☐

En el caso de que sí haya tomado alguna sustancia tóxica, especifique cuales: _____

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

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

ACTIVIDAD FÍSICA (durante el embarazo)

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

Horas/semana

- Mi trabajo es básicamente de estar sentada y caminar poco (estudiante, docente, conductora de vehículos, dependienta, administrativa)..... ☐ _____

- En mi trabajo ando bastante pero no hago ningún esfuerzo vigoroso (ama de casa, fábrica, vendedora, cartera)..... ☐ _____

- Mi trabajo es básicamente de mucha actividad física (deportista) ..... ☐ _____

- ¿Qué actividad hace en el tiempo libre? (anotar la prioritaria si dos actividades coinciden en horas)

Horas/semana

- Lectura, televisión y actividades que no requieran actividad física importante..... ☐ _____

- Caminar, ir en bicicleta, jardinería (no se incluye el transporte de ir y volver del trabajo)..... ☐ _____

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

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

- Durante los últimos 12 meses

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

Nom: Data:

PLANIFICACIÓN DEL EMBARAZO

- ¿Ha buscado / planificado este embarazo? **Sí** ☐ **No** ☐

- Durante los 6 meses antes del embarazo

	Ninguno	DIU	Anticonceptivos orales	Pegados anticonceptivos	Anillo vaginal	Preservativo
¿Que método anticonceptivo ha utilizado?	☐	☐	☐	☐	☐	☐

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

DATOS SOCIODEMOGRÁFICOS

- Cual es su trabajo actual y que nivel de estudios ha completado

	Mare	Pare
Trabajo actual	☐	☐
Nivel de estudios	Primarios sin finalizar ☐ Primarios (ESO, EGB, ...) ☐ Secundarios (BUP, Bachillerato, FP, ...) ☐ Superiores (Universitarios) ☐	Primarios sin finalizar ☐ Primarios (ESO, EGB, ...) ☐ Secundarios (BUP, Bachillerato, FP, ...) ☐ Superiores (Universitarios) ☐ No aplicable (Familia monoparental) ☐

- Numero de personas que forman la unidad familiar _____

- Ingresos netos anuales totales en el hogar

Ejemplo, si la mujer tiene un sueldo de 20000 €, el hombre uno de 18000€ y hay un abuelo que vive con la familia y recibe una pensión de 6000 €

Menos de 9000 €	9000 € - 19000 €	19000 € - 25000 €	25000 € - 35000 €	Más de 35000 €
☐	☐	☐	☐	■

Menos de 9000 €	>9000 € - 19000 €	>19000 € - 25000 €	>25000 € - 35000 €	Más de 35000 €
☐	☐	☐	☐	☐

Anote cualquier duda relacionada con esta encuesta:

32 semanas

Nom: Data:

ENCUESTA 2 SOBRE HÁBITOS Y ESTILO DE VIDA
(referida a la segunda mitad del embarazo)

ANOTE LAS RESPUESTAS EN LOS ESPACIOS CORRESPONDIENTES A CADA PREGUNTA.

Estos datos servirán a la Universitat Rovira i Virgili para realizar un estudio comparativo entre diferentes poblaciones. En los resultados nunca aparecerá su nombre.

USO DE SUPLEMENTOS DE VITAMINAS / MINERALES

Por diferentes motivos, los suplementos de vitaminas y minerales recomendados no se toman siempre: por olvido, por sentimiento de que no son necesarios, por no encontrarse bien, porque dan molestias, etc. Por favor, conteste sinceramente estas preguntes para ayudarnos a valorar la realidad del seguimiento de los suplementos.

- ¿Ha tomado por iniciativa propia o recetado por un médico algún tipo de suplemento vitamínico / mineral?

  Nunca he tomado ☐ **Si he tomado** ☐

En el caso que sí, escriba el nombre del preparado e indique las veces a la semana que lo ha tomado. Rellene el cuadrado correspondiente a los meses del embarazo que lo ha tomado.

Ejemplo, una mujer que ha tomado la mayoría de los días FERPLEX durante los meses 6, 7, 8 y 9, escribiría:

Nombre del preparado	¿Cuántas veces a la semana?	Meses del embarazo6			
			7	8	9
HIERRO	☐ Cada día	☐	☐	☐	☐
	■ La mayoría de los días (4-6 veces)	■	■	■	■
¿Cuál?: FERPLEX	☐ Algunos días (1-3 veces)	☐	☐	☐	☐

Nombre del preparado	¿Cuántas veces a la semana?	Meses del embarazo			
		6	7	8	9
HIERRO	☐ Cada día	☐	☐	☐	☐
	☐ La mayoría de los días (4-6 veces)	☐	☐	☐	☐
¿Cuál?:_____	☐ Algunos días (1-3 veces)	☐	☐	☐	☐
ÁCIDO FÓLICO	☐ Cada día	☐	☐	☐	☐
	☐ La mayoría de los días (4-6 veces)	☐	☐	☐	☐
¿Cuál?:_____	☐ Algunos días (1-3 veces)	☐	☐	☐	☐
MULTI-VITAMINAS	☐ Cada día	☐	☐	☐	☐
	☐ La mayoría de los días (4-6 veces)	☐	☐	☐	☐
¿Cuál?:_____	☐ Algunos días (1-3 veces)	☐	☐	☐	☐

- Si ha dejado de tomar el hierro, ¿cuál ha sido el motivo o motivos?
  - ☐ Olvido
  - ☐ Le causaba molestias
  - ☐ No lo consideraba muy importante para la salud
  - ☐ Otros (especificar) _____

DESAYUNO (durante el embarazo)

	Sí	No
¿Tiene la costumbre de desayunar?	☐	☐
¿Desayuna cereales inflados habitualmente (p.ej. tipo Kelloggs / Nestlé etc) ?	☐	☐
¿Toma café con cafeína?	☐	☐

Nom: Data:

TABACO

- ¿Es fumadora pasiva (expuesta al humo habitualmente en casa o en el trabajo)? **Sí** ☐ **No** ☐
- ¿Es fumadora activa? **Sí** ☐ **No** ☐

Sólo para fumadoras en los últimos 5 años

	0 cigs/día	1-5 cigs/día	6-10 cigs/día	>10 cigs/día
Actualmente fuma	☐	☐	☐	☐
Fumaba durante los 12 meses antes del embarazo	☐	☐	☐	☐

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

ALCOHOL

	Nunca / Ocasionalmente	<3 copas / semana	Cada día como aperitivo y/o con las comidas	>7 copas / semana
Actualmente bebe alcohol	☐	☐	☐	☐
En los 12 meses antes del embarazo	☐	☐	☐	☐

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

PESO, ALTURA, EDAD, ORIGEN Y PARTICIPACIÓN EN ESTUDIOS

(ORIGEN Y PARTICIPACIÓN SOLO SI NO SE DISPONE DE ENCUESTA 1)

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

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

Origen padres:
Origen abuelos:

NOM: DATA:

SUSTANCIAS TÓXICAS

- ¿Ha tomado algún otro tipo de sustancia tóxica (p.ej. marihuana, cocaína, heroína, etc...) en los últimos 5 años?

  Sí ☐ **No** ☐

En el caso de que sí haya tomado alguna sustancia tóxica, especifique cuales: _____

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

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

ACTIVIDAD FÍSICA (durante el embarazo)

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

Horas/semana

- Mi trabajo es básicamente de estar sentada y caminar poco (estudiante, docente, conductora de vehículos, dependienta, administrativa) ..... ☐ _____

- En mi trabajo ando bastante pero no hago ningún esfuerzo vigoroso (ama de casa, fábrica, vendedora, cartera)..... ☐ _____

- Mi trabajo es básicamente de mucha actividad física (deportista)..... ☐ _____

- ¿Qué actividad hace en el tiempo libre? (anotar la prioritaria si dos actividades coinciden en horas)

Horas/semana

- Lectura, televisión y actividades que no requieran actividad física importante ..... ☐ _____

- Caminar, ir en bicicleta, jardinería (no se incluye el transporte de ir y volver del trabajo)..... ☐ _____

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

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

- Durante los últimos 12 meses

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

NOM: DATA:

DATOS SOCIODEMOGRÁFICOS (SOLO SI NO SE DISPONE DE LA ENCUESTA 1)

- Cual es su trabajo actual y que nivel de estudios ha completado

	Mare	Pare
Trabajo actual	☐	☐
Nivel de estudios	Primarios sin finalizar ☐ Primarios (ESO, EGB, …) ☐ Secundarios (BUP, Bachillerato, FP, …) ☐ Superiores (Universitarios) ☐	Primarios sin finalizar ☐ Primarios (ESO, EGB, …) ☐ Secundarios (BUP, Bachillerato, FP, …) ☐ Superiores (Universitarios) ☐ No aplicable (Familia monoparental) ☐

- Numero de personas que forman la unidad familiar _____

- Ingresos netos anuales totales en el hogar

Ejemplo, si la mujer tiene un sueldo de 20000 €, el hombre uno de 18000€ y hay un abuelo que vive con la familia y recibe una pensión de 6000 €

Menos de 9000 €	9000 € - 19000 €	19000 € - 25000 €	25000 € - 35000 €	Más de 35000 €
☐	☐	☐	☐	■

Menos de 9000 €	>9000 € - 19000 €	>19000 € - 25000 €	>25000 € - 35000 €	Más d e 35000 €
☐	☐	☐	☐	☐

Anote cualquier duda relacionada con esta encuesta:

Estudio NUTCIR 2

Nombre..... *Fecha ....*

CUESTIONARIO DE FRECUENCIA DE CONSUMO ALIMENTARIO 2

INSTRUCCIONES PARA CONTESTAR

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

Este cuestionario le pregunta la frecuencia con que usted consume de forma **habitual** determinados alimentos durante el embarazo.

La frecuencia de consumo se tiene que especificar en los recuadros de la derecha del listado de alimentos de este cuestionario. Para cada alimento del listado debe apuntar el **número de veces** que lo consume.

- Si lo consume **todos los días de la semana**, escriba un 7 en la columna **A LA SEMANA.**
- Si el consume **alguna vez a la semana,** escriba las veces: 1-2-3-4-5 o 6 en la columna **A LA SEMANA.**

  Piense siempre en sumar el consumo de todas las comidas del día (desayuno, almuerzo, merienda, cena, otros,…). Por ejemplo, si toma todos los días leche para desayunar y alguna vez a la semana para cenar: 7 + 4 = 11 veces a la semana.
- Si consume el alimento **alguna vez al mes**, escriba las veces: 1-2-3 etc… en la columna: **AL MES**
- Si no lo consume **nunca** o casi nunca, deje la casilla en blanco, sin escribir nada.

Ejemplo: Una mujer desayuna habitualmente un vaso de leche (7 veces) con magdalenas (7 veces), y para cenar a veces toma leche (4 veces) y a veces toma yogur (3 veces) de postres. Además, toma pescado algunas veces a la semana para almorzar (2 veces) y otras veces para cenar (4 veces). De legumbres consume alguna vez al mes (aproximadamente 4 veces). Si no consume nunca un alimento deje la casilla en blanco, sin contestar nada.
Este consumo lo apuntaría de la siguiente manera:

LISTADO DE ALIMENTOS	**¿CUÁNTAS VECES COME…?**	
	A LA SEMANA	**AL MES**
Leche	**11**	
Yogur	**3**	
Bizcocho, madalenas, …	**7**	
...		
Pescado	**6**	
...		
Legumbre		**4**
…		
Queso de régimen		

CUESTIONARIO DE FRECUENCIA DE CONSUMO ALIMENTARIO

LISTADO DE ALIMENTOS	¿CUANTAS VECES COME...?	
	A LA SEMANA	AL MES
Leche		
Yogur		
Chocolate: tableta, bombones, "Kit-Kat", "Mars",…		
Cereales de desayuno ("Corn-Flakes", "Kellog's")		
Galletas tipo "maría"		
Galletas con chocolate, crema...		
Magdalenas, bizcocho ...		
Ensaimada, Donut, croissant...		

	A LA SEMANA	AL MES
Ensalada: lechuga, tomate, escarola…		
Judías verdes, acelgas, o espinacas		
Verduras de guarnición: berenjena, calabacín, champiñones…		
Patatas al horno, fritas o hervidas		
Legumbres: lentejas, garbanzos, judías blancas…		
Arroz blanco, paella		
Pasta: fideos, macarrones, espaguetis …		
Sopas y cremas		

	A LA SEMANA	AL MES
Huevos		
Pollo o pavo		
Ternera, cerdo, cordero (bistec, empanada…)		
Carne picada: longaniza, hamburguesa …		
Pescado blanco: merluza, mero…		
Pescado azul: sardinas, atún, salmón …		
Marisco: mejillones, gambas, langostinos, pulpo, calamares …		
Croquetas, empanadillas, pizza		
Pan (en bocadillos, en las comidas)		

CUESTIONARIO DE FRECUENCIA DE CONSUMO ALIMENTARIO

	¿CUÁNTAS VECES COME...?	
	A LA SEMANA	AL MES
Jamón, jamón dulce, embutidos		
Queso fresco (Burgos…) o bajo en calorías		
Quesos curados o semicurados, cremosos		

	A LA SEMANA	AL MES
Frutas cítricas: naranja, mandarina		
Otras frutas: manzana, pera, melocotón, albaricoque, plátano		
Frutas en conserva (en almíbar...)		
Zumos de fruta natural		
Zumos de fruta comercial		
Frutos secos: cacahuetes, avellanas, almendras		
Postres lácteos: natillas, flan, requesón		
Pasteles de crema o chocolate		
Bolsas de aperitivo (“chips”, “cheetos”, “fritos”)		
Golosinas: gominolas, caramelos,…		
Helados		

	A LA SEMANA	AL MES
Bebidas azucaradas (“coca-cola”, “Fanta”)		
Bebidas bajas en calorías (coca-cola light…)		
Vino, sangría		
Cerveza		
Cerveza sin alcohol		
Bebidas destiladas (Whisky, ginebra, coñac…)		

Indique con una X la respuesta que usted desee señalar:

1.- ¿En la mesa, se añade sal a las comidas?

Nunca_ / Alguna vez__ / Frecuentemente_/ Casi siempre_

2.- ¿Cómo definiría su apetito? Mucho__ Bastante_ Normal__ Poco__ Ninguno_

3.- ¿Qué tipo de leche toma habitualmente?: Entera_ Semidesnatada_ Desnatada_

4.-¿Qué tipo de yogur toma habitualmente?

a) Natural ___ b) Natural desnatado ___
c) De sabores ___ d) De sabores desnatado ___
e) Con trozos de fruta ___ f) Con trozos de frutas desnatado ___

5.- ¿Qué tipo de pan toma habitualmente?: Blanco Integral _

6.- ¿Unta el pan con tomate y aceite en los bocadillos?:

Siempre_ Habitualmente_ Alguna vez_ Casi nunca_

7.- ¿Cómo acostumbra a tomar el suplemento de hierro durante este embarazo? *(marque con una x)*

- ☐ No lo he tomado nunca
- ☐ Con agua
- ☐ Con zumo de naranja
- ☐ Con leche
- ☐ Otros (especificar)_____

8.- ¿Durante el embarazo ha tenido náuseas? ☐ Si ☐ No Y vómitos? ☐ Si ☐ No

En caso que sí haya sufrido vómitos durante el embarazo, especifique en qué meses:

☐ **1-3** ☐ **4** ☐ **5** ☐ **6** ☐ **7** ☐ **8**

¿Con qué frecuencia ha tenido estos vómitos?

- ☐ Regularmente
- ☐ De vez en cuando
- ☐ Muy pocas veces

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CLINICAL RESEARCH ARTICLE

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

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

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IMPACT:

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

INTRODUCTION

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

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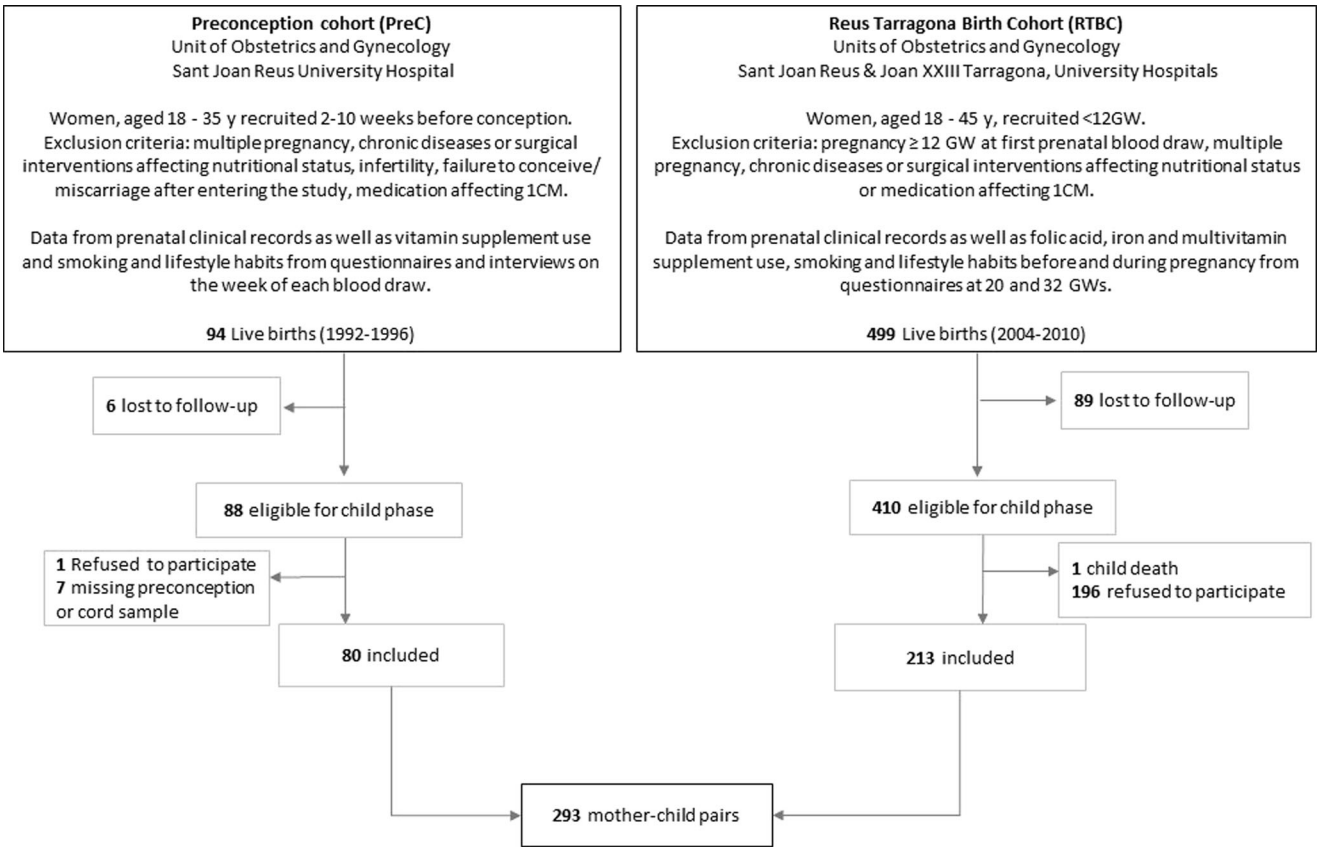

Fig. 1 Participant recruitment and follow-up. 1CM: 1 carbon metabolism.

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

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

METHODS

Participants

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

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

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

Health check-up at 6–8 years

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

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

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

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

For $y \leq 35$ mm:

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

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

For $y > 35$ mm:

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

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

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

Blood sample collection, processing and storage

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

Biochemical determinations

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

Metabolic score

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

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

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

Sample size calculation

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

Statistical analysis

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

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

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

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

RESULTS

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

Table 1. Participant characteristics according to pregnancy tHcy status.

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

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

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

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

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

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

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

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

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

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

DISCUSSION

Principal findings

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

Comparison with previous studies

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

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

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

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

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

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

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

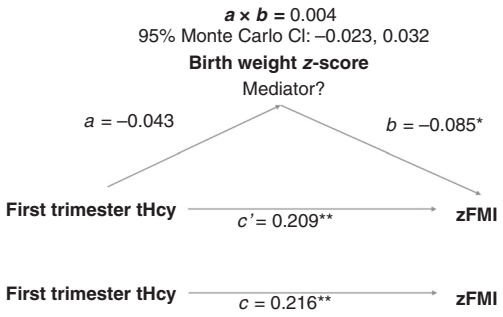

Fig. 2 Mediation analysis[34]: association between first trimester tHcy–child *z*-fat mass index via birth weight *z*-score. *a*, *b*, *c*′, and *c* (linear regression analysis *B* coefficients adjusting for maternal age, BMI, socioeconomic status, smoking (*a*), and birth weight *z*-score, breastfeeding,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

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

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

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

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

Interpretation

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

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

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

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

Strengths and limitations

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

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

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

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

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

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

CONCLUSIONS

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

DATA AVAILABILITY

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

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AUTHOR CONTRIBUTIONS

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

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

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

ADDITIONAL INFORMATION

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Research paper

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

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ABSTRACT

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

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

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

1. Introduction

1.1. Background

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

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

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

1.3. Hypothesis and aims

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

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

2. Materials and methods

2.1. Study participants

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

2.2. Blood sample collection

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

2.3. Pregnancy confirmation and miscarriage diagnosis

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

2.4. Statistical analysis

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

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

3. Results

3.1. Cases included

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

3.2. Participant characteristics according to first trimester tHcy category

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

3.3. Exposure to first trimester tHcy and risk of miscarriage

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

3.4. Participant characteristics according to pregnancy outcome

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

3.5. Factors predicting first trimester tHcy

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

3.6. Factors influencing first trimester RBC folate status

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4. Discussion

4.1. Principal findings

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

4.2. Comparison with previous studies

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

4.3. Interpretation

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

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

4.4. Strengths and limitations

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

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

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

4.5. Implications

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

5. Conclusions

Moderately elevated early pregnancy tHcy is associated with 2.5

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

Author contributions

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

Declaration of competing interest

None to declare.

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Abstract

Background: Methylenetetrahydrofolate reductase (*MTHFR)* C677T genotype and elevated fasting plasma total homocysteine (tHcy) have been associated with hypertension. Whether interaction with the L-Arginine pathway is involved, is unclear. **Methods:** We determined plasma tHcy, folate, cobalamin, asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), erythrocyte glutathionine reductase activation coefficient (EGRAC), rs1801133 and rs1799983 genotypes, in a representative population sample of 788 adults (aged 18 to 75) and recorded their clinical history and medication use at a medical checkup. **Results:** In participants not on medication, tHcy was associated with ADMA (B=0.002; *P*= 0.048) and SDMA (B=0.007; *P*<0.001). Plasma L-Arginine pathway metabolite concentrations did not differ between rs1801133 genotypes. Having ADMA in the mid-high tertiles, versus the lowest tertile, was associated with increased risk of hypertension in people above 50 years of age (OR [95%CI], 2.0 [1.0, 4.1]). This was also true for the low-mid versus the highest L-Arginine/ADMA Ratio tertile (OR [95%CI], 2.4 [1.1, 5.3]). Carriers of the variant rs1799983 allele had a greater risk of hypertension compared to participants with the homozygote normal genotype, (OR [95%CI], 3.3 [1.2, 9.4]), only when ADMA was in the mid-high tertile group. Mediation analyses showed a direct inverse association between the variant compared to the homozygote normal rs1801133 genotypes and ADMA and a mediation effect via homocysteine. THcy was indirectly associated, via ADMA, with hypertension in the population above 50 years old.

Conclusion: THcy, but not rs1801133 genotype was associated with L-Arginine pathway metabolites. ADMA, L-Arginine/ADMA Ratio and variant rs1799983 genotype were only associated with hypertension in adults >50 years old.

Keywords

Homocysteine, Methylenetetrahydrofolate reductase C677T, Asymmetric dimethylarginine, Symmetric dimethylarginine, Endothelial nitric oxide synthase G894T, Hypertension

Introduction

Endothelial dysfunction occurs in the early stage of the pathogenesis of atherosclerosis and hypertension [1]. Age, sex and genetics, modifiable factors including smoking, alcohol consumption and physical activity [2] as well as anomalies in one carbon metabolism (1CM) including deficient B vitamin status, high plasma tHcy concentrations or the homozygote variant genotype for the methylenetetrahydrofolate reductase (*MTHFR*) 677 C>T polymorphism [3] have all been associated with hypertension .

Previously we reported that 18.1 % of adults from our local population had the *MTHFR* 677 TT genotype, and had lower folate status and higher tHcy compared to the CC genotype [4]. The *MTHFR* TT genotype has been associated with higher blood pressure when folate or riboflavin status are low [5]. Riboflavin supplementation lowered systolic blood pressure in hypertensive adults with this genotype in different studies from the same Northern Irish group [6, 7] but the mechanism remains unexplained to date. We recently reported that the *MTHFR* 677TT compared to CC genotype is associated with increased risk of hypertension in adults under 50, but not over 50 years of age, and that elevated tHcy is associated with increased hypertension risk in adults over 50 [8].

During the methionine cycle, S-Adenosylmethionine (SAM), donates two methyl groups to reactions involving Protein-Arginine N-Methyltransferase (PRMT) resulting in L-Arginine methylation and the formation of asymmetric dimethylarginine (ADMA) or its structural

isomer symmetric dimethylarginine (SDMA). Following proteolysis, free ADMA or SDMA are released. Free ADMA can inhibit endothelial nitric oxide synthase (eNOS), that catalyzes the conversion of L-Arginine into nitric oxide (NO) [9] and SDMA may cause L-Arginine depletion by competing for the L-Arginine transporter [10]. Elevated ADMA has been recognised as a risk factor for endothelial dysfunction [11] and has been associated with hypertension [12], stroke [13] and myocardial infarction [14]. SDMA is eliminated by the renal system and has been associated with renal dysfunction [15]. Studies investigating its association with cardiovascular diseases are scarce and its role is not clear. A study by Kiechl et al, postulated that ADMA and SDMA each predict cardiovascular risk [16]. NO prevents platelet aggregation and smooth muscle cell proliferation, so is essential for regulating vascular tone, vasodilation and blood pressure and reduced bioavailability can lead to endothelial dysfunction [17]. The *NOS* G894T polymorphism is associated with reduced enzymatic activity [18] and subsequently reduced NO synthesis [19]. The variant T allele of the polymorphism has been associated with coronary artery disease, myocardial infarction and essential hypertension [20–22].

Hyperhomocysteinemia has been linked to endothelial dysfunction through alteration of redox state, plasma ADMA accumulation and impaired nitric oxide (NO) bioavailability in bovine aortic endothelial cells *in vitro [23]*, in mice [24] and in both normotensive and hypertensive patients [25].

In randomized control trials, supplementation with folic acid or its active form 5-methyltetrahydrofolate (5-MTHF) has been associated with increased NO bioavailability and vasodilation in dyslipidemic conditions [26, 27]. However, endothelium dependent vasodilation did not improve following 8 weeks of supplementation with B vitamins, despite

tHcy reduction, in hyperhomocysteinemic peripheral arterial occlusion patients. In contrast, L-Arginine supplementation did not reduce tHcy but was associated with improved endothelium-dependent vasodilation and reduced oxidative stress [28]. Therefore, while 1CM and the L-Arginine pathway are closely related, their association in the development of endothelial dysfunction is unclear and studies to date may be influenced by variations in stages of disease progression and underlying medication and supplement use.

We hypothesized that impaired 1CM is associated with hypertension via the L-Arginine pathway. Elevated homocysteine may lead to increased risk of hypertension through inhibition of eNOS activity by ADMA and SDMA. The aim was to explore these associations, in a representative sample of an adult population, unexposed to mandatory fortification of flour with folic acid or B vitamin supplement use.

Material and methods

Subjects and procedure

The regional population-based cross-sectional study including 788 adults aged 18-75 years, randomly selected from age and sex stratified populations records in accordance with the population distribution from two towns in Tarragona province, Spain has been described previously [4, 8, 29]. It was carried out between 1998 and 2002 and was approved by the Sant Joan University Hospital, Reus and Jordi Gol Gorina Foundation ethics committees.

Briefly, participants taking B-vitamin supplements or medication known to affect folate or cobalamin status or with impaired renal function (plasma creatinine >97 mmol/L for women and >124 mmol/L for men) were excluded from the study. Additionally, 59 participants

whose blood samples were not processed in less than 2 hours after their collection were excluded from the analyses to prevent artefacts in tHcy determinations.

Clinical history and lifestyle data (including sex, age, BMI, previous and current illness, medication use, smoking, alcohol and drug use, socioeconomic status) were recorded at the medical check-up, and blood pressure measured, as previously described [8]. Participants with previously diagnosed hypertension were classified as hypertension cases, even if they were normotensive at the visit due to medical control of their condition.

Briefly, the blood pressure was measured following a standardized protocol and by a trained clinician. At least 15 minutes before the measurements, the participants remained seated. During the measurement, the participants were seated with their back supported, feet on the floor and arm resting on the arm rest of the chair with the palm of their hand up and the cubital fossa at the level of the heart. Using a mercury column sphygmomanometer (Riester), the mean of two measurements (2 minutes apart) was recorded. Participants with no previously detected hypertension and a blood pressure measurement >140/90 mmHg, at the check-up, were recommended to consult their doctor and were excluded from the analysis, to avoid misclassification. Participants with no history of hypertension and with normal blood pressure (<140/90 mmHg) at the check-up were categorized as controls. Participants with normal blood pressure but taking antihypertensives medication were selected as cases.

Collection and classification of data on medication use

Medical treatments recorded at the check-up were coded according to the WHO ATC/DDD index [30]. A PubMed search of literature linking the active ingredients of any medication recorded by clinicians with components of the L-Arginine pathway was carried out.

Treatments were classified into three categories (**Supplementary Table 1**): Group 1) none or sporadic, Group 2) chronic, not affecting the L-Arginine pathway (eg. pain treatment, insulin, depression, asthma) and Group 3) medical treatment known to affect the L-Arginine pathway (eg. anti-hypercholesterolemia, anti-hypertensive or thyroid treatments).

Blood samples

Fasting venous blood samples were collected into EDTA-K3 vacutainers and kept chilled until they were processed in less than 2 hours after collection. Plasma whole blood diluted in 1% ascorbic acid solution for Red Blood Cell Folate, washed erythrocytes and Leukocyte fractions, were kept at -80ºC for later determinations as previously described [4, 31].

Total plasma homocysteine was determined by fluorescence polarization immunoassay using the IMx autoanalyzer (Abbott Laboratories). L-Arginine, ADMA and SDMA were measured by HPLC-MS/MS [32]. Plasma folate and red blood cell folate were measured by microbiological assay with *Lactobacillus casei* [33]and plasma cobalamin with *L. leichmannii [34]*. Functional riboflavin status (erythrocyte glutathionine reductase activation coefficient (EGRAC)[31]) and plasma creatinine concentration (Jaffé reaction (Quimica Clinica Aplicada) [4, 31]) were determined using the COBAS MIRA autoanalyzer (Roche Diagnostics). Total and HDL-cholesterol and triglycerides were determined as previously described [35] and the Friedewald equation used to estimate LDL-cholesterol [36].

MTHFR C677T (rs1801133) and *NOS* G894T (rs1799983) genotypes were determined from leukocyte extracted DNA by matrix-assisted laser desorption/ionization/time-of-flight mass spectrometry [37].

Statistical analysis

Descriptive data are reported as means and 95% confidence interval (CI) for normally distributed variables and as geometric means and 95% CI when variables with skewed distributions were ln-transformed for the application of parametric statistical tests. Categorical variables are reported as percentages and 95% CI. Comparison between over 50 and under 50 age groups was by ANOVA for continuous variables and χ^2 for categorical variables.

The associations between 1) tHcy, 2) *MTHFR* 677CT vs CC and TT vs CC genotypes, and L-Arginine pathway metabolites were assessed using multiple linear regression analysis. All models were adjusted for sex, age, smoking, alcohol intake, socio-economic status, body mass index (BMI), plasma LDL cholesterol and medical treatment (reference: none/sporadic). The *MTHFR* genotype models were further adjusted for plasma creatinine, plasma cobalamin, plasma folate and EGRAC. The models included the interaction terms *tHcy x medical treatment category* or *MTHFR genotype x medical treatment category*, as appropriate. Stratified analyses by age group (≤ 50 years, > 50 years) were also performed, adjusting for the same variables as the total population models except age.

The probability of having hypertension was explored using multiple logistic regression analysis. Basic models explored L-Arginine pathway metabolite status (low-mid tertile versus the highest tertile for L-Arginine and L-Arginine/ADMA Ratio and mid-high tertile compared with the lowest tertile for ADMA, SDMA and ADMA/SDMA Ratio) as predictors of hypertension. All tertiles were sex and age group specific (≤ 50 years, > 50 years). Models were adjusted for socio-economic status, smoking, alcohol intake, BMI, total cholesterol,

diabetes and plasma creatinine. The *NOS* 894 TT and GT versus GG genotype models were stratified by ADMA or SDMA lowest and mid-high tertiles and adjusted for the same variables as above.

To test whether the relationship between 1CM and diagnosed hypertension was via the L-Arginine pathway, we performed three mediation analyses following the principles of Hayes [38]. 1) The potential association of the *MTHFR* 677 TT vs CC genotype with ADMA via tHcy, the mediation effect of ADMA tertiles in the association of 2) *MTHFR* 677 TT vs CC genotype and 3) high versus mid-low tHcy tertiles with hypertension. The same mediations were performed substituting ADMA for SDMA.

All mediation models were stratified by age group and adjusted for sex, smoking, alcohol intake, socio-economic status, BMI and medication category (reference: none/sporadic). Models including *MTHFR* 677 TT vs CC polymorphism were also adjusted for plasma cobalamin, plasma folate and EGRAC. Models including diagnosed hypertension were also adjusted for total cholesterol, diabetes and *NOS* GT+TT vs GG genotypes.

All models met the requirements for assumptions in linear regressions (normality of errors, multicollinearity, homogeneity of variance (homoscedasticity)). Bonferroni corrected *P* values to account for multiple comparisons in linear regressions are reported. Logistic regression diagnostics were performed by examining box plots to identify outliers and Cook's distance ($>4/\sqrt{n}$) to identify influential cases. The difference in the number of participants included in each analysis was due to missing data for some of the biochemical or clinical variables. Data were analyzed using SPSS software version 25.0. The mediation analyses were performed using macro PROCESS software version 3.5.3. Significance was

accepted at $P < 0.05$ and the indirect effects of mediation analysis were considered as statistically significant when the 95% CI did not include the value 0.

Results

The characteristics of the participants are described in **Table 1**. Participants over 50 years of age had higher BMI, folate, cobalamin and riboflavin status, and a greater prevalence of low socioeconomic status compared to those under 50. In addition, plasma tHcy, ADMA, SDMA and cholesterol concentrations were also higher in the older age group. Overall, the older group smoked less, but 22.8% (95% CI:21.3, 39.3) of men were smokers compared to 6.1 % (95% CI:3.3, 12.3) of women in the oldest age group (data not shown). Alcohol intake was higher in the older than in the younger group. *MTHFR* C677T and *NOS* G894T polymorphisms were in Hardy-Weinberg equilibrium, and 17.9% (95% CI:15.3, 20.7) and 17.4% (95% CI:14.9, 20.3) of the participants had the homozygous variant genotypes for these polymorphisms, respectively.

The prevalence of diagnosed hypertension and diabetes in the population was 14.1% and 4.2%, respectively. Regarding hypertension, 4 out of 5 cases were from the over 50 age group. Of the confirmed cases, 8.4% were on blood pressure lowering medication and only 12.6% of these were on medication known to affect the L-Arginine pathway.

The associations between tHcy and L-Arginine and its metabolites are reported in **Figure 1** and **Supplemental Figure 1.** The interaction terms for tHcy x medication category were significant in the ADMA model for adults over 50 ($P < 0.001$) and in the SDMA model for adults ≤ 50 ($P = 0.002$), so stratified analyses by medication category were carried out.

Figure 1 illustrates the associations between tHcy and the different L-Arginine pathway metabolites in the participants not on medication. tHcy was positively associated with plasma ADMA and SDMA concentrations in the entire population. In the under 50 age group, the association was only observed between tHcy and SDMA.

In the *MTHFR* models (**Figure 2**) the interaction term for *MTHFR* genotype x medication category was significant (*P*=0.038) in the L-Arginine/ADMA ratio model in adults <= 50 y. As statistical power was lost after stratification by medication category, and no significant results were found in the global model nor in the stratified model, no stratification is shown in **Figure 2**. The *MTHFR* 677 CT and TT genotypes were associated with lower plasma ADMA and SDMA concentrations compared to the CC genotype, but these associations were not significant after posthoc Bonferroni correction for multiple comparisons in the unstratified models. In participants not taking medication, CT vs CC and TT vs CC genotypes were negatively associated with ADMA (B=-0.011; SD=0.007; *P*=0.18) and (B=-0.011; SD=0.01; *P*=0.26), respectively. The same tendency was seen with SDMA for both CT (B=-0.012; SD=0.013; *P*=0.36) and TT (B=-0.02; SD=0.017; *P*=0.23) compared with CC genotypes.

The risk of hypertension associated with the different L-Arginine pathway metabolites is reported in **Table 2**. In participants over 50 years of age, mid-high compared with lowest ADMA tertiles and low-mid vs high L-Arginine/ADMA ratio tertiles were associated with increased risk of having hypertension (OR=2.3; 95% CI=1.1, 5.0) and (OR=2.4; 95% CI=1.1, 5.3), respectively.

No significant association was observed between the *NOS* 894 T allele and hypertension risk when ADMA was in the lowest tertile (**Table 3**). However, the *NOS* 894 TT and GT compared to GG genotypes, were associated with triple the risk of having hypertension in participants over 50 with ADMA in the mid-high tertiles (OR=3.3; 95% CI=1.2, 9.4). However, no significant association was seen between the *NOS* G894T polymorphism and hypertension according to SDMA tertiles.

The results of the mediation analyses are shown in **Figure 3**. The effect of *MTHFR* 677 TT vs CC genotype on ADMA is mediated by tHcy in the younger (B=0.011; 95% CI=0.002, 0.021) and the older (B=0.011; 95% CI=0.002, 0.025) adults **(Figure 3A)**. However, in those under 50 there is a stronger direct negative effect between *MTHFR* 677 TT vs CC genotypes and ADMA (B_c=-0.028; *P*=0.009).

ADMA was not a mediator in the association between the *MTHFR* TT genotype (**Figure 3B**) and diagnosed hypertension in either age group. The direct association between *MTHFR* 677 TT versus CC genotypes and hypertension was significant in the adults under 50.

In those over 50 years of age, the association between mid-high versus lowest tHcy tertiles with hypertension was mediated by ADMA tertile (B=0.286; 95% CI=0.045, 0.629). In those under 50, neither tHcy nor ADMA were significantly associated with risk of hypertension (**Figure 3C**).

Discussion

Major findings

In people not taking any medication, tHcy was positively associated with ADMA and SDMA. None of the L-Arginine pathway metabolites differed between the different *MTHFR* C677T genotypes. Mid-high tertiles of plasma ADMA and low-mid tertiles of L-Arginine/ADMA Ratio were associated with increased risk of hypertension in people over 50 years of age. The T allele of the *NOS* G894T polymorphism triples the risk of having hypertension when plasma ADMA concentration is in the middle or highest tertiles. In both age groups, the association between the *MTHFR* TT genotype and plasma ADMA showed a direct negative effect but a positive indirect effect via tHcy. The association between mid-high tHcy tertile and hypertension was mediated by ADMA.

One-Carbon metabolism and L-Arginine pathway association

The results regarding the association between tHcy and plasma ADMA and SDMA are in line with other studies. In monkeys with diet-induced hyperhomocyst(e)inemia, mean ADMA concentration was threefold higher than controls (P <0.05), but plasma SDMA concentrations did not differ between hyperhomocysteinemic and control monkeys (1.9 ± 0.6 µmol/L) (1.9 ± 0.6 µmol/L) [39]. In other observational human studies, such as the Framingham study, a positive correlation between tHcy and SDMA was reported [40–42]. tHcy and ADMA have been reported to be positively associated in various studies [43].

Few studies have examined the association between the *MTHFR* C677T polymorphism and L-Arginine pathway metabolites. Dimitroulas et al, reported higher serum ADMA concentrations in rheumatoid arthritis patients with the *MTHFR* 677 TT genotype compared to the CT or CC genotypes in a univariate analysis (P = 0.042). However, when tHcy and other covariables were considered in the multivariate analysis, no differences were observed [44]. In a study of epileptic patients, plasma ADMA concentrations were higher (P =0.002) and L-Arginine/ADMA ratio was lower (P <0.001) in patients (but not in controls) with the

MTHFR 677CT compared to CC genotype [45]. To the best of our knowledge, the possible association between the *MTHFR* C677T polymorphism and SDMA has not been studied previously.

Interestingly, our mediation analysis indicates that there is a direct inverse association between the *MTHFR* 677TT genotype and ADMA but a positive association is seen when tHcy is the mediator.

The association between the *MTHFR* 677TT genotype and DNA hypomethylation reported by some authors may explain the inverse association between the *MTHFR* 677TT genotype and ADMA in our population. This genotype may limit the availability of methyl groups from 5-methyltetrahydrofolate for SAM synthesis, which may reduce cellular capacity to methylate, decreasing ADMA and SDMA concentrations [46, 47]. In the positive association between tHcy and ADMA, homocysteine has been shown to inhibit the enzyme dimethylarginine dimethylaminohydrolase (DDAH), the enzyme responsible for the degradation of ADMA to L-Citrulline. In humans, 80% of ADMA is degraded by this enzyme [48, 49]. Inhibition of L-Citrulline synthesis by homocysteine would increase intraendothelial ADMA concentrations. A hypothesis is that the *MTHFR* TT genotype may lead to ADMA reduction but its increasing effect on tHcy and subsequent inhibition of DDAH, might explain why other studies report higher plasma ADMA concentrations in patients with the TT compared to CC genotype. We only observe this in our adult population when we consider homocysteine as a mediator, in the mediation analysis.

Association between the L-Arginine pathway and hypertension

We reported an increased risk of hypertension when plasma ADMA was in the middle or high tertile, only adults over 50. This is consistent with studies that observed a relationship between high plasma ADMA concentrations, hypertension and aging [50, 51]. However, our

results differ from those of Sonmez et al, that observed in a study of young men, higher plasma ADMA concentrations in hypertensive patients compared to controls [52]. In a study of adults undergoing routine medical checkups, ADMA and age were positively correlated, leading the authors to suggest that ADMA may reflect a vascular degenerative process associated with aging [51]. In fact, endothelium-dependent coronary microvascular dysfunction was associated with aging in 34 patients (27 to 73 years old) with no coronary risk factors [53]. Both ADMA and SDMA were associated with increased risk of cardiovascular disease in an adult, population-based, cohort after adjusting for several cofounders [16]. L-Arginine/ADMA Ratio and ADMA/SDMA ratio have been postulated to be better predictors of endothelial function than the metabolites separately [54, 55]. As observed in our population, the L-Arginine/ADMA ratio has been reported to be associated with blood pressure [56]. However, we did not observe that the L-Arginine/ADMA Ratio was a better predictor than ADMA alone, in fact the observations were similar for either of these.

Few studies take into account not only the potential lowering effect on NO of the *NOS* G894T polymorphism, but also, the decrease in NO synthesis due to low production by NOS in the presence of high ADMA concentrations (its inhibitor). In patients with renal disease, the combination of the *NOS* 894T allele and ADMA above the 75th percentile more than doubled the risk of cardiovascular mortality compared to the GG genotype and plasma ADMA below the 75th percentile [57]. Others concluded that both the *NOS* G894T allele and ADMA are independent factors in the development of atherosclerosis [58]. As ADMA competes with L-Arginine for eNOS, the accumulation of this inhibitor could amplify the genetic defects caused by the polymorphism in NO synthesis [57].

One possible mechanism by which ADMA and age are associated is the effect of LDL cholesterol on ADMA accumulation. In our population, the highest concentrations of plasma LDL cholesterol occurred in adults over 50. In human endothelial cells, LDL cholesterol increased PRMT gene expression [59] and DDAH enzyme activity was decreased to almost 60% of baseline values [60]. This may contribute to increased ADMA synthesis in hypercholesterolemia by increasing its synthesis by PRMT and decreasing its degradation by DDAH.

1CM-hypertension link via L-Arginine pathway

Our group recently reported, from the same population study, that the *MTHFR* 677TT genotype is associated with hypertension in people under 50 years of age, and moderately elevated tHcy was associated with hypertension in those over 50 [8]. The mediation analyses, carried out here, support the L-Arginine pathway as a mediator in the development of hypertension by hyperhomocysteinemia but not by the presence of mutant variant of the *MTHFR* C677T polymorphism.

NO synthesis can be also altered by tetrahydrobiopterin (BH4). The MTHFR enzyme produces 5-methyltetrahydrofolate, which interacts with BH4 (cofactor of the eNOS enzyme) to reduce the uncoupling of eNOS [61]. It has been proposed that smokers with the *MTHFR* TT genotype have reduced NO levels due to eNOS uncoupling and the higher affinity of the enzyme for O_2 rather than L-Arginine [62]. However, neither NO or BH4 values were determined in our study.

Strengths and limitations

Reverse causality is a potential limitation in cross-sectional studies. However, studying genotypes helps to overcome this limitation, in the case of the analyses that considered the *MTHFR* C677T genotype. In the case of the tHcy - ADMA analyses, while we excluded

medication use that affects the arginine pathway, reverse causation cannot be ruled out so further studies are required to clarify the directionality of the observations. While, previous B vitamin intervention studies have been inconclusive with regard to changes in plasma L-arginine, ADMA and SDMA concentrations after tHcy lowering in cardiovascular patients, there is evidence from a long-term intervention study with folic acid alone, in hyperhomocysteinaemic patients, that tHcy, plasma ADMA and arginine were all reduced after 6 weeks and 12 months [63].

In Spain there is no mandatory fortification of flour with folic acid or any B vitamins, so the population was not influenced by B-vitamin supplementation or mandatory fortification, thus avoiding their confounding effect in the association between the *MTHFR* C677T polymorphism and tHcy.

Conclusions

THcy and L-Arginine pathway metabolites concentrations were positively associated in a representative sample of an adult population unexposed to B vitamin fortification and supplementation. Plasma ADMA, L-Arginine/ADMA Ratio, and the *NOS* G894T polymorphism were associated with increased risk of hypertension. Our data support the L-Arginine pathway as a potential mechanistic link between moderately elevated tHcy and hypertension but not the *MTHFR* 677TT genotype.

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M. M. M. and J. D. F.-B. conducted the research. P. M. U. shared responsibility for the genotyping. C. R. -R., M. M. M., A. R. -G. and J. D. F.-B. analysed the data. C. R. -R. and M. M. M. wrote the manuscript. M. M. M. had primary responsibility for the final content. All authors read and approved the final manuscript. The authors declare that there are no conflicts of interest. C. R. -R. is a predoctoral fellow under the URV Martí Franques programme.

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Figure legends:

Figure 1: Multiple linear regressions testing the association between tHcy and L-Arginine pathway metabolites in plasma. tHcy, total fasting plasma homocysteine; ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine. Only participants not taking medication/sporadic medication (Group 1) are shown. All models were adjusted for sex, age (only in total population models), smoking (cigarettes/day), alcohol intake category (moderate (<16 g/d in women and <24 g/d in men) versus none; high versus none (≥16 g/d in women and ≥24 g/d in men)), low versus mid-high socio-economic status, BMI and LDL cholesterol. Participants not on medication are shown. Unstandardized B-coefficients, Standard error (error bars), are reported. * P <0.05; ** P <0.01; *** P <0.001. L-Arginine model N= 524 (total: R^2=0.011, P=0.095; ≤ 50 y: R^2=0.028, P=0.015; > 50 y R^2=0.013, P =0.299); ADMA model N=523 (total: R^2=0.120, P <0.001; ≤ 50 y: R^2=0.063, P <0.001; > 50 y R^2=0.00, P =0.442); SDMA model N= 523 (total: R^2=0.106, P <0.001; ≤ 50 y: R^2=0.035, P =0.005; > 50 y R^2=0.087, P=0.015); L-Arginine/ADMA Ratio model N=523 (total: R^2=0.055, P <0.001; ≤ 50 y: R^2=0.052, P <0.001; > 50 y R^2=-0.023, P =0.733); ADMA/SDMA Ratio model N=522 (total: R^2=0.062, P <0.001; ≤ 50 y: R^2=0.052, P <0.001; > 50 y R^2=0.105, P =0.006).

Figure 2: Multiple linear regressions testing the association of *MTHFR* C677T genotype and L-Arginine pathway metabolites, stratified by age. MTHFR, methylenetetrahydrofolate reductase; EGRAC, erythrocyte glutathione reductase activation coefficient; BMI, body mass index; ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine. All models were adjusted for sex, age (only in the total population models), smoking (cigarettes/day), alcohol intake category (moderate (<16 g/d in women and <24 g/d in men) versus none; high versus none (≥16 g/d in women and ≥24 g/d in men)), low versus mid-high socio-economic status, BMI, LDL cholesterol, creatinine, plasma cobalamin (µmol/l), plasma folate (nmol/l), EGRAC, and category of medication use. Unstandardized B-coefficients, SD, error bars, are reported. *P <0.05; ** P <0.01; *** P <0.001.. L-Arginine N= 677 (total: R^2=0.050, P <0.001; ≤ 50 y: R^2=0.046, P =0.001; > 50 y R^2=0.073, P =0.010); ADMA N=676 (total: R^2=0.137, P <0.001; ≤ 50 y: R^2=0.051, P <0.001; > 50 y R^2=0.016, P

=0.253); SDMA N= 676 (total: R^2=0.216, *P* <0.001; ≤ 50 y: R^2=0.102, *P* <0.001; > 50 y R^2=0.264, *P* <0.001); and L-Arginine/ADMA Ratio N=676 (total: R^2=0.106, *P* <0.001; ≤ 50 y: R^2=0.076, *P* <0.001; > 50 y R^2=0.044, *P* =0.061); ADMA/SDMA Ratio N=675 (total: R^2=0.195, *P* <0.001; ≤ 50 y: R^2=0.159, *P* <0.001; > 50 y R^2=0.236, *P* <0.001).

Figure 3: Mediation analysis to test **(A)** tHcy as a mediator of the relationship between *MTHFR* 677 TT vs CC and ADMA, **(B)** ADMA tertiles as the mediator of the relationship between *MTHFR* 677 TT vs CC and diagnosed hypertension and **(C)** ADMA tertiles as the mediator of the relationship between high versus low-mid tertiles of tHcy and diagnosed hypertension; stratified by age group. *MTHFR*, methylenetetrahydrofolate reductase; tHcy, total fasting plasma homocysteine; BMI, body mass index; ADMA, Asymmetric dimethylarginine; *NOS,* Nitric oxide synthase. All mediation models were adjusted for sex, smoking habits, alcohol intake (moderate (<16 g/d in women and <24 g/d in men) versus none; high versus none (≥16 g/d in women and ≥24 g/d in men)), socio-economic status, BMI and medication category (reference: none/sporadic). Models including *MTHFR* 677 TT vs CC polymorphism were also adjusted for plasma cobalamin, plasma folate and erythrocyte glutathione reductase activation coefficient. Models including diagnosed hypertension were also adjusted for total cholesterol, diabetes and NOS G894T GT+TT vs GG polymorphism. The indirect (a X b) and the direct (c') effects reported indicate the association (B coefficients) of the independent variable with the outcome including and and not including the mediator, respectively. In dichotomous outcomes (hypertension), B coefficients of the indirect effect are expressed in log-odd metric. * *P* <0.05; ** *P* <0.01; *** *P* <0.001.

Supplementary Figure 1: Multiple linear regressions testing the association between tHcy and L-Arginine pathway metabolites in plasma. tHcy, total fasting plasma homocysteine; ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine. Only participants taking medication not affecting L-Arginine pathway and participants taking medication affecting L-Arginine pathway. All models were adjusted for sex, age (only in total population models), smoking (cigarettes/day), alcohol intake category (moderate (<16 g/d in women and <24 g/d in men) versus none; high versus none (≥16 g/d in women and

≥24 g/d in men)), low versus mid-high socio-economic status, BMI and LDL cholesterol. Participants not on medication are shown. Unstandardized B-coefficients, Standard error (error bars), are reported. * P <0.05; ** P <0.01; *** P <0.001. Participant taking medication not affecting L-Arginine pathway N: All population N=94, ≤ 50 y/o N=56 and >50 N=38. L-Arginine (All: R^2=-0.008, ≤ 50 y: R^2=-0.0081, > 50 y R^2=-0.073); ADMA (All: R^2=-0.009, ≤ 50 y: R^2=-0.085, > 50 y R^2=0.277***); SDMA (All: R^2=0.020, ≤ 50 y: R^2=-0.005 > 50 y R^2=0.085); L-Arginine/ADMA Ratio (All: R^2=0.009, ≤ 50 y: R^2=0.004, > 50 y R^2=0.040); ADMA/SDMA Ratio (All: R^2=-0.030, ≤ 50 y: R^2=-0.042, > 50 y R^2=-0.025). Participant taking medication affecting L-Arginine pathway N: All population N=81, ≤ 50 y/o N=23 and >50 N=58. L-Arginine (All: R^2=0.004, ≤ 50 y: R^2=-0.224, > 50 y R^2=-0.002); ADMA (All: R^2=0.198***, ≤ 50 y: R^2=0.097, > 50 y R^2=0.151***); SDMA (All: R^2=0.067, ≤ 50 y: R^2=-0.192 > 50 y R^2=0.266***); L-Arginine/ADMA Ratio (All: R^2=0.096, ≤ 50 y: R^2=-0.290, > 50 y R^2=0.106); ADMA/SDMA Ratio (All: R^2=0.115, ≤ 50 y: R^2=-0.064, > 50 y R^2=0.120).

Table 1. Characteristics of the study population according to age group.

	All participants	≤ 50 years old	>50 years old	*P*
	n/N	n/N	n/N	
Age	788/788	534/534	254/254	
Arithmetic Mean	43.0	34.2	61.5	
95% CI	41.9, 44.1	33.5, 34.9	60.6, 62.4	
Women	407/788	276/534	131/254	
%	51.6	51.7	51.6	
95% CI	48.2, 55.1	47.5, 55.9	45.5, 57.7	
BMI (kg/m^2)	773/788	524/534	249/254	
Arithmetic Mean	27.0	25.7	29.9	<0.001
95% CI	26.7, 27.4	25.3, 26.1	29.3, 30.5	
Smokers	263/788	227/534	180/254	
%	33.4	42.5	14.2	<0.001
95% CI	30.2, 36.7	38.4, 46.7	10.4, 19.0	
Alcohol consumption*				
None	468/788	320/534	148/254	
%	59.4	59.9	58.3	
95% CI	55.9, 62.8	55.7, 64.0	52.1, 64.2	
Moderate intake	196/788	141/534	55/254	
%	24.9	26.4	21.7	0.047
95% CI	22.0, 28.0	22.8, 30.3	17.0, 27.1	
High intake	124/788	73/534	51/254	
%	15.7	13.7	20.1	
95% CI	13.4, 18.4	11.0, 16.8	15.6, 25.4	
Low socioeconomic status	296/788	116/534	180/254	
%	37.6	21.7	70.9	<0.001
95% CI	34.3, 41.0	18.4, 25.4	65.0, 76.1	
Plasma folate (nmol/l)	787/788	533/534	254/254	
Geometric Mean	11.5	10.0	15.5	<0.001
95% CI	11.1, 11.9	9.5, 10.4	14.5, 16.5	
Red blood cell folate (nmol/l)	787/788	533/534	254/254	
Geometric Mean	812.3	747.5	967.4	<0.001
95% CI	790.9, 834.4	724.5, 771.2	926.0, 1073.2	

Plasma cobalamin (pmol/l)	786/788		533/534		253/254	
Geometric Mean		346.7		339.7	361.8	0.013
95 % CI		337.8, 355.7		329.4, 350.3	345.0, 379.4	
EGRAC	776/788		526/534		250/254	
Geometric Mean		1.35		1.39	1.29	<0.001
95% CI		1.34, 1.37		1.37, 1.41	1.26, 1.31	
tHcy (µmol/l)	788/788		534/534		254/254	
Geometric Mean		9.6		9.3	10.2	<0.001
95% CI		9.4, 9.8		9.1, 9.5	9.9, 11.0	
L, Arginine (µmol/l)	784/788		531/534		253/254	
Arithmetic Mean		65.8		66.6	64.3	0.118
95% CI		64.5, 67.2		64.9, 68.2	62.3, 66.4	
Plasma ADMA (µmol/l)	783/788		530/534		253/254	
Arithmetic Mean		0.53		0.52	0.57	<0.001
95% CI		0.52, 0.54		0.51, 0.52	0.56, 0.58	
Plasma SDMA (µmol/l)	783/788		530/534		253/254	
Arithmetic Mean		0.51		0.48	0.56	<0.001
95% CI		0.50, 0.52		0.47, 0.50	0.53, 0.58	
Plasma L, Arginine/ADMA Ratio	783/788		530/534		253/254	
Arithmetic Mean		125.0		130.2	114.1	<0.001
95% CI		122.3, 127.6		126.9, 133.5	110.1, 118.1	
Plasma ADMA/SDMA Ratio	782/788		529/534		253/254	
Arithmetic Mean		1.10		1.11	1.09	0.286
95% CI		1.09, 1.12		1.09, 1.14	1.05, 1.13	
Plasma creatinine (µmol/l)	784/788		531/534		253/254	
Arithmetic Mean		71.9		72.0	71.7	0.815
95% CI		70.8, 72.9		70.7, 73.2	69.8, 73.6	
Plasma total cholesterol (mmol/l)	786/788		533/534		253/254	
Arithmetic Mean		5.3		5.1	5.7	<0.001
95% CI		5.2, 5.4		5.0, 5.2	5.6, 5.9	
Plasma LDL cholesterol	774/788		525/534		249/254	
Arithmetic Mean		3.2		3.0	3.5	<0.001
95% CI		3.1, 3.2		2.9, 3.1	3.4, 3.7	
MTHFR 677 CC	278/778		185/529		93/249	0.693

%		35.7	35.0	37.3	
95% CI		32.4, 39.2	31.0, 39.1	31.6, 43.5	
MTHFR 677 CT	361/778		251/529	110/249	
%		46.4	47.4	44.2	
95 % CI		42.9, 49.9	43.2, 51.7	38.1, 50.4	
MTHFR 677 TT	139/778		93/529	46/249	
%		17.9	17.6	18.5	
95% CI		15.3, 20.7	14.6, 21.1	14.1, 23.8	
NOS 894 GG	273/753		182/514	91/239	
%		36.3	35.4	38.1	
95% CI		32.9, 39.8	31.4, 39.6	32.2, 44.4	
NOS 894 GT	349/753		243/514	106/239	
%		46.3	47.3	44.4	0.731
95% CI		42.8, 49.9	43.0, 51.6	38.2, 50.7	
NOS 894 TT	131/753		89/514	42/239	
%		17.4	17.3	17.6	
95% CI		14.9, 20.3	14.3, 20.8	13.3, 22.9	
Diagnosed hypertension					
%	111/656		24/466	87/190	<0.001
95% CI		16.9	5.2	45.8	
Diabetes		14.3, 20.0	3.5, 7.6	38.9, 52.9	
%	33/788		8/534	25/254	<0.001
95% CI		4.2	1.5	9.8	
Medication use		3.0, 5.8	0.8, 2.9	6.8, 14.1	
Group 1: none/sporadic	585/788		448/534	137/254	
%		74.2	83.9	53.9	
95% CI		71.1, 77.2	80.5, 86.8	47.8, 60.0	
Group 2: Medication not affecting L-Arginine pathway	104/788		60/534	44/254	<0.001
%		13.2	11.2	17.3	
95% CI		11.0, 15.7	8.8, 14.2	13.2, 22.5	
Group 3: Medication affecting L-Arginine pathway	99/788		26/534	73/254	
%		12.6	4.9	28.7	

95% CI	10.4, 15.1	3.3, 7.0	23.5, 34.6

BMI, body mass index; tHcy, total plasma homocysteine; ADMA, Asymmetric Dimethylarginine; SDMA, Symmetric Dimethylarginine; EGRAC, erythrocyte glutathione reductase activation coefficient; *MTHFR*, methylenetetrahydrofolate reductase; *NOS*, Nitric oxide synthase. *MTHFR* 677CT and *NOS* 894GT polymorphisms were in Hardy-Weinberg equilibrium. Comparison between age groups was by ANOVA for continuous variables and χ^2 for categorical variables. Twenty-four participants were excluded after the medical check-up due to declared B vitamin supplement use. A further 51 participants were excluded from all analyses involving tHcy because their blood samples were not processed within 2 h of collection and 5 participants because they had suspected altered renal function (plasma creatinine >97 mmol/l for women and >124 mmol/l for men). Two participants were also excluded for having outlier values of ADMA and SDMA. *Category of habitual alcohol intake: moderate (< 16 g/d in women and < 24 g/d in men) and high (≥ 16 g/d in women and ≥ 24 g/d in men).

Table 2: Association between L-Arginine pathway metabolites and diagnosed hypertension

		All participants				≤ 50 years old				> 50 years old			
	Model	n	R^2	OR	95%CI	n	R^2	OR	95%CI	n	R^2	OR	95%CI
L-Arginine	1	599	0.002	1.8	0.7, 1.9	430	0.003	1.4	0.6, 3.2	169	0.004	1.3	0.7, 2.5
	2		0.193***	1.3	0.8, 2.2		0.087**	1.4	0.6, 3.4		0.054*	1.4	0.7, 2.7
	3		0.428***	1.4	0.8, 2.4		0.308***	2.0	0.7, 5.2		0.308***	1.3	0.6, 2.8
ADMA	1	599	0.011	1.6	1.0, 2.5	430	0.003	0.7	0.3, 1.9	169	0.082**	3.0	1.5, 5.9
	2		0.196***	1.5	0.9, 2.4		0.089**	0.6	0.2, 1.6		0.132***	3.2	1.6, 6.3
	3		0.426***	0.9	0.5, 1.7		0.344***	0.2	0.1, 0.7		0.334***	2.3	1.1, 5.0
SDMA	1	598	0.004	1.3	0.8, 2.0	429	0.000	1.1	0.4, 2.6	169	0.017	1.6	0.9, 3.1
	2		0.195***	1.4	0.8, 2.2		0.085**	1.1	0.5, 2.8		0.061*	1.6	0.8, 3.0
	3		0.426***	1.0	0.6, 1.9		0.299***	0.8	0.3, 2.3		0.309***	1.3	0.6, 2.8
L-Arginine/ADMA Ratio	1	599	0.016*	1.7	1.1, 2.7	430	0.011	1.7	0.7, 4.1	169	0.058**	2.5	1.3, 5.0
	2		0.205***	1.9	1.1, 3.0		0.031**	1.6	0.7, 3.9		0.110**	2.7	1.4, 5.5
	3		0.434***	1.8	1.0, 3.1		0.303***	1.6	0.6, 4.2		0.333***	2.4	1.1, 5.3
ADMA/SDMA Ratio	1	598	0.000	1.0	0.6, 1.6	429	0.002	0.8	0.3, 2.0	169	0.000	1.0	0.5, 1.9
	2		0.191***	0.9	0.5, 1.5		0.088**	0.7	0.3, 1.8		0.048*	1.0	0.5, 2.0
	3		0.427***	0.8	0.4, 1.4		0.307***	0.5	0.2, 1.6		0.306***	1.0	0.4, 2.2

BMI, body mass index; ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine. Multiple logistic regression analysis was used. Sex and age specific tertiles for L-arginine, AMA and SDMA were derived. Cut-offs for low-mid L-Arginine tertiles were ≤73.3 μmol/l in women ≤50 years, ≤72.3 μmol/l in women >50, ≤ 75.6 μmol/l in men ≤50 years, ≤ 70.3 μmol/l in men >50. Cut-offs for mid-high ADMA tertiles were ≥0.485 μmol/l in women ≤50 years, ≥0.546 μmol/l in women >50, ≥0.489 μmol/l in men ≤50 years, ≥0.382 μmol/l in men >50. Cut-offs for mid-high SDMA tertiles were ≥0.404 μmol/l in women ≤50 years, ≥0.450 μmol/l in women >50, ≥0.442 μmol/l in men ≤50 years, ≥0.484 μmol/l in men >50. Cut-offs for low-mid L-Arginine/ADMA Ratio tertiles were ≤142.89 in women ≤50 years, ≤130.38 in women >50, ≤ 151.30 in men ≤50 years, ≤ 127.74 in men >50. Cut-offs for mid-high ADMA/SDMA Ratio tertiles were ≥1.03 in women ≤50 years, ≥1.03 in women >50, ≥0.95 in men ≤50 years, ≥0.89 in men >50. Participants without diagnosed hypertension but with once off point blood pressure measurements >140/90mmHg, at the study check-up, were excluded from the analysis.

Model 1: L-Arginine, L-Arginine/ADMA Ratio low-mid versus high tertile; ADMA, SDMA and ADMA/SDMA Ratio mid-high versus low tertile.

Model 2: included the same variables as model 1 as well as low versus mid-high socio-economic status.

Model 3: included the same variables as model 2 as well as BMI, category of regular alcohol intake (moderate (<16 g/d in women and <24 g/d in men) versus none; high versus none (≥16 g/d in women and ≥24 g/d in men)), smoking habits (cigarettes/day), total cholesterol, plasma creatinine and diabetes.

Nagelkerke R^2. * $P<0.05$; ** $P<0.01$; *** $P<0.001$

Table 3: Association between *NOS* G894T and diagnosed hypertension stratified by age group according to ADMA or SDMA tertiles.

		All participants				≤ 50 years old				> 50 years old			
	Model	n	R^2	OR	95%CI	n	R^2	OR	95%CI	n	R^2	OR	95%CI
Low ADMA tertile	1	205	0.005	1.4	0.5, 3.7	145	0.000	1.1	0.2, 6.2	60	0.006	1.4	0.4, 5.1
	2		0.360***	1.7	0.5, 5.2		0.001	1.1	0.2, 6.2		0.031	1.4	0.4, 5.1
	3		0.529***	1.5	0.4, 6.1		0.375	1.6	0.1, 18.8		0.485**	1.1	0.2, 6.5
Mid-High ADMA tertile	1	368	0.001	1.1	0.6, 1.9	270	0.028	0.4	0.2, 1.1	98	0.065*	2.6	1.1, 6.0
	2		0.435***	1.3	0.6, 2.5		0.055	0.4	0.2, 1.1		0.097*	3.0	1.2, 7.2
	3		0.524***	1.4	0.7, 3.0		0.396** *	0.5	0.2, 1.8		0.283** *	3.3	1.2, 9.4
Low SDMA tertile	1	187	0.001	1.2	0.5, 2.8	136	0.013	0.6	0.1, 2.3	51	0.029	1.9	0.6, 6.6
	2		0.360***	1.4	0.5, 3.8		0.141*	0.5	0.1, 2.0		0.099	2.6	0.7, 9.6
	3		0.609***	0.9	0.2, 3.2		0.491**	0.3	0.1, 3.1		0.498*	1.1	0.1, 8.1
Mid-High SDMA tertile	1	386	0.001	1.2	0.6, 2.1	279	0.013	0.5	0.2, 1.5	107	0.029	1.9	0.8, 4.4
	2		0.436***	1.3	0.6, 2.6		0.013	0.5	0.2, 1.5		0.043	2.0	0.8, 4.5
	3		0.561***	1.4	0.6, 3.0		0.515** *	0.7	0.2, 2.7		0.321**	1.9	0.7, 5.1

BMI, body mass index; NOS, Nitric oxide synthase; ADMA, Asymmetric dimethylarginine; SDMA, Symmetric dimethylarginine. Multiple logistic regression analysis was used. Cut-offs for mid-high ADMA tertiles were ≥0.485 μmol/l in women ≤50 years, ≥0.546 μmol/l in women >50, ≥0.489 μmol/l in men ≤50 years, ≥0.382 μmol/l in men >50. Cut-offs for mid-high SDMA tertiles were ≥0.404 μmol/l in women ≤50 years, ≥0.450 μmol/l in women >50, ≥0.442 μmol/l in men ≤50 years, ≥0.484 μmol/l in men >50. Participants without diagnosed hypertension but with point blood pressure measurements >140/90mmHg, at the study check-up, were referred for blood pressure monitoring and excluded from the analysis.

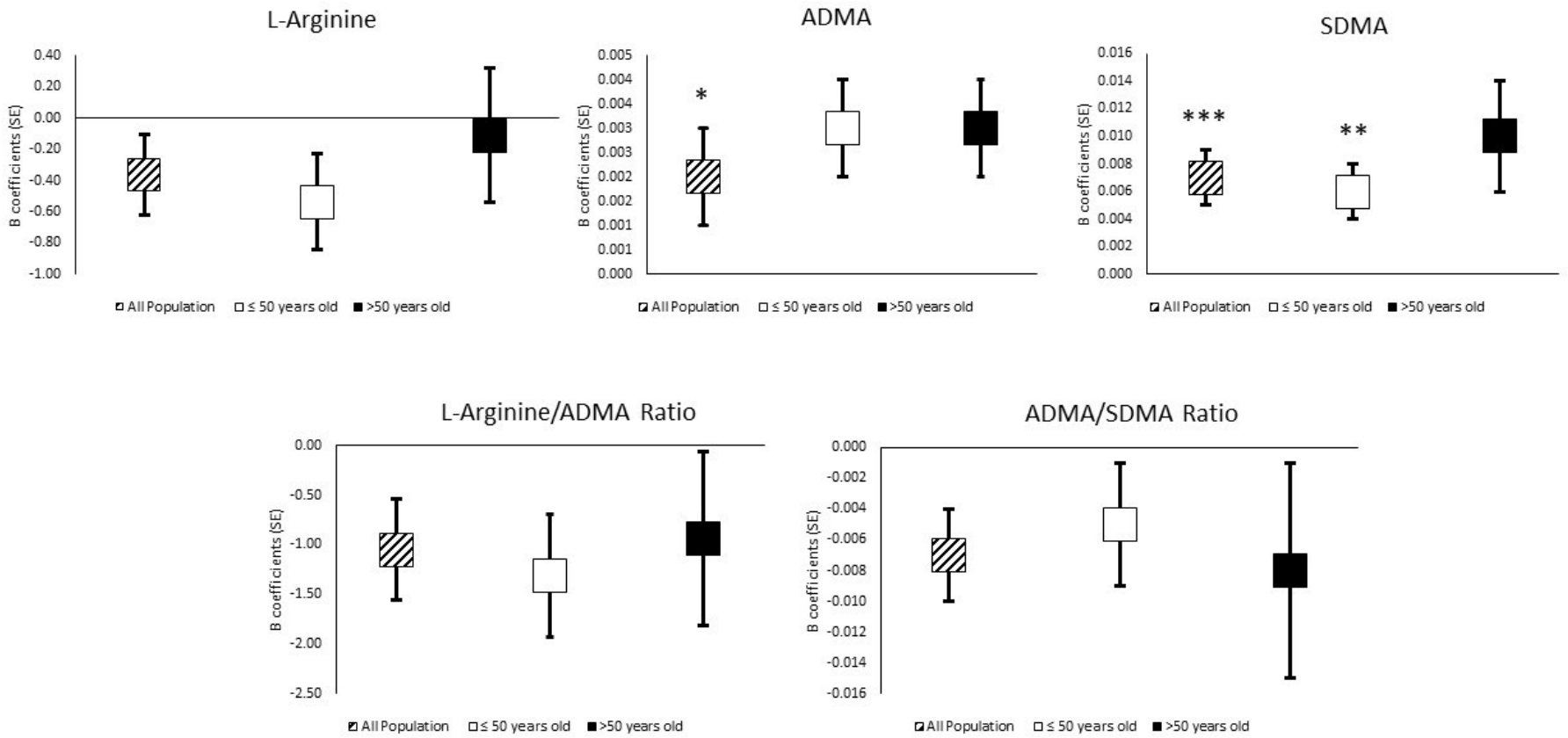
Model 1: *NOS* 894 GT+TT compared with *NOS* 894 GG

Model 2: included the same variables as model 1 as well as age (only in the models of the entire population) and sex.

Model 3: included the same variables as model 2 as well as low versus mid-high socio-economic status, smoking habits (cigarettes/day), category of regular alcohol intake (moderate (<16 g/d in women and <24 g/d in men) versus none; high versus none (≥16 g/d in women and ≥24 g/d in men)), BMI, total cholesterol, plasma creatinine and diabetes.

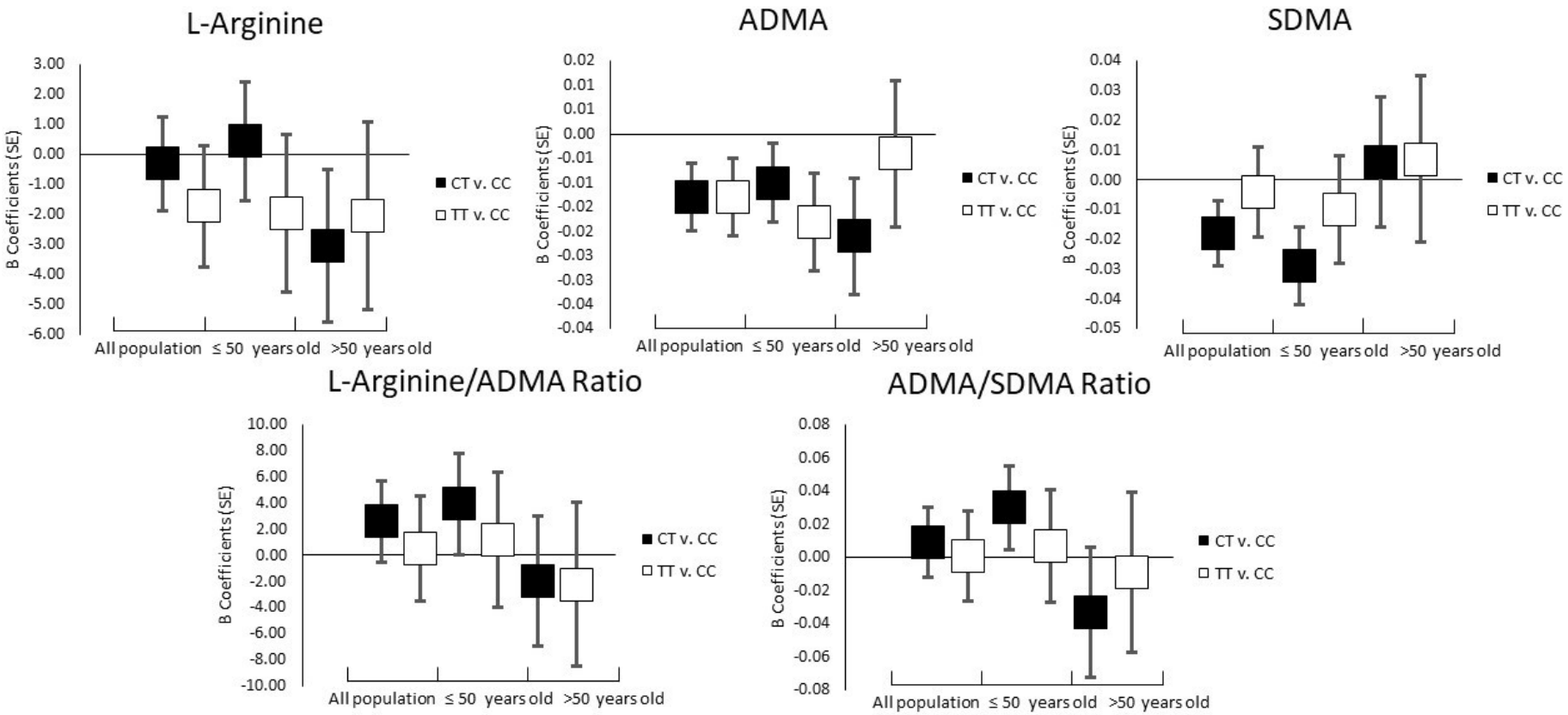
Nagelkerke R^2. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

2 Figure 1


L-Arginine
B coefficients (SE)
0.40
0.20
0.00
-0.20
-0.40
-0.60
-0.80
-1.00
All Population
≤ 50 years old
>50 years old
ADMA
B coefficients (SE)
0.005
0.004
0.004
0.003
0.003
0.002
0.002
0.001
0.001
0.000
*
All Population
≤ 50 years old
>50 years old
SDMA
B coefficients (SE)
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
***
**
All Population
≤ 50 years old
>50 years old
L-Arginine/ADMA Ratio
B coefficients (SE)
0.00
-0.50
-1.00
-1.50
-2.00
-2.50
All Population
≤ 50 years old
>50 years old
ADMA/SDMA Ratio
B coefficients (SE)
0.000
-0.002
-0.004
-0.006
-0.008
-0.010
-0.012
-0.014
-0.016
All Population
≤ 50 years old
>50 years old

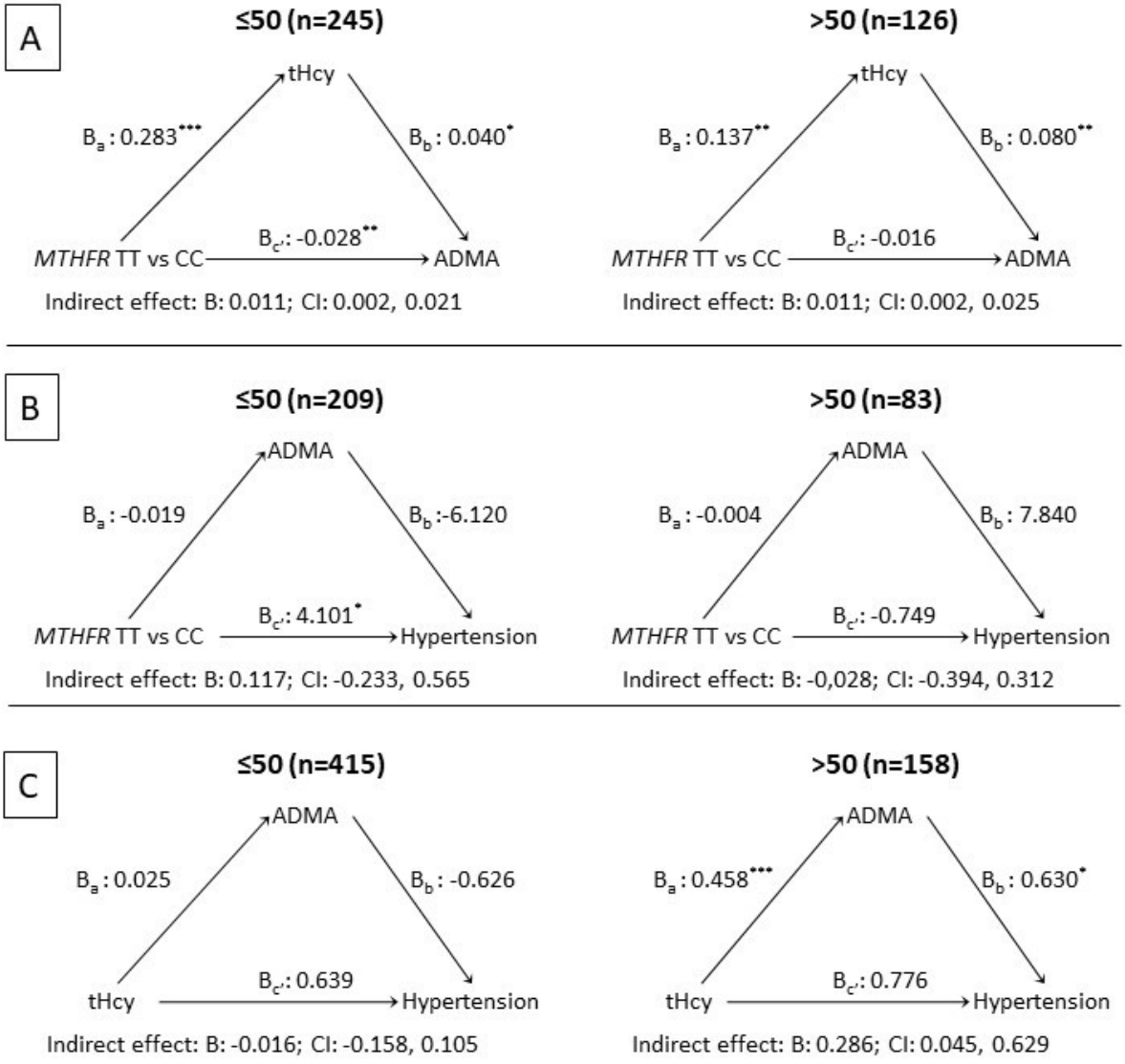
3

4 Figure 2


L-Arginine
B Coefficients (SE)
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
-6.00
All population
≤ 50 years old
>50 years old
CT v. CC
TT v. CC
ADMA
B Coefficients (SE)
0.02
0.01
0.01
0.00
-0.01
-0.01
-0.02
-0.02
-0.03
-0.03
-0.04
-0.04
All population
≤ 50 years old
>50 years old
CT v. CC
TT v. CC
SDMA
B Coefficients (SE)
0.04
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
-0.04
-0.05
All population
≤ 50 years old
>50 years old
CT v. CC
TT v. CC
L-Arginine/ADMA Ratio
B Coefficients (SE)
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
-4.00
-6.00
-8.00
-10.00
All population
≤ 50 years old
>50 years old
CT v. CC
TT v. CC
ADMA/SDMA Ratio
B Coefficients (SE)
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.08
All population
≤ 50 years old
>50 years old
CT v. CC
TT v. CC

5

6 Figure 3


A
≤50 (n=245)
tHcy
Ba : 0.283***
Bb : 0.040*
MTHFR TT vs CC
Bc': -0.028**
ADMA
Indirect effect: B: 0.011; CI: 0.002, 0.021
>50 (n=126)
tHcy
Ba : 0.137**
Bb : 0.080**
MTHFR TT vs CC
Bc': -0.016
ADMA
Indirect effect: B: 0.011; CI: 0.002, 0.025
B
≤50 (n=209)
ADMA
Ba : -0.019
Bb :-6.120
MTHFR TT vs CC
Bc': 4.101*
Hypertension
Indirect effect: B: 0.117; CI: -0.233, 0.565
>50 (n=83)
ADMA
Ba : -0.004
Bb : 7.840
MTHFR TT vs CC
Bc': -0.749
Hypertension
Indirect effect: B: -0,028; CI: -0.394, 0.312
C
≤50 (n=415)
ADMA
Ba : 0.025
Bb : -0.626
tHcy
Bc': 0.639
Hypertension
Indirect effect: B: -0.016; CI: -0.158, 0.105
>50 (n=158)
ADMA
Ba : 0.458***
Bb : 0.630*
tHcy
Bc': 0.776
Hypertension
Indirect effect: B: 0.286; CI: 0.045, 0.629

Supplementary Table

Supplementary table: Participant medication use (code, active pharmaceutical ingredient and category)

Participants	Medication code (CI-9)	Active ingredient	Treatment for	Medication category
1	A02	-	Antacid	No medication use/Sporadic medication
2	A02	Aluminium hydroxide	Antacid	No medication use/Sporadic medication
3	A02	Aluminium hydroxide	Antacid	No medication use/Sporadic medication
4	A02	Aluminium hydroxide	Antacid	No medication use/Sporadic medication
5	A02	Aluminium hydroxide	Antacid	No medication use/Sporadic medication
6	A02 + M01 + N02B	-	Antacid + Pain, inflammation & fever	No medication use/Sporadic medication
7	A02A	-	Antacid	No medication use/Sporadic medication
8	A02B	-	Antacid	No medication use/Sporadic medication
9	A02B	-	Antacid	No medication use/Sporadic medication
10	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
11	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
12	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
13	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
14	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
15	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
16	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
17	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
18	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
19	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
20	M01	-	Pain, inflammation and fever	No medication use/Sporadic medication
21	M01	Clonixin	Pain, inflammation and fever	No medication use/Sporadic medication
22	M01	Ibuprofen	Pain, inflammation and fever	No medication use/Sporadic medication
23	M01A	Naproxen	Pain, inflammation and fever	No medication use/Sporadic medication
24	N02	-	Pain, inflammation and fever	No medication use/Sporadic medication
25	N02	-	Pain, inflammation and fever	No medication use/Sporadic medication

26	N02	Acetaminophen	Pain, inflammation and fever	No medication use/Sporadic medication
27	N02 + N02B	-	Pain, inflammation and fever	No medication use/Sporadic medication
28	N02B	-	Pain, inflammation and fever	No medication use/Sporadic medication
29	N02B	Metamizole	Pain, inflammation and fever	No medication use/Sporadic medication
30	N02B	Metamizole	Pain, inflammation and fever	No medication use/Sporadic medication
31	N02B	Acetaminophen	Pain, inflammation and fever	No medication use/Sporadic medication
32	N02B	Acetaminophen	Pain, inflammation and fever	No medication use/Sporadic medication
33	N02B	Acetaminophen	Pain, inflammation and fever	No medication use/Sporadic medication
34	N02B	Acetaminophen	Pain, inflammation and fever	No medication use/Sporadic medication
35	N02B	Acetaminophen	Pain, inflammation and fever	No medication use/Sporadic medication
36	N02B	Tramadol	Pain, inflammation and fever	No medication use/Sporadic medication
37	N02B	Acetaminophen	Pain, inflammation and fever	No medication use/Sporadic medication
38	R03	Fluticasone	Nasal congestion	No medication use/Sporadic medication
39	R06	-	Allergies	No medication use/Sporadic medication
40	R06	-	Allergies	No medication use/Sporadic medication
41	R06 + A03F	Cinitapride	Allergies + Reflux	No medication use/Sporadic medication
42	R06 + A07E	Budesonide	Allergies	No medication use/Sporadic medication
43	R06A	Ebastine	Allergies	No medication use/Sporadic medication
44	R06A	Loratadine	Allergies	No medication use/Sporadic medication
45	R06A	Mizolastine	Allergies	No medication use/Sporadic medication
46	A02 + A02B	Aluminium hydroxide + Omeprazole	Antiacid	Medication not affecting L-Arginine pathway
47	A02 + N06A	Aluminium hydroxide + Sertraline	Antacid + Anxiety	Medication not affecting L-Arginine pathway
48	A02A	Ranitidine	Ulcer	Medication not affecting L-Arginine pathway
49	A02B	Omeprazole	Antacid	Medication not affecting L-Arginine pathway
50	A02B	Omeprazole	Antacid	Medication not affecting L-Arginine pathway
51	A02B	Omeprazole	Antacid	Medication not affecting L-Arginine pathway
52	A02B	Omeprazole	Antacid	Medication not affecting L-Arginine pathway
53	A02B	Omeprazole	Antacid	Medication not affecting L-Arginine pathway

54	A02B + C07	Omeprazole + Atenolol	Antacid + Hypertension	Medication not affecting L-Arginine pathway
55	A10B	Gliclazide	Diabetes	Medication not affecting L-Arginine pathway
56	A10B + A10A	Glyburide + Insulin human	Diabetes	Medication not affecting L-Arginine pathway
57	A10B + H04	Glyburide + Insulin human	Diabetes	Medication not affecting L-Arginine pathway
58	A12A	Ossein-hydroxyapatite + Raloxifene + Fluoxetine	Osteoposthorosis	Medication not affecting L-Arginine pathway
59	A12A + A02B	Ossein-hydroxyapatite + Famotidine	Osteoposthorosis + Ulcer	Medication not affecting L-Arginine pathway
60	B01A	Acenocoumarol	Stroke	Medication not affecting L-Arginine pathway
61	B04	Gemfibrozil	Hypertriglyceridemia	Medication not affecting L-Arginine pathway
62	B04	Gemfibrozil	Hypertriglyceridemia	Medication not affecting L-Arginine pathway
63	B04A	Lovastatin	Hypercholesterolemia	Medication not affecting L-Arginine pathway
64	C02	Nifedipine	Hypertension	Medication not affecting L-Arginine pathway
65	C07	Atenolol	Hypertension	Medication not affecting L-Arginine pathway
66	C07A	Bisoprolol	Hypertension	Medication not affecting L-Arginine pathway
67	C10A	Fenofibrate	Dyslipemia	Medication not affecting L-Arginine pathway
68	C10A	Bezafibrate	Hypercholesterolemia	Medication not affecting L-Arginine pathway
69	C10A	Simvastatin	Hypercholesterolemia	Medication not affecting L-Arginine pathway
70	C10A + C07	Lovastatin + Atenolol	Hypercholesterolemia + Hypertension	Medication not affecting L-Arginine pathway
71	G03	-	Contraceptive	Medication not affecting L-Arginine pathway
72	G03 + A03	Ethinylestradiol + Otilonium	Contraceptive + Spasms	Medication not affecting L-Arginine pathway
73	G03G	Raloxifene	Osteoposthorosis	Medication not affecting L-Arginine pathway
74	H03B	Carbimazole	Thyroid disorder	Medication not affecting L-Arginine pathway
75	H03B	Methimazole	Thyroid disorder	Medication not affecting L-Arginine pathway

76	H04	Insulin human	Diabetes	Medication not affecting L-Arginine pathway
77	H04	Insulin human	Diabetes	Medication not affecting L-Arginine pathway
78	H04	Insulin human	Diabetes	Medication not affecting L-Arginine pathway
79	H04 + G04C	Insulin human + Tamsulosin	Diabetes + Benign hyperplasia	Medication not affecting L-Arginine pathway
80	L01G	Tamoxifen	Cancer	Medication not affecting L-Arginine pathway
81	M01 + A02B	Omeprazole	Pain, inflammation and fever + Ulcer	Medication not affecting L-Arginine pathway
82	M01 + B04	Pravastatin	Pain, inflammation and fever + Hypercholesterolemia	Medication not affecting L-Arginine pathway
83	M01 + N05B + N06A	Alprazolam + Sertraline	Pain, inflammation and fever + Anxiety + Depression	Medication not affecting L-Arginine pathway
84	M01 + N06	-	Pain, inflammation and fever + Depression	Medication not affecting L-Arginine pathway
85	M03	Tetrazepam	Rehabilitation or functional re-education	Medication not affecting L-Arginine pathway
86	M03	Tetrazepam	Rehabilitation or functional re-education	Medication not affecting L-Arginine pathway
87	M03 + N06A	Tetrazepam + Paroxetine	Rehabilitation or functional re-education + Depression	Medication not affecting L-Arginine pathway
88	M03 + N06A + H04	Tetrazepam + Celecoxib + Venlafaxine + Calcitonin	Pain, inflammation and fever + Depression + Osteoposthorosis	Medication not affecting L-Arginine pathway
89	M05	Alendronic acid	Osteoposthorosis	Medication not affecting L-Arginine pathway
90	M05	Alendronic acid	Osteoposthorosis	Medication not affecting L-Arginine pathway
91	M05 + G02C + C02	Alendronic acid+ Raloxifene + Bisoprolol	Osteoposthorosis + Hypertension	Medication not affecting L-Arginine pathway
92	N02 + G03H	Flutamide	Pain, inflammation and fever + Cancer	Medication not affecting L-Arginine pathway
93	N02B + C02E	Bisoprolol	Pain, inflammation and fever + Hypertension	Medication not affecting L-Arginine pathway

94	N02B + G03C	Acetaminophen + Tibolone	Pain, inflammation and fever + Menopause	Medication not affecting L-Arginine pathway
95	N02B + M01A	Acetaminophen + Diclofenac	Pain, inflammation and fever + Arthrosis	Medication not affecting L-Arginine pathway
96	N02B + N05B + M05	Bromazepam + Calcitonin	Pain, inflammation and fever + Depression + Osteoposthorosis	Medication not affecting L-Arginine pathway
97	N02B + N06	Metamizole + Reboxetine	Pain, inflammation and fever + Depression	Medication not affecting L-Arginine pathway
98	N02B + R03A	Acetaminophen + Salbutamol	Pain, inflammation and fever + Asthma	Medication not affecting L-Arginine pathway
99	N02C	Sumatriptan	Migraine	Medication not affecting L-Arginine pathway
100	N02C	Sumatriptan	Migraine	Medication not affecting L-Arginine pathway
101	N02C	Sumatriptan	Migraine	Medication not affecting L-Arginine pathway
102	N02C	Sumatriptan	Migraine	Medication not affecting L-Arginine pathway
103	N02C	Dihydroergotamine	Migraines and vertigo	Medication not affecting L-Arginine pathway
104	N02C	Dihydroergotamine	Migraines and vertigo	Medication not affecting L-Arginine pathway
105	N03	Phenytoin	Epilepsy	Medication not affecting L-Arginine pathway
106	N03A	Carbamazepine	epilepsies	Medication not affecting L-Arginine pathway
107	N05B	Alprazolam	Anxiety	Medication not affecting L-Arginine pathway
108	N05B	Alprazolam	Anxiety	Medication not affecting L-Arginine pathway
109	N05B	Alprazolam	Anxiety	Medication not affecting L-Arginine pathway
110	N05B	Alprazolam	Anxiety	Medication not affecting L-Arginine pathway
111	N05B	Alprazolam	Anxiety	Medication not affecting L-Arginine pathway
112	N05B	Lorazepam	Anxiety	Medication not affecting L-Arginine pathway
113	N05B	Bromazepam	Depression	Medication not affecting L-Arginine pathway
114	N05B	Citalopram	Depression	Medication not affecting L-Arginine pathway
115	N05B	Diazepam	Depression	Medication not affecting L-Arginine pathway
116	N05B	Diazepam	Depression	Medication not affecting L-Arginine pathway
117	N05B	Lormetazepam	Insomnia	Medication not affecting L-Arginine pathway
118	N05B + A10B	Alprazolam + Miglitol	Anxiety + Diabetes	Medication not affecting L-Arginine pathway
119	N05B + C07	Diazepam + Atenolol	Anxiety + Hypertension	Medication not affecting L-Arginine pathway

120	N05B + C10A	Alprazolam + Lansoprazole	Anxiety + Ulcer	Medication not affecting L-Arginine pathway
121	N05B + G03A	Hydroxyzine + Ethinylestradiol + Gestodene	Anxiety + Contraceptive	Medication not affecting L-Arginine pathway
122	N05B + M03 + C07	Hydroxyzine + Tetrazepam + Atenolol	Anxiety + Rehabilitation or functional re-education + Hypertension	Medication not affecting L-Arginine pathway
123	N05B + N02C	Alprazolam + Dihydroergotamine	Anxiety + Migraine	Medication not affecting L-Arginine pathway
124	N05B + N02C	Diazepam + Sumatriptan	Depression + Migraine	Medication not affecting L-Arginine pathway
125	N05B + N07 + S01	Alprazolam + Insulin human	Anxiety + Diabetes	Medication not affecting L-Arginine pathway
126	N05C + C10A	Clomethiazole + Pravastatin	Anxiety + Hypercholesterolemia	Medication not affecting L-Arginine pathway
127	N06	-	Depression	Medication not affecting L-Arginine pathway
128	N06A	Paroxetine	Anxiety	Medication not affecting L-Arginine pathway
129	N06A	Paroxetine	Depression	Medication not affecting L-Arginine pathway
130	N06A	Paroxetine	Depression	Medication not affecting L-Arginine pathway
131	N06A	Paroxetine	Depression	Medication not affecting L-Arginine pathway
132	N06A	Sertraline	Depression	Medication not affecting L-Arginine pathway
133	N06A	Venlafaxine	Depression	Medication not affecting L-Arginine pathway
134	N06A + B04	Paroxetine + Simvastatin	Depression + Hypercholesterolemia	Medication not affecting L-Arginine pathway
135	N06A + N02C	Venlafaxine + Dihydroergotamine	Depression + Migraine	Medication not affecting L-Arginine pathway
136	N07C	Flunarizine	Migraines and vertigo	Medication not affecting L-Arginine pathway
137	R03A	Salbutamol	Asthma	Medication not affecting L-Arginine pathway
138	R03A	Salbutamol	Asthma	Medication not affecting L-Arginine pathway
139	R03A	Salbutamol	Asthma	Medication not affecting L-Arginine pathway
140	R03A	Salbutamol	Asthma	Medication not affecting L-Arginine pathway

141	R03A	Salbutamol	Asthma	Medication not affecting L-Arginine pathway
142	R03A	Salbutamol	Asthma	Medication not affecting L-Arginine pathway
143	R03A	Salbutamol	Asthma	Medication not affecting L-Arginine pathway
144	R03A	Terbutaline	Asthma	Medication not affecting L-Arginine pathway
145	R03A	-	Bronchi	Medication not affecting L-Arginine pathway
146	R03A	-	Bronchi	Medication not affecting L-Arginine pathway
147	R03A + H04	Terbutaline + Insulin human	Asthma + Diabetes	Medication not affecting L-Arginine pathway
148	R03B	-	Asthma	Medication not affecting L-Arginine pathway
149	R06 + R03A	-	Allergies + Bronchi	Medication not affecting L-Arginine pathway
150	A02 + C02 + H03A	Omeprazole + Quinapril + Levothyroxine	Ulcer + Hypertension + Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
151	A02 + N02 + N06A	Aluminium hydroxide + Acetaminophen + Fluoxetine	Antiacid + Pain, inflammation and fever + Bulimia	Hypertensive medication/ Medication affecting L-Arginine pathway
152	A02A + C03	Ranitidine + Chlorthalidone	Ulcer + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
153	A02A + M05	Glucosamine	Vomiting + Arthrosis	Hypertensive medication/ Medication affecting L-Arginine pathway
154	A02B + C02 + M04	Omeprazole + Indapamide + Allopurinol	Antiacid + Hypertension + Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
155	A10 + C01D + C02	Metformin + Repaglinide + Acetylsalicylic acid + Telmisartan	Diabetes + Atherosclerosis + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
156	A10A + C02B4	Insulin human + Irbesartan	Diabetes + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
157	A10B	Glimepiride	Diabetes	Hypertensive medication/ Medication affecting L-Arginine pathway
158	A10B	Glimepiride	Diabetes	Hypertensive medication/ Medication affecting L-Arginine pathway

159	A10B + B04A	Acarbose + Glimepiride + Atorvastatin	Diabetes + Hypercholesterolemia	Hypertensive medication/ Medication affecting L-Arginine pathway
160	A10B + C02	-	Diabetes + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
161	A10B + C02	Gliclazide + Candesartan	Diabetes + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
162	A10B + C02	Acarbose + Indapamide	Diabetes + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
163	A10B + C02 + R03A	Gliclazide + Enalapril	Diabetes + Hypertension + Bronchi	Hypertensive medication/ Medication affecting L-Arginine pathway
164	A10B + C05A	Gliclazide + Nicardipine	Diabetes + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
165	A10B + C09C	Glimepiride + Losartan	Diabetes + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
166	A10B + C09C	Losartan	Diabetes + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
167	A10B + S01	Glyburide + Acarbose + Metformin + Latanoprost	Diabetes + Ocular hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
168	A12A + G02C +N06A	Ossein-hydroxyapatite + Raloxifene + Fluoxetine	Osteoposthorosis + Bulimia	Hypertensive medication/ Medication affecting L-Arginine pathway
169	B04 + C02	Gemfibrozil+ Indapamide	Hypertriglyceridemia + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
170	B04 + C02B4	Atorvastatin + Enalapril	Hypercholesterolemia + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
171	B04H	Atorvastatin	Hypercholesterolemia	Hypertensive medication/ Medication affecting L-Arginine pathway
172	B04H	Atorvastatin	Hypercholesterolemia	Hypertensive medication/ Medication affecting L-Arginine pathway
173	C02	Amlodipine	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway

174	C02	Amlodipine	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
175	C02	Amlodipine	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
176	C02	Enalapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
177	C02	Indapamide	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
178	C02	Indapamide	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
179	C02	Lacidipine	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
180	C02	Quinapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
181	C02	Quinapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
182	C02	Quinapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
183	C02 + C02B4	Doxazosin + Enalapril + Indapamide	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
184	C02 + C02B4	Indapamide + Irbesartan	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
185	C02B + C02B4	Diltiazem + Irbesartan	Hypertension + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
186	C02B4	Captopril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
187	C02B4	Captopril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
188	C02B4	Enalapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
189	C02B4	Enalapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
190	C02B4	Enalapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway

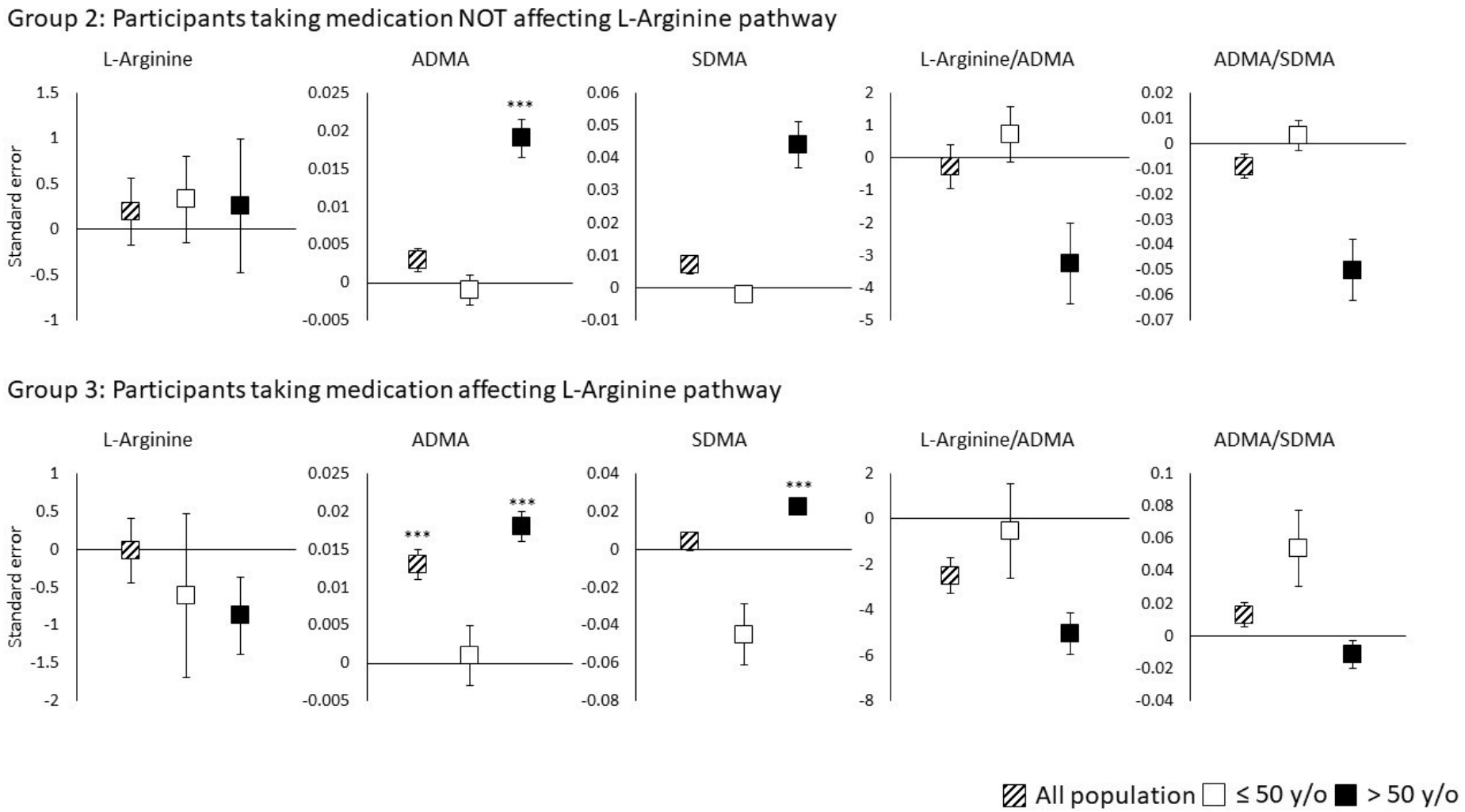
191	C02B4	Irbesartan	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
192	C02B4 + M04	Captopril + Allopurinol	Hypertension + Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
193	C02B4 + M04A	Enalapril + Allopurinol	Hypertension + Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
194	C03A	Midamor	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
195	C03A	Midamor	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
196	C03A	Midamor	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
197	C03A	Enalapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
198	C03A	Enalapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
199	C03A	Enalapril	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
200	C03A	Valsartan	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
201	C03A + M04A	Enalapril + Allopurinol	Hypertension + Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
202	C03B	Furosemide	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
203	C03B + H03	Furosemide + Levothyroxine	Hypertension + Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
204	C04A + B04A + C02	Hidrosmin + Atorvastatin + Lisinopril	Edema + Hypercholesterolemia + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
205	C05C	Ruscogenin	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
206	C05C + C02	Troxerutin + Doxazosin	Atherosclerosis + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
207	C07A	Propranolol	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway

208	C09A + M04A	Lisinopril + Allopurinol	Hypertension + Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
209	C09C	Losartan	Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
210	C10A + C07A + C09A	Pravastatin + Sotalol + Lisinopril	Hypercholesterolemia + Tachyarrhythmias + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
211	G04 + B04A	Alfuzosin + Atorvastatin	Prostate + Hypercholesterolemia	Hypertensive medication/ Medication affecting L-Arginine pathway
212	H02A	Deflazacort	Arthrosis	Hypertensive medication/ Medication affecting L-Arginine pathway
213	H03	Levothyroxine	Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
214	H03A	Levothyroxine	Atherosclerosis	Hypertensive medication/ Medication affecting L-Arginine pathway
215	H03A	Levothyroxine	Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
216	H03A	Levothyroxine	Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
217	H03A	Levothyroxine	Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
218	H03A	Levothyroxine	Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
219	H04 + B04 + C02	Insulin human + Gemfibrozil + Quinapril	Diabetes + Hypertriglyceridemia + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
220	J04A + M04	Rifampicin + Allopurinol	Tuberculosis + Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
221	M04	Allopurinol	Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
222	M04A	Allopurinol	Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
223	M05	Glucosamine	Arthrosis	Hypertensive medication/ Medication affecting L-Arginine pathway

224	M05 + B04A + C03A	Elcatonin + Atorvastatin + Midamor	Osteoposthorosis + Hypercholesterolemia + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
225	M05 + C05A	Glucosamine + Escin	Arthrosis + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
226	N02	Troxerutin	Atherosclerosis	Hypertensive medication/ Medication affecting L-Arginine pathway
227	N02 + C02 + H03	Enalapril + Levothyroxine	Pain, inflammation and fever + Hypertension + Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
228	N02 + C05A	-	Pain, inflammation and fever + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
229	N02B + C02	Acetaminophen + Indapamide	Pain, inflammation and fever + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
230	N02B + C02 + M04	Doxazosin + Allopurinol	Pain, inflammation and fever + Hypertension + Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
231	N02B + C02B + M04Z	Acetaminophen + Amlodipine + Allopurinol	Pain, inflammation and fever + Hypertension + Idiopathic gout or lithiasis	Hypertensive medication/ Medication affecting L-Arginine pathway
232	N02B + C02B4	Acetaminophen + Cilazapril + Hydrochlorothiazide	Pain, inflammation and fever + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
233	N02B + D05 + C07A	Acetaminophen + Tacalcitol + Propranolol	Pain, inflammation and fever + Psoriasis + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
234	N02B + M05	Acetaminophen + Glucosamine	Pain, inflammation and fever + Arthrosis	Hypertensive medication/ Medication affecting L-Arginine pathway
235	N02B + N05B + C02B4	Acetaminophen + Diazepam + Valsartan	Pain, inflammation and fever + Depression + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
236	N02B + N05B + H03A	Lorazepam + Levothyroxine	Pain, inflammation and fever + Anxiety + Thyroid disorder	Hypertensive medication/ Medication affecting L-Arginine pathway
237	N03A + B01B	Phenytoin + Ticlopidine	Epilepsy + Atherosclerosis	Hypertensive medication/ Medication affecting L-Arginine pathway

238	N05	Amitriptyline	Depression	Hypertensive medication/ Medication affecting L-Arginine pathway
239	N05 + C02	Loprazolam + Quinapril	Insomnia + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
240	N05B + A16 + C03B	Alprazolam + Atorvastatin + Furosemide	Anxiety + Hypercholesterolemia + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
241	N05B + C02 + C07	Bromazepam + Indapamide + Timolol	Anxiety + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
242	N05B + N06A	Zolpidem + Fluoxetine	Insomnia + Bulimia	Hypertensive medication/ Medication affecting L-Arginine pathway
243	N06A	Amitriptyline	Depression	Hypertensive medication/ Medication affecting L-Arginine pathway
244	N06A	Fluoxetine	Bulimia	Hypertensive medication/ Medication affecting L-Arginine pathway
245	N06A	Fluoxetine	Bulimia	Hypertensive medication/ Medication affecting L-Arginine pathway
246	R03A + C02	Formoterol + Budesonide +Theophylline	Asthma + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway
247	R05D + A02 + B01B	Dextromethorphan + Pantoprazole + Clopidogrel	Coughing + Esophageal problems + Heart attack	Hypertensive medication/ Medication affecting L-Arginine pathway
248	R06 + M05 + C02	Cetirizine + Glucosamine + Amlodipine	Allergies + Arthrosis + Hypertension	Hypertensive medication/ Medication affecting L-Arginine pathway

12 Supplementary Figure



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Carla Ramos Rodríguez

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