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The L-Arginine pathway may act as a mediator in the association between impaired one-carbon metabolism and hypertension

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ABSTRACT

Elevated fasting plasma total homocysteine (tHcy) and the methylenetetrahydrofolate reductase C677T polymorphism (rs1801133) have been associated with hypertension. Whether the L-Arginine pathway is involved, is unclear. We aimed to investigate whether the association between tHcy, the rs1801133 polymorphism and hypertension involves the L-Arginine pathway. THcy, plasma folate and cobalamin, erythrocyte glutathionine reductase activation coefficient, rs1801133 genotype, plasma L-Arginine, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) were determined in a cross-sectional study of 788 adults (aged 18 to 75), randomly selected from 2 town population registers. Participants participated in a medical checkup and provided a fasting blood sample. Associations between tHcy, rs1801133 genotype and L-Arginine pathway metabolites were assessed by multiple linear regression analysis and whether the tHcy and rs1801133 genotype are associated with hypertension via the L-Arginine pathway was investigated using mediation analysis. tHcy was positively associated with ADMA (B = 0.003; SE = 0.001; $P < 0.001$) and SDMA (B = 0.007; SE = 0.002; $P < 0.001$) and negatively associated with the L-Arginine/ADMA (B = −1.140; SE = 0.451; $P < 0.05$) and ADMA/SDMA (B = −0.006; SE = 0.003; $P < 0.05$) ratios. The *MTHFR* 677 CT vs CC genotype was negatively associated with ADMA (B = −0.013; SE = 0.007; $P < 0.05$) and with SDMA (B = −0.029; SE = 0.013; $P < 0.05$) in participants under 50 years. Each standard deviation increase (37.6) in the L-Arginine/ADMA ratio was associated with reduced hypertension risk (OR [95%CI], 0.6 [0.5, 0.8]). Mediation analysis showed that tHcy and ADMA were mediators in the association between the rs1801133 TT vs CC genotypes and hypertension. Our results support the L-Arginine pathway as a mediator in the association of impaired One-Carbon metabolism and hypertension.

Abbreviations: 5-MTHF, 5-methyl-tetrahydrofolate; ADMA, Asymmetric dimethylarginine; ANOVA, Analysis of variance; BMI, Body mass index; CI, Confidence interval; DDAH, Dimethylarginine dimethylaminohydrolase; EGRAC, Erythrocyte glutathionine reductase activation coefficient; HPLC-MS/MS, Liquid chromatography–mass spectrometry; LDL, Low-density lipoprotein; MTHFR, Methylenetetrahydrofolate reductase; NO, Nitric oxide; OR, Odd Ratio; RBCF, Red blood cell folate; SD, Standard deviation; SDMA, Symmetric dimethylarginine; tHcy, Fasting plasma total homocysteine; WHO, World Health Organization; y, Years old.

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1. Introduction

Endothelial dysfunction occurs in the early stage of the pathogenesis of atherosclerosis and hypertension [1].

Alterations in one-carbon metabolism, involving elevated tHcy or the homozygote variant genotype for the methylenetetrahydrofolate reductase (*MTHFR*) 677C > T polymorphism (rs1801133) [2], have been associated with hypertension [3], but the underlying pathophysiological mechanism is unclear. It has been proposed that elevated tHcy is associated with endothelial dysfunction [4]. The *MTHFR* 677C > T polymorphism causes partial dissociation of its flavine adenine dinucleotide cofactor rendering the enzyme thermolabile [5] and the TT genotype increases susceptibility to elevated tHcy [6] and to hypertension [7,8]. We previously reported, from a study of a representative sample of an adult population recruited from population records, that the *MTHFR* 677 TT genotype was associated with increased risk of hypertension only in participants under 50 years of age [9]. In those over 50, elevated tHcy, but not the *MTHFR* 677 TT genotype, was associated with increased hypertension risk.

Homocysteine participates directly in the methionine cycle and the *MTHFR* 677C > T polymorphism affects homocysteine remethylation to methionine. This cycle intersects with the L-Arginine pathway when *S*-Adenosylmethionine donates two methyl groups to reactions involving Protein-Arginine *N*-Methyltransferase 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 into the cytosol to regulate nitric oxide (NO) production [10,11]. ADMA can inhibit endothelial NO synthase, which catalyses the conversion of L-Arginine to NO [12] and SDMA may cause L-Arginine depletion by competing for the L-Arginine transporter [13]. Elevated ADMA has been recognized as a risk factor for cardiovascular diseases [14] and has been associated with hypertension [15], stroke [16] and myocardial infarction [17]. NO prevents platelet aggregation and smooth muscle cell proliferation, so is essential for regulating vascular tone, vasodilation and blood pressure. Reduced NO bioavailability can lead to endothelial dysfunction [18].

In randomized control trials, supplementation with folic acid or 5-methyltetrahydrofolate (5-MTHF) has been associated with increased NO bioavailability and vasodilation in dyslipidemic conditions [19,20]. However, endothelium dependent vasodilation did not improve following 8 weeks of supplementation with B vitamins, despite tHcy reduction, in hyperhomocysteinemic peripheral arterial occlusion patients [21]. In contrast, L-Arginine supplementation did not reduce tHcy but was associated with improved endothelium-dependent vasodilation and reduced oxidative stress [21]. In premature cardiovascular disease patients with the *MTHFR* 677 TT genotype, on antihypertensive medication, riboflavin supplementation reduced tHcy and both systolic and diastolic blood pressure [7,22]. The same intervention regime in medicated hypertensive individuals with the *MTHFR* 677 TT genotype led to a reduction in systolic blood pressure [8]. The mechanism linking tHcy and the *MTHFR* 677C > T polymorphism to elevated blood pressure remains to be determined.

Therefore, while one-carbon metabolism and the L-Arginine pathway are closely related, whether they interact in the development of hypertension is unclear. Studies to date may be influenced by participant recruitment (e.g., convenience work setting or clinical based recruitment), differing stages of disease progression and co-morbidities as well as underlying medication in patients, vitamin supplement use or even inappropriate sample processing leading to artifacts in tHcy measurements [23].

We hypothesized that impaired one-carbon metabolism is associated with hypertension via the L-Arginine pathway. Elevated homocysteine or the *MTHFR* 677 TT genotype may lead to increased ADMA and SDMA concentrations and subsequently increased hypertension risk. The aim was to explore these associations, in a representative sample of an adult population, unexposed to mandatory fortification with folic acid or B vitamin supplement use and controlling for underlying medication.

2. Materials and methods

2.1. Subjects and procedure

The cross-sectional study of adults aged 18–75 years, is representative of 2 towns from Tarragona province, Southern Catalonia, Spain. The sample was randomly selected from the population registers of these towns and has been described previously [6,9,24]. Out of 812 adults that were recruited, the analysis for the current paper was based on 788 participants with complete information and viable blood samples. The study was approved by the *Hospital Universitari Sant Joan* (Reus) and *Fundació Jordi Gol Gorina* ethical committees. All participants provided written consent to participate in the study, in accordance with the Declaration of Helsinki. Participants were invited to attend a medical check-up in which clinical history and lifestyle data including sex, age, body mass index, previous and current illness, medication use, smoking, alcohol and drug use as well as socioeconomic status based on Spanish Epidemiology Society guidelines [25] were recorded. Blood pressure was measured following a standardized protocol and by a trained clinician. Participants were seated for at least 15 min before measurements. During the measurements, they 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 heart level. The mean of two measurements (2 min apart) by mercury column sphygmomanometer (Riester, Germany, 1998) was recorded. Participants with no previously detected hypertension and a blood pressure measurement ≥140/90 mmHg, for the first time at the check-up, were recommended to consult their doctor and were excluded from the analysis, to avoid misclassification to the hypertensive group. Participants with no history of hypertension and with blood pressure <140/90 mmHg at the check-up were categorized as controls. Participants with previously diagnosed hypertension or on hypertensive medication were categorized as cases even if their blood pressure was normal at the check-up [9].

2.2. Collection and classification of data on medication use

Medical treatment recorded at the check-up was coded using the WHO ATC/DDD index [26]. The active ingredients of the treatments were classified into three categories (Supplementary Table 1): Group 1) none or sporadic, Group 2) chronic, not affecting the L-Arginine pathway and Group 3) medical treatment known to affect the L-Arginine pathway. This last group included treatment for arthrosis, atherosclerosis, epilepsy, hypercholesterolemia, hypertension, hypertriglyceridemia, thyroid disorders as well as non-insulin diabetes treatments. Groups 1 and 2 were joined, to create the "*no medication group*". Treatments known to affect the L-Arginine pathway constituted the "*medication group*".

2.3. Blood samples

Fasting venous blood samples were collected into EDTA-K3 vacutainers and kept chilled until plasma was separated, strictly within 2 h of collection. Whole blood diluted in 1 % ascorbic acid solution (for red blood cell folate determinations), washed erythrocytes and plasma were kept at −80 °C for later determinations

and DNA was extracted from leukocyte pellets as previously described [6,27].

Total plasma homocysteine was determined by the fluorescence polarization immunoassay on the IMx autoanalyzer (Abbott Laboratories). L-Arginine, ADMA and SDMA were measured by HPLC-MS/MS [28]. Plasma folate was measured by the gold standard microbiological assay with *Lactobacillus casei* [29] and plasma cobalamin with *L. leichmannii* [30] respectively. Riboflavin status was determined using the gold standard functional (erythrocyte glutathionine reductase activation coefficient (EGRAC) assay [27] and plasma creatinine concentration (Jaffé reaction (Quimica Clinica Aplicada)) [27] were determined using the COBAS MIRA autoanalyzer (Roche Diagnostics). Total and HDL-cholesterol and triglycerides were determined as previously described [31] and the Friedewald equation was used to estimate LDL-cholesterol [32]. *MTHFR* C677T (rs1801133) genotype was determined from leukocyte extracted DNA as previously described [31].

2.4. Statistical analysis

Variables with skewed distributions were ln-transformed before applying parametric statistical tests. Descriptive data are reported as arithmetic means (95 % confidence intervals (CI)) for untransformed continuous variables and as geometric means (95 % CI) for ln-transformed variables. Categorical variables are reported as percentages (95 % CI). Means between age groups were compared by ANOVA and categorical variables by χ^2.

Missing data, due to incomplete/unreturned questionnaires or incomplete records or insufficient blood sample, occurred randomly and data analysis was performed without imputation. Reasons for any missing data are provided in the footnotes to the tables where relevant. 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. The probability of having hypertension was explored using multiple logistic regression analysis. Basic models explored a 1 standard deviation increase in L-Arginine pathway metabolites as predictors of hypertension. To test whether the relationship between one-carbon metabolism and diagnosed hypertension was via the L-Arginine pathway, a mediation analysis following the principles of Hayes [33] was performed. tHcy and ADMA as mediators in the association between *MTHFR* 677CT vs CC and TT vs CC genotype and hypertension was tested. All models met the requirements for assumptions in linear regressions (normality of errors, no multicollinearity, homogeneity of variance (homoscedasticity)). Logistic regression diagnostics were performed and included box plot examination to identify outliers and Cook's distance ($>4/\sqrt{n}$) to identify influential cases. All multivariate analyses were adjusted for sex assigned at birth and tested as male versus female (reference category). Data analysis was with SPSS software version 28.0 (IBM Corporation, USA). The mediation analyses were performed by PROCESS macro software version 4.2 by Hayes for SPSS [33]. Significance was accepted at *P*-value <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. Results

The characteristics of the participants are described in Table 1. Participants over 50 years of age had higher body mass index, and a greater prevalence of low socioeconomic status compared to those under 50. Alcohol intake was higher in the older than in the younger group. The *MTHFR* C677T polymorphism was in Hardy-Weinberg equilibrium, and 17.9 % (95 % CI:15.3, 20.7) of the participants had the homozygous variant genotype. The prevalence of diagnosed hypertension was 32.0 % and 3 out of 4 cases were from the over 50 age group. In addition, 15.0 % were on medication known to affect the L-Arginine pathway, two-thirds of which were on blood pressure lowering medication.

Plasma 1CM and L-Arginine pathway metabolite concentrations, and erythrocyte glutathione reductase activation coefficient concentrations, stratified by age group, are reported in Fig. 1. Participants over 50 years of age had higher folate, cobalamin and riboflavin 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 (Supplementary Fig. 1).

Plasma L-Arginine, the L-Arginine/ADMA ratio and the ADMA/SDMA ratio were inversely correlated, while ADMA and SDMA were positively correlated with tHcy (Table 2). ADMA was positively correlated with total and LDL cholesterol. All metabolites, except ADMA were positively correlated with plasma creatinine.

Table 3 illustrates the associations between tHcy and the different L-Arginine pathway metabolites. tHcy was positively associated with plasma ADMA and SDMA concentrations and negatively associated with L-Arginine/ADMA and ADMA/SDMA ratios in the entire population and participants over 50. In the under 50 age group, the association was only observed between tHcy and ADMA, and SDMA.

The associations between *MTHFR* C677T genotypes and L-Arginine and its metabolites are reported in Table 4. In the entire population, the *MTHFR* 677 CT genotype was associated with lower plasma ADMA concentrations compared to the CC genotype. In the under 50 years group, this association was maintained for SDMA.

The risk of hypertension associated with the different L-Arginine pathway metabolites is reported in Table 5. An increase of 1 standard deviation of plasma ADMA concentrations (0.08 μmol/l) was associated with a twofold risk of hypertension in the entire population (OR = 2.3; 95 % CI = 1.0, 1.6) and in the over 50 years group (OR = 1.7; 95 % CI = 1.2, 2.4). In addition, an increase of 1 standard deviation of plasma L-Arginine and L-Arginine/ADMA ratio, was associated with a 30–50 % reduction in hypertension risk, regardless of age group.

The association between *MTHFR C*677T genotype and hypertension, mediated by tHcy and ADMA is represented in Fig. 2. In the first step of the analysis, the *MTHFR* 677 CT vs CC genotype did not show an association with homocysteine (B coefficient, 0.31; p, 0.216). The *MTHFR* 677 TT (vs CC) genotype was positively associated with tHcy (B coefficient, 2.82; p, <0.001) and this in turn with increased plasma ADMA concentrations (B coefficient, 0.044; p, <0.001). An increase of 1 standard deviation in plasma ADMA concentrations was positively associated with hypertension risk (B coefficient, 0.039; p, <0.05). The indirect effect showed a positive association between *MTHFR* 677 TT vs CC and hypertension via tHcy and ADMA (B = 0.05; 95 % CI = 0.02, 0.10). When stratifying by age group, the same indirect effect was only observed in participants over 50 (Supplementary Fig. 2).

4. Discussion

4.1. One-carbon metabolism and L-Arginine pathway association

The observed associations between tHcy and L-Arginine pathway metabolites add to evidence from previous studies and confirm that these occur in adults recruited from non-clinical settings, in the absence of B vitamin supplementation or mandatory fortification with folic acid and riboflavin, and in an analysis controlling for medication affecting the L-Arginine pathway. ADMA and

Table 1
Characteristics of the study population according to age group.

	All (n = 788)		≤50 years (n = 534)		>50 years (n = 254)		*P*
	n		n		n		
Age, y[a]	788	43.0 (41.9, 44.1)	534	34.2 (33.5, 34.9)	254	61.5 (60.6, 62.4)	<0.001
Women[b]	788	51.6 (48.2, 55.1)	534	51.7 (47.5, 55.9)	254	51.6 (45.5, 57.7)	0.519
BMI (kg/m^2)[a]	773	27.1 (26.7, 27.4)	524	25.7 (25.3, 26.1)	249	29.9 (29.3, 30.5)	<0.001
Smokers[b]	788	33.4 (30.2, 36.7)	534	42.5 (38.4, 46.7)	254	14.2 (10.4, 19.0)	<0.001
Cigarettes per day[a]	786	5.3 (4.6, 6.0)	534	6.8 (6.0, 7.7)	252	2.1 (1.2, 2.9)	<0.001
Alcohol consumption[b,c]	788		534		254		
None		59.4 (55.9, 62.8)		59.9 (55.7, 64.0)		58.3 (52.1, 64.2)	0.047
Moderate intake		24.9 (22.0, 28.0)		26.4 (22.8, 30.3)		21.7 (17.0, 27.1)	
High intake		15.7 (13.4, 18.4)		13.7 (11.0, 16.8)		20.1 (15.6, 25.4)	
Low socio-economic status[b,d]	788	37.6 (34.3, 41.0)	534	21.7 (18.4, 25.4)	254	70.9 (65.0, 76.1)	<0.001
MTHFR C677T polymorphism[b]	778		529		249		
CC genotype		35.7 (32.4, 39.2)		35.0 (31.0, 39.1)		37.4 (31.6, 43.5)	0.693
CT genotype		46.4 (42.9, 49.9)		47.4 (43.2, 51.7)		44.2 (38.1, 50.4)	
TT genotype		17.9 (15.3, 20.7)		17.6 (14.6, 21.1)		18.5 (14.1, 23.8)	
Diagnosed hypertension[b]	788	32.0 (28.8, 25.3)	534	18.2 (15.1, 21.7)	254	61.0 (54.9, 66.8)	<0.001
Antihypertensive medication[b]	788	10.0 (8.1, 12.3)	534	3.0 (1.9, 4.8)	254	24.8 (19.9, 30.5)	<0.001
Medication affecting L-Arginine pathway[b]	788	15.0 (12.7, 17.6)	534	6.2 (4.4, 8.6)	254	33.5 (27.9, 39.5)	<0.001

BMI, body mass index; *MTHFR*, methylenetetrahydrofolate reductase. *MTHFR* 677CT polymorphism was in Hardy-Weinberg equilibrium. Comparison between age group 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] Arithmetic Mean (95 % CI).
[b] Percentage (95 % CI).
[c] 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).
[d] Socioeconomic status, based on household income, educational level and occupation.

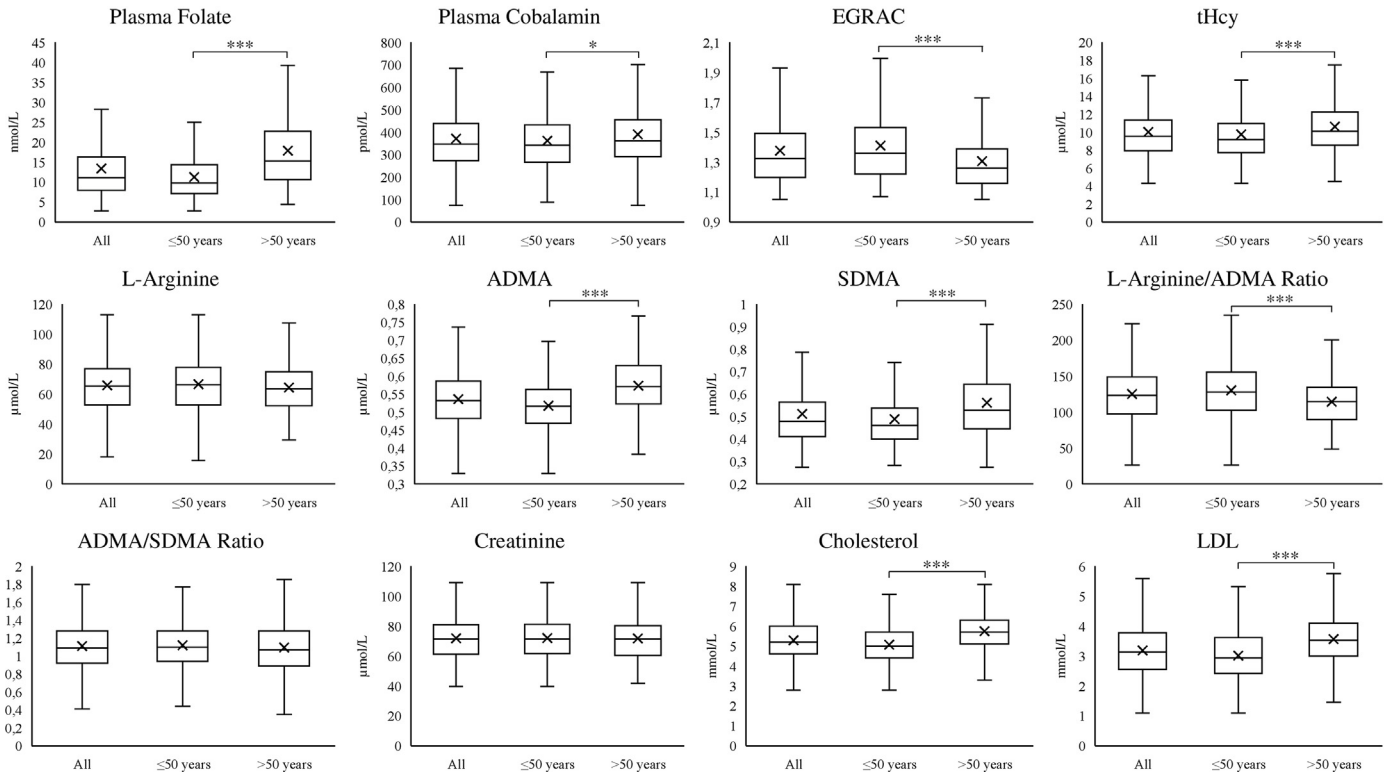

Fig. 1. Box plot median (P25, P75) and means (x) for plasma 1CM and L-Arginine pathway metabolites, as well as erythrocyte glutathione reductase activation coefficient, stratified by age group. Comparisons between age groups were by ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001.

SDMA have previously been positively associated with aging [34,35]. Miyasaki et al. proposed that ADMA may reflect a vascular degenerative process associated with aging [34].

THcy was the strongest modifiable predictor of ADMA, or second strongest after body mass index, in all of the age-stratified regression models (Supplementary Table 2). Homocysteine was one of the strongest predictors of ADMA concentrations in a previous study in rheumatoid arthritis patients [36]. In a separate study, patients with post-methionine load hyperhomocysteinemia, had higher plasma ADMA concentration and a lower L-Arginine/

Table 2
Correlations for 1CM and L-Arginine pathway.

	L-Arginine	ADMA	SDMA	L-Arginine/ADMA Ratio	ADMA/SDMA Ratio	tHcy	Plasma folate	Plasma B12	EGRAC	Creatinine	Cholesterol	LDL
L-Arginine	1.000 [784]	0.131[b] [783]	0.070[a] [783]	0.851[b] [784]	0.0369 [784]	−0.092[b] [784]	−0,061 [783]	0.069 [782]	0.238[b] [772]	0.092[a] [784]	0.022 [782]	0.001 [768]
ADMA		1.000 [783]	0.266[b] [782]	−0.357[b] [783]	0.304[b] [783]	0.198[b] [783]	0.125[b] [782]	0.018 [781]	−0.110[b] [771]	−0.046 [783]	0.129[b] [781]	0.156[b] [767]
SDMA			1.000 [783]	−0.073[a] [783]	−0.796[b] [783]	0.259[b] [783]	0.128[b] [782]	0.050 [781]	−0.037 [771]	0.365[b] [783]	0.056 [781]	0.054 [767]
L-Arginine/ADMA Ratio				1.000 [784]	−0.120[b] [784]	−0.184[b] [784]	−0.115[b] [783]	0.057 [782]	0.281[b] [772]	0.124[b] [784]	−0.029 [782]	−0.061 [768]
ADMA/SDMA Ratio					1.000 [784]	−0.147[b] [784]	−0.045 [783]	−0.017 [782]	−0.043 [772]	−0.392[b] [784]	0.020 [782]	0.033 [768]
tHcy						1.000 [788]	−0.241[b] [787]	−0.225[b] [786]	−0.127[b] [776]	0.295[b] [784]	0.052 [786]	0.083[a] [772]
Plasma folate							1.000 [787]	0.135[b] [786]	−0.167[b] [775]	−0.111[b] [783]	0.160[b] [785]	0.124[b] [771]
Plasma B12								1.000 [786]	−0.126[b] [774]	0.004 [782]	0.109[b] [784]	0.117[b] [770]
EGRAC									1.000 [776]	0.025 [772]	−0.145[b] [775]	−0.159[b] [761]
Creatinine										1.000 [784]	0.023 [782]	0.053 [768]
Cholesterol											1.000 [786]	0.920[b] [772]
LDL												1.000 [772]

Spearman correlation coefficients between plasma 1CM and L-Arginine pathway metabolites, as well as erythrocyte glutathione reductase activation coefficient (EGRAC). Rho [n]. ADMA, Asymmetric Dimethylarginine; SDMA, Symmetric Dimethylarginine; tHcy, total plasma homocysteine.
[a] $P < 0.05$.
[b] $P < 0.01$.

Table 3
Multiple linear regression analysis of tHcy and L-Arginine pathway metabolites, stratified by age group.

	All (n = 698)		≤50 years old (n = 475)		>50 years old (n = 223)	
	R^2	B (SE)	R^2	B (SE)	R^2	B (SE)
L-Arginine	0.010	−0.320 (0.229)	0.014	−0.394 (0.285)	0.018	−0.218 (0.381)
ADMA	0.143[d]	0.003 (0.001)[d]	0.053[d]	0.002 (0.001)[a]	0.074[d]	0.007 (0.002)[d]
SDMA	0.106[d]	0.007 (0.002)[d]	0.023[b]	0.004 (0.002)[b]	0.108[d]	0.015 (0.004)[d]
L-Arginine/ADMA ratio	0.069[d]	−1.140 (0.451)[b]	0.038[c]	−1.021 (0.564)	0.041[b]	−1.775 (0.740)[b]
ADMA/SDMA ratio	0.069[d]	−0.006 (0.003)[b]	0.052[d]	−0.003 (0.004)	0.092[d]	−0.013 (0.006)[b]

Multiple linear regressions testing the association between tHcy and L-Arginine pathway metabolites in plasma, stratified by age group. ADMA, Asymmetric Dimethylarginine; BMI, body mass index; SDMA, Symmetric Dimethylarginine; tHcy, total plasma homocysteine. Of the 788 participants, 59 were excluded from all analyses because their blood samples were not processed within 2 h of collection, 4 because they had suspected altered renal function (plasma creatinine >97 μmol/l for women and >124 μmol/l for men), 27 had insufficient blood sample for all of the determinations or unreturned questionnaires. All models were adjusted for age (only in the all-participant model), sex, 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 and medication category.
[a] $P < 0.07$.
[b] $P < 0.05$.
[c] $P < 0.01$.
[d] $P < 0.001$.

ADMA ratio compared to baseline. However, no difference in plasma SDMA concentration was observed between post-methionine load and baseline [37]. In a study of The Framingham Offspring Cohort that excluded unhealthy participants and those on antihypertensive or antidiabetic medication, among other exclusion criteria, positive associations between tHcy and SDMA (as well as ADMA), were reported [38,39]. This same study, along with others, reported an inverse association between tHcy concentrations and the L-Arginine/ADMA ratio [40,41].

The *MTHFR* 677 TT genotype has been associated with increased tHcy and decreased genomic methylation (especially at low folate concentrations), due to the limited availability of 5-MTHF for homocysteine remethylation and *S*-Adenosylmethionine synthesis [42,43], the universal methyl group donor essential for ADMA and SDMA synthesis by Protein-Arginine *N*-Methyltransferase [11]. The observed negative association between the *MTHFR* 677 CT genotype and SDMA (reported for the first time) and ADMA support this. The negative association was also observed in participants with the *MTHFR* 677 TT genotype but was not statistically significant. This may be due to lack of sufficient statistical power due to the relatively low number of TT individuals. This observation should be confirmed in future studies. However, the few previous studies that have examined the association between the *MTHFR* C677T polymorphism and L-Arginine pathway metabolites have reported higher serum ADMA concentrations in patients and healthy individuals with the *MTHFR* 677 TT or CT genotypes compared to the CC genotype [36,44,45]. These studies had some limitations. In one, while participants on medication were excluded, healthy women had been exposed to an environmental pollutant [45] previously associated with increased serum ADMA concentrations [46]. This pollutant interacted with the *MTHFR* C677T polymorphism and was a stronger predictor of ADMA than the polymorphism [45]. Another

Table 4
Multiple linear regression analysis of the *MTHFR* C677T genotype and L-Arginine pathway metabolites, by age.

		All (n = 677)		≤50 years old (n = 463)		>50 years old (n = 214)	
		R^2	B (SE)	R^2	B (SE)	R^2	B (SE)
L-Arginine	CT vs CC	0.050[d]	−0.402 (1.568)	0.047[c]	0.513 (1.973)	0.071[b]	−3.208 (2.523)
	TT vs CC		−1.818 (2.041)		−1.970 (2.608)		−2.186 (3.155)
ADMA	CT vs CC	0.138[d]	−0.013 (0.007)[b]	0.051[d]	−0.010 (0.008)	0.031	−0.021 (0.012)
	TT vs CC		−0.013 (0.009)		−0.018 (0.010)[a]		−0.002 (0.015)
SDMA	CT vs CC	0.217[d]	−0.017 (0.011)	0.105[d]	−0.029 (0.013)[b]	0.268[d]	0.006 (0.022)
	TT vs CC		−0.004 (0.015)		−0.009 (0.018)		0.007 (0.028)
L-Arginine/ADMA ratio	CT vs CC	0.108[d]	2.561 (3.091)	0.080[d]	4.076 (3.891)	0.047[b]	−2.277 (4.998)
	TT vs CC		−0.167 (4.025)		0.602 (5.141)		−2.905 (6.251)
ADMA/SDMA ratio	CT vs CC	0.205[d]	0.008 (0.021)	0.174[d]	0.031 (0.025)	0.234[d]	−0.035 (0.039)
	TT vs CC		0.005 (0.027)		0.012 (0.034)		−0.008 (0.048)

Multiple linear regressions testing the association between the *MTHFR* C677T genotype and L-Arginine pathway metabolites in plasma, stratified by age group. ADMA, Asymmetric Dimethylarginine; BMI, body mass index; SDMA, Symmetric Dimethylarginine; tHcy, total plasma homocysteine. Of the 788 participants, 59 were excluded from all analyses because their blood samples were not processed within 2 h of collection, 4 because they had suspected altered renal function (plasma creatinine >97 μmol/l for women and >124 μmol/l for men), 48 had insufficient blood sample for all of the determinations or unreturned questionnaires. All models were adjusted for age (only in the all-participant model), sex, 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, medication category, plasma folate, plasma cobalamin and EGRAC.
[a] $P < 0.09$.
[b] $P < 0.05$.
[c] $P < 0.01$.
[d] $P < 0.001$.

Table 5
Association between L-Arginine pathway metabolites and diagnosed hypertension, stratified age group.

Increase of 1 SD	All (n = 672)		≤50 years old (n = 471)		>50 years old (n = 201)	
	R^2	OR (95 % CI)	R^2	OR (95 % CI)	R^2	OR (95 % CI)
L-Arginine (65.8 μmol/l)	0.399[a]	0.7 (0.5–0.9)	0.274[a]	0.7 (0.5–0.9)	0.204[a]	0.7 (0.5–0.9)
ADMA (0.08 μmol/l)	0.388[a]	2.3 (1.0–1.6)	0.246[a]	1.0 (0.7–1.4)	0.235[a]	1.7 (1.2–2.4)
SDMA (0.15 μmol/l)	0.381[a]	1.1 (0.8–1.3)	0.248[a]	1.1 (0.8–1.5)	0.182[a]	1.1 (0.8–1.6)
L-Arginine/ADMA (37.6)	0.408[a]	0.6 (0.5–0.8)	0.274[a]	0.6 (0.5–0.9)	0.240[a]	0.5 (0.4–0.8)
ADMA/SDMA (0.28)	0.381[a]	1.1 (0.8–1.4)	0.248[a]	0.9 (0.6–1.3)	0.183[a]	1.2 (0.8–1.8)

Multiple logistic regression analysis was used. ADMA, Asymmetric dimethylarginine; BMI, body mass index; SD, Standard deviation SDMA, Symmetric dimethylarginine. Of the 788 participants, 59 were excluded from all analyses because their blood samples were not processed within 2 h of collection, 4 because they had suspected altered renal function (plasma creatinine >97 μmol/l for women and >124 μmol/l for men), 16 had insufficient blood sample for all of the determinations or unreturned questionnaires. 37 participants had an elevated blood pressure (≥140/90 mmHg) for the first time at the check-up and were excluded from the analysis. Models were adjusted for age (only in the all-participant model), sex, 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, BMI, total cholesterol, plasma creatinine and medication category (antihypertensive medication not included). Nagelkerke R^2.
[a] $P < 0.001$.

study included participants on methotrexate and while it did not interact with the association between the *MTHFR* C677T polymorphism and serum ADMA concentration, both the polymorphism and the drug were negatively associated with ADMA and the associations were lost in multivariate analysis [36]. In another case control study of epileptic patients, higher plasma ADMA concentrations were observed in epileptic cases with the CT genotype only [44]. Patients were being treated with antiepileptic drugs (such as carbamazepine), reported to be associated with increased plasma ADMA concentrations [47]. Therefore, interactions between medication affecting the L-Arginine pathway and one-carbon metabolism may explain why these studies observed higher ADMA concentrations in the presence of the *MTHFR* 677CT or TT compared to CC genotype, while lower plasma ADMA and SDMA concentrations were observed in the present study, after adjusting for medication use.

4.2. Association between the L-Arginine pathway and hypertension

We observed an increased risk of hypertension for each SD increase in plasma ADMA, in the entire population and in adults over 50. This is consistent with studies that observed a relationship between high plasma ADMA concentrations, hypertension and aging [34,48]. As mentioned above, ADMA may reflect a vascular degenerative process associated with aging. In fact, endothelium-dependent coronary microvascular dysfunction was associated with aging in 34 patients (27–73 years old) with no coronary risk factors [49]. Previously we proposed that age-associated and cumulative lifetime risk factors such as body mass index, socioeconomic status or homocysteine (and now we add ADMA) are important factors associated with hypertension in the older population [9]. We proposed that genetic mutations (such as *MTHFR* 677CT or TT) may have a stronger association with the disease in the younger population, in which age-associated risk factors would not yet have taken their physiological toll. Both ADMA and SDMA were associated with increased risk of cardiovascular disease in an adult, population-based, cohort after adjusting for several cofounders [50]. However, the association of SDMA and hypertension risk is not observed in this population.

Elevated plasma ADMA concentrations have been associated with several cardiovascular diseases, even when circulating L-Arginine is in the normal range, the so-called "L-Arginine paradox" [51]. L-Arginine supplementation has been proposed as treatment for cardiovascular disease, since an increase in the L-Arginine/ADMA ratio would reduce the inhibitory effect of ADMA on NO synthesis [51]. In addition, this ratio provides information that may be missed when ADMA alone is considered [51]. The results in our population are consistent with studies that reported low flow

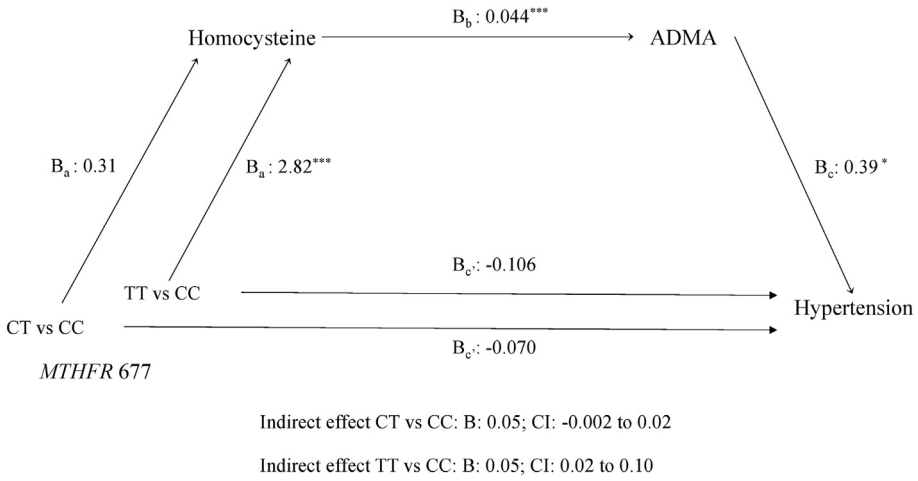

Fig. 2. Mediation analysis to test tHcy and ADMA as mediators of the relationship between *MTHFR* 677 CT vs CC and TT vs CC with hypertension. ADMA, Asymmetric dimethylarginine; BMI, body mass index; *MTHFR*, methylenetetrahydrofolate reductase; tHcy, total fasting plasma homocysteine. 671 participants were included. Of the 788 participants, 59 were excluded from all analyses because their blood samples were not processed within 2 h of collection, 4 because they had suspected altered renal function (plasma creatinine >97 μmol/l for women and >124 μmol/l for men), 17 had insufficient blood sample for all of the determinations or unreturned questionnaires. 37 participants had an elevated blood pressure (≥140/90 mmHg) for the first time at the check-up and were excluded from the analysis. Model adjusted for age, sex, smoking habits (cigarettes per day), 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)), socio-economic status, BMI, total cholesterol and medication category (antihypertensive medication not included). B_a represents the B coefficient of the association between *MTHFR* C667T genotypes and tHcy. B_b represents the B coefficient of the association between tHcy and ADMA. B_c represents the B coefficient of the association between ADMA and hypertension. $B_{c'}$ represents the B coefficient of the direct association between *MTHFR* C667T genotypes and hypertension. Direct and indirect effects on hypertension were estimated using a log-odds scale. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

mediated dilation, low coronary flow [52] and elevated blood pressure [41] when the L-Arginine/ADMA ratio is low.

4.3. One-carbon metabolism -hypertension link via L-Arginine pathway

Homocysteine has been shown to inhibit the enzyme dimethylarginine dimethylaminohydrolase (DDAH), responsible for the degradation of 80 % of ADMA in humans [53,54]. We report a negative association between tHcy and ADMA/SDMA ratio. An increase in this ratio may indicate impaired DDAH activity [55].

We also report a negative association between the *MTHFR* 677 TT (vs CC) genotype and plasma ADMA concentrations in a multiple linear regression analysis, but a positive association, mediated by tHcy in a mediation analysis (Supplementary Fig. 3).

The results of the mediation analysis, carried out here, support the L-Arginine pathway as a mediator in the development of hypertension by altered one-carbon metabolism. The *MTHFR* 677 TT genotype may lead to ADMA reduction by hypomethylation, but its effect on homocysteine accumulation and subsequent inhibition of DDAH, might increase plasma ADMA concentrations, leading to an inhibition of NO synthesis and eventually, hypertension.

A placebo controlled randomized control trial testing the B-vitamin lowering effect of tHcy on the L-Arginine pathway and change in blood pressure before and after intervention in pre-hypertensive individuals, would increase the evidence for this proposed mechanism. It would be especially interesting to also test this in individuals with the *MTHFR* 677 TT versus the *MTHFR* CC genotype.

4.4. Strengths and limitations

The primary aim of the study was to investigate the association between tHcy and the *MTHFR* C677T polymorphism with risk of hypertension in the absence of the influences of mandatory fortification of flour with folic acid or other micronutrients and B vitamin supplement use. Unlike other studies conducted in countries with mandatory fortification with folic acid and widespread B vitamin use, the potential overriding effects of folic acid, riboflavin and other B vitamins on the associations between the *MTHFR* C677T polymorphism and tHcy or on blood pressure, were absent. In addition, it has been proven that tHcy on a population level was decreased following the introduction of mandatory folic acid fortification [56]. Conceivably, this may affect circulating ADMA concentrations via the proposed route in our paper. However, a placebo controlled RCT in participants with moderately elevated tHcy reported no effect of B vitamin supplementation on ADMA [57]. Folic acid alone was not specifically tested. A separate study reported a reduction in tHcy and in ADMA, in hyperhomocysteinaemic patients following 6 weeks intervention with 5 mg/d of folic acid [57,58].

Furthermore, our study was of a representative sample from local population records. The study was not affected by the inherent biases in studies that recruit participants from clinical settings or from the workplace etc. The influence of medication affecting the L-Arginine pathway on the studied pathways was considered by adjusting the analyses for medication use. The associations of interest were also explored in women and men separately and the results and conclusions were the same. Therefore, we report the results combined for both sexes, so as not to lose statistical power in the subgroup analyses. All our multivariate analyses were adjusted for sex. Lastly, sample processing protocols to prevent artifacts in tHcy measurements were strictly adhered to.

Reverse causality and residual confounding are potential limitations in cross-sectional studies. However, studying the *MTHFR* C677T genotype helped overcome the limitation of reverse causation. In the case of the tHcy - L-Arginine pathway metabolites analyses, the possibility of reverse causation cannot be ruled out. Regarding residual confounding, our models were adjusted for known potential confounding factors of the associations of interest.

5. Conclusions

Impaired One-carbon metabolism was associated with L-Arginine pathway metabolites. Enhanced L-Arginine/ADMA ratio may have a protective effect against hypertension. Our results support

the L-Arginine pathway as a mediator in the association of impaired One-Carbon metabolism and hypertension. Age and age-related factors may play an important role in these associations.

CRediT authorship contribution statement

Carla Ramos-Rodríguez: Writing – original draft, Formal analysis, Conceptualization. **Alejandra Rojas-Gomez:** Writing – review & editing, Formal analysis. **Luis A. Santos-Calderón:** Writing – review & editing. **Santiago Ceruelo:** Resources. **Lídia Ríos:** Resources. **Per M. Ueland:** Resources. **Joan D. Fernandez-Ballart:** Project administration. **Albert Salas-Huetos:** Writing – review & editing. **Michelle M. Murphy:** Writing – original draft, Supervision, Project administration.

Ethics approval and consent to participate

The study was approved by the *Hospital Universitari Sant Joan* (Reus) and *Fundació Jordi Gol Gorina* ethical committees. All participants provided written consent to participate in the study, in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

No further biological material from this population cohort is available. Data described in the manuscript can be made available upon reasonable request subject to compliance with the signed ethical agreement by the participants and pending application, review and approval by the HOMFOL-GENUP Steering Committee and IISPV CEIm. In the event of approval, signed data sharing and access agreement is required.

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Conflict of interest

We have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2024.11.006.

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