Eur J Nutr (2006) 45:383–390
DOI 10.1007/s00394-006-0611-1

ORIGINAL CONTRIBUTION

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Medium-term methionine supplementation increases plasma homocysteine but not ADMA and improves blood pressure control in rats fed a diet rich in protein and adequate in folate and choline

Received: 1 April 2006
Accepted: 26 June 2006
Published online: 1 September 2006

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Summary *Background* Hyperhomocysteinemia (HHcy) is associated with cardiovascular risk, possibly because it increases asymmetric dimethyl-arginine (ADMA), but the general association remains unclear and may vary with nutritional and physiological conditions. *Aim of the study* We aimed to monitor the effect of methionine supplementation, and subsequent HHcy, on plasma ADMA and hemodynamics in the context of a diet rich in protein and adequate in folic acid and choline. *Methods* For 6 weeks, rats were fed a 29% protein diet supplemented (M) or not (C) with 8 g/kg L-methionine. Blood pressure and plasma amino acids, including homocysteine and ADMA, were measured throughout the experiment and additional parameters, including in vivo hemodynamic response to acetylcholine, were measured at week 5–6. *Results* As compared to the C diet, the M diet induced a marked HHcy during the first 3 weeks, which lessened at week 5. In contrast, plasma ADMA stayed similar in the C and M diet. Paradoxically, M rats had lower mean and diastolic blood pressure values over the experiment, together with a lower left ventricular mass at week 6, when compared with C rats. No difference was observed between groups regarding vascular reactivity and plasma NO*x* at week 6. *Conclusions* In a context of a diet rich in protein and adequate in methyl donors, rats exhibit a complex adaptation to the medium-term methionine supplementation, with improvement in blood pressure control despite marked HHcy. The lack of increase in plasma ADMA may account for the absence of detrimental effects of HHcy on hemodynamics.

Key words methionine – homocysteine – sulfur amino acids – asymmetric dimethyl-arginine – blood pressure – hemodynamics – diet

Introduction

Plasma homocysteine (Hcy) has been shown to be strongly associated with cardiovascular risk. However, it is still uncertain whether Hcy is a direct causal factor, a fellow traveler, or a marker of a pathogenic mechanism [1–6]. In addition, the mechanism whereby Hcy may increase the cardiovascular risk remains unclear, and may differ depending on the period of exposure to hyperhomocysteinemia (HHcy), nutritional status, and other risk factors [5–7].

In animals, experimental HHcy has been shown to induce atherosclerosis, in particular in susceptible animal models [8] and can be achieved via a variety of dietary manipulations [9], which include methionine supplementation and/or vitamin and choline depletion [10] and, in a few cases, Hcy supplementation [8]. Even though they result in similar HHcy from a quantitative viewpoint, these manipulations differ largely with respect to the metabolic challenge imposed on sulfur amino acid pathways, interconnected metabolism [particularly one carbon metabolism, 11], and vascular homeostasis.

In humans, the effect of Hcy is studied after acute induction of a transient HHcy by a supra-physiological oral load of methionine. Using this HHcy experimental model, most, but not all [12–14], studies, have reported acute impairment of vascular function, particularly of the endothelium-dependent function, suggesting that an early alteration to nitric oxide (NO) production/bioavailability is central to the initiation of Hcy-induced atherogenesis [14–17].

Numerous mechanisms have been advocated but recently particular emphasis has been laid on the role of asymmetric dimethyl-arginine (ADMA), an endogenous inhibitor of NO synthase. Although a large body of evidence supports ADMA as being central to the physiopathology of CVD and other diseases [18], it is inconclusive whether ADMA is increased in human, experimentally induced HHcy [19–22], and recently it has been reported that lowering Hcy concentrations does not decrease plasma ADMA [21, 23]. In animals, the effect of methionine supplementation on plasma ADMA concentration has only been reported once, in the context of a folic acid and choline-depleted diet in monkeys [24].

Indeed, the effects of so-called experimental HHcy models are varied. The nature of the basal diet, in particular as regards the intake of other amino acids (such as glycine and serine), has been shown to modulate HHcy [25, 26] by affecting sulfur amino acid pathways. Furthermore, experimental HHcy models may indeed differ considerably regarding their effects on vascular function, as illustrated by the fact that hyperhomocysteinemic humans free of other risk markers and/or with normal folate status exhibit no altered vascular reactivity [7, 27, 28] and that the effect of folate intake on vascular reactivity may be independent of reduced plasma Hcy [29]. Thus the effects of methionine intake on HHcy and hemodynamics and vascular reactivity may vary according to many factors (including the basal or experimental diet and nutritional status) so that it remains unclear whether the adverse effects are directly related to dietary methionine and plasma Hcy or indirectly related to other limiting dietary factors, including protein, folate, and choline.

Our aim was therefore to test the sequential relationship in rats between methionine, Hcy, ADMA, and hemodynamics by monitoring the effect of a simple chronic methionine challenging of sulfur amino acid metabolism in a situation where no nutrient is supposed to limit the capacity of the sulfur amino acid metabolism. For that purpose, rats were fed for 6 weeks a diet rich in protein (twice the standard amount of protein, mimicking spontaneous protein intake in western countries) and containing standard levels of folic acid and choline (to ensure a normal methyl donor status) and supplemented or not with methionine. Changes to plasma amino acids, including methionine, total cysteine, total Hcy, and ADMA, were examined after methionine supplementation. The systolic and diastolic blood pressures (DBP) of the rats were monitored during the 6 weeks of methionine supplementation, and additional parameters were measured at week 5–6 to check for the potential impact on hemodynamics and NO production.

Materials and methods

Animals and diets

The "Principles of laboratory animal care" (NIH publication No. 85–23, revised 1985) and French guidelines concerning the care and use of laboratory animals were followed. A total of 16 outbred Wistar-Hanover male rats (Harlan, France), weighing 130–140 g on arrival, were housed two by two, with free access to food and tap water, on a 12-h light:dark cycle (lights on at 08h00). The experimental diets were prepared under strict laboratory conditions by UPAE, an Experimental Food Preparation Unit (INRA, Jouy-en-Josas, France). During the first week, animals were fed with the Control diet (Table 1), which was a modified AIN-93 diet with an increased protein content (29% of total energy). The animals were then ("Week 0") split into two groups, the first (Control Group, C) continuing with the control diet and the second (Methionine group, M) switching to the methionine-enriched diet (Table 1), for the next 6 weeks. Animals were weighed every week during the experimental period and food intake was checked on the first 2 days after the rats started to receive their specific diets. On the last week, animals received nitrate-free water (Wattwiller, Cernay, France) prior to the analysis of plasma nitrate + nitrite, a surrogate measure for NO production.

Experimental design

During the first week before experimental diets, rats were adapted to laboratory conditions and

Table 1 Composition of control and methionine-supplemented diets

g per kg diet	Control diet (C diet)	Methionine-enriched diet (M diet)
Total milk protein	290	282
Corn starch	493.2	493.2
Sucrose	79.5	79.5
Soybean oil	40	40
AIN-93M-MX mineral mix	35	35
AIN-93-VX vitamin mix	10	10
Alpha-cellulose	50	50
Choline	2.3	2.3
L-Methionine	0	8
Methionine content	5.6	13.6
Folic acid content	0.002	0.002

experimental sessions, including blood pressure measurements as described below. Rats in both groups were processed in a randomized order, on the same day of each week, in the fasted state.

Systolic blood pressure (SBP), DBP, and heart rate (HR) were determined weekly by volume–pressure recordings using an automated tail-cuff plethysmographic device (XBP 1000, Kent Scientific, Torrington, CT). After 10 min of adaptation to inflation and deflation of the occlusion cuff in the restrainer, 20 recordings per rat and per session were used to obtain average values. The Mean Arterial Pressure (MAP) was calculated as MAP = DBP + (SBP−DBP)/3. Increments in volume during deflation of the occlusion cuff from minimum (SBP) to maximum (DBP) volume were computed and taken as the blood flow index (BFI).

During week 5, an indwelling catheter was inserted in a distal lateral tail vein and flushed with heparinized saline. After baseline measurements, SBP, DBP, HR, and BFI were computed after the injection of acetylcholine chloride (10 μg kg^{-1}, Sigma, France). The effects of acetylcholine were expressed as an absolute variation in blood pressure and a relative variation in BFI (i.e., (BFI_after−BFI_basal)/BFI_basal), as a percentage). At week 0, 1, 3, and 5, 500 μl blood drawn from a tail catheter was dropped into prechilled tubes containing 30-μl of an EDTA and aprotinin solution (Trasylol, 10000 U; Bayer, Leverkusen, Germany). Plasma aliquots were stored at −80°C. At the end of the experiment (week 6), the rats were killed with an overdose of pentobarbital and the heart was dissected out, cleaned with saline and stored in alcohol formalin-acetic acid fixative (AFA, ethylic acid 50%, acetic acid 4%, and formaldehyde 6%). The left ventricle was dissected out and weighed both immediately and then 24 h later to take account of the AFA (i.e., after near-complete AFA evaporation). The two measurements proved to be highly correlated (R^2 = 0.95, $P < 0.0001$) and resulted in a similar statistical inference. The first measurement was chosen to calculate the left ventricular mass index (left ventricular mass index = left ventricular weight/body weight).

Biochemical analysis

As previously described [30], plasma amino acids, including total Hcy and total cysteine, were analyzed together in a classical aminogram using ion-exchange-chromatography under a pre-reduction method [31] and plasma ADMA concentrations were determined using an ELISA assay (Cardiovasics Medical Science Laboratory). Plasma nitrite + nitrate was determined by a fluorimetric method [32].

Statistical analysis of results

Data are expressed as means ± SEM. Repeated (i.e., weekly) measurements were analyzed using a mixed model with diet and time (i.e. weeks) as independent, fixed factors and the rat as a random factor, nested in the diet factor (SAS institute, Cary, NC). Multiple comparisons were made using ad hoc contrasts under the mixed models. Selective measurements (at week 5 or 6, or collapsed data) were analyzed with a two-tail Student *T*-test. A *P*-value <0.05 was considered to be significant.

Results

Animals

The weight of the animals remained very similar between groups throughout the experiment (data not shown), being 186.1 ± 2.8 g in the M group and 189.4 ± 3.0 g in the C group at week 0 and 334.9 ± 10.3 g in the M group and 333.0 ± 9.9 g in the C group at the end of the experiment. Food intake at day 1–2 after introduction of the diet was similar in the two groups (amount consumed per pair of rats: 44.0 ± 3.1 g in the C group and 44.9 ± 6.5 g in the M group).

Plasma amino acids

Plasma amino acids concentrations are reported in Table 2 and Fig. 1. Methionine supplementation did not affect plasma methionine but induced a major increase in plasma Hcy at weeks 1 and 3. Plasma Hcy decreased in the M group at week 5, resulting in a

Table 2 Plasma concentrations of methionine, total cysteine, taurine, serine, glycine, arginine and citrulline during 5 weeks in rats fed a control (C) or methionine-supplemented (M) diet

Amino acid	Diet	Week									Time	Diet	Time × Diet
		Week 0	Week 1		Week 3			Week 5					
Methionine	C	60 ± 6	82 ± 5	††	62 ± 7			63 ± 5			$P < 0.01$	NS	NS
	M	58 ± 4	71 ± 6		69 ± 8			65 ± 73					
Total cysteine	C	163 ± 15	191 ± 8		218 ± 22		††	209 ± 23		†	$P < 0.05$	$P < 0.05$	NS
	M	157 ± 8	147 ± 16	*	167 ± 13	*		149 ± 14	*				
Taurine	C	109 ± 14	85 ± 15		95 ± 9			96 ± 12			NS	$P < 0.01$	NS
	M	134 ± 17	123 ± 15		123 ± 9			160 ± 20	**				
Serine	C	118 ± 7	168 ± 7	††	182 ± 8		††	182 ± 15		††	$P < 0.001$	$P < 0.05$	$P < 0.05$
	M	118 ± 6	142 ± 12		147 ± 10	*		142 ± 9	**				
Glycine	C	118 ± 14	136 ± 8		130 ± 12			146 ± 18			NS	NS	NS
	M	143 ± 8	136 ± 12		142 ± 10			129 ± 9					
Arginine	C	86 ± 5	106 ± 2	††	112 ± 7		††	116 ± 8		††	$P < 0.001$	NS	NS
	M	83 ± 8	101 ± 10		103 ± 4			98 ± 8					
Citrulline	C	57 ± 3	66 ± 2	††	60 ± 5			52 ± 3			$P < 0.001$	NS	NS
	M	60 ± 2	73 ± 7		59 ± 2			53 ± 5					

Values are means ± SEM. † and ††: significantly different from week 0 (collapsing groups), $P < 0.05$ and $P < 0.01$, respectively. * and **: significantly different from the control group, $P < 0.05$ and $P < 0.01$, respectively

non-significant difference at week 5 between groups and as compared together with baseline. Total cysteine was significantly lower in the M group at weeks 1, 3, and 5 when compared with the C group, exhibiting a smaller increase at week 3 when compared to baseline. Plasma serine was higher than baseline in both groups as early as week 1 ($P < 0.0001$), but was significantly lower in the M group than in the C group overall ($P < 0.05$) and at weeks 3 ($P < 0.05$) and 5 ($P < 0.01$). In contrast, the concentrations of other amino acids such as arginine and citrulline, which exhibited slight but significant changes over time, did not differ as a function of diet. Glycine (Table 2) was affected neither by time nor by the nature of the diet. Plasma ADMA concentrations significantly increased over time, being higher at weeks 3 and 5 than at baseline ($P < 0.01$), but they were not affected by the nature of the diet.

Blood pressure and left ventricular mass

Systolic blood pressure values tended to be lower in the M group ($0.05 < P < 0.10$) and DBP and MAP were significantly lower in the M group when compared to the C group (Fig. 2). When the values from week 1 to week 6 were taken together, the SBP was 127.2 ± 2.0 mmHg in the M group vs. 135.0 ± 1.6 mmHg in the C group ($P < 0.01$), the DBP was 67.2 ± 0.9 mmHg (M group) vs. 71.6 ± 1.4 mmHg (C group) ($P < 0.01$), and the MAP was 87.5 ± 1.1 mmHg (M group) vs. 93.2 ± 1.2 mmHg (C group) ($P < 0.01$). The left ventricular mass index, measured at week 6, was significantly lower in the M group than in the C group (Table 3).

Hemodynamics and plasma NO*x* at week 5–6

At the end of the experiment, acetylcholine injection (Table 3) resulted in a similar decrease in blood pressure (average 18.0 ± 3.8 mmHg—decrease in MAP compared to baseline value) and the tail BFI (average—43.3 ± 5.4% of the baseline value). The HR also increased similarly after the acetylcholine injection (13 ± 2% increment over baseline value, Table 3). Plasma nitrite + nitrate concentration did not differ between groups (Table 3).

Discussion

As expected, methionine supplementation resulted in a marked HHcy. Interestingly, methionine supplementation did not result in steady-state HHcy. Starting from the marked HHcy at weeks 1 and 3 after supplementation, the decrease in HHcy evidenced in week 5 clearly formed part of a complex adaptation of the sulfur amino acid metabolism. Glycine and serine are closely implicated in sulfur amino acid metabolism, with serine being both a major methyl donor for Hcy remethylation (generating glycine) and an obligatory substrate for transsulfuration [11]. Plasma glycine and serine are good markers of changes to the sulfur amino acid metabolism [33]. In the present study, the decrease in plasma serine observed in the methionine group (when compared to the control group), together with a lack of difference in plasma glycine, strongly suggests an increase in the transsulfuration pathway that would have favored the disposal of Hcy. Indeed, transsulfuration is expected

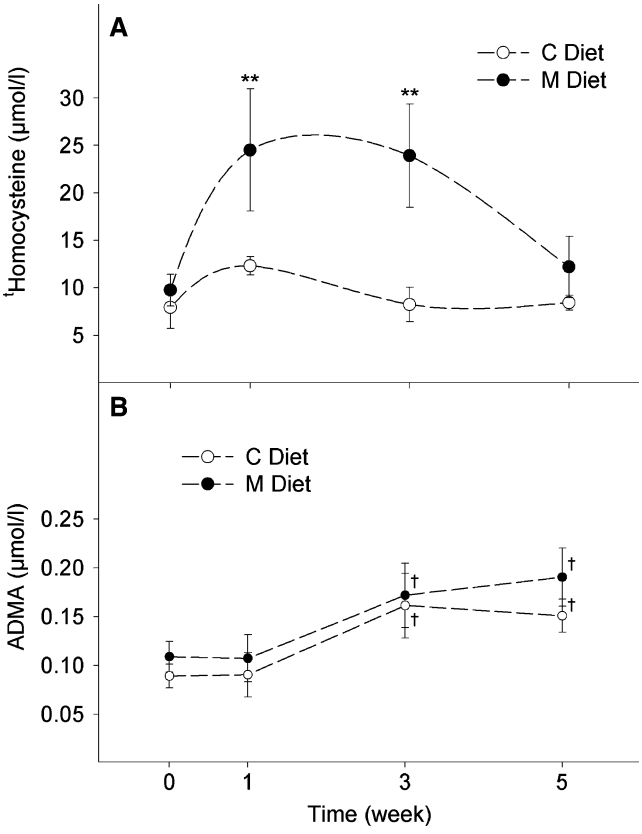

Fig. 1 Plasma concentrations of (total) homocysteine (panel A) and asymmetric dimethyl-arginine (ADMA, panel B) during 5 weeks in rats fed a control (C) or methionine-supplemented (M) diet. Values are means ± SEM. Plasma homocysteine was affected by both time and diet ($P < 0.05$) and plasma ADMA was affected by time ($P < 0.0001$). **: significantly different from control group, $P < 0.01$. †: significantly different from week 0, $P < 0.05$

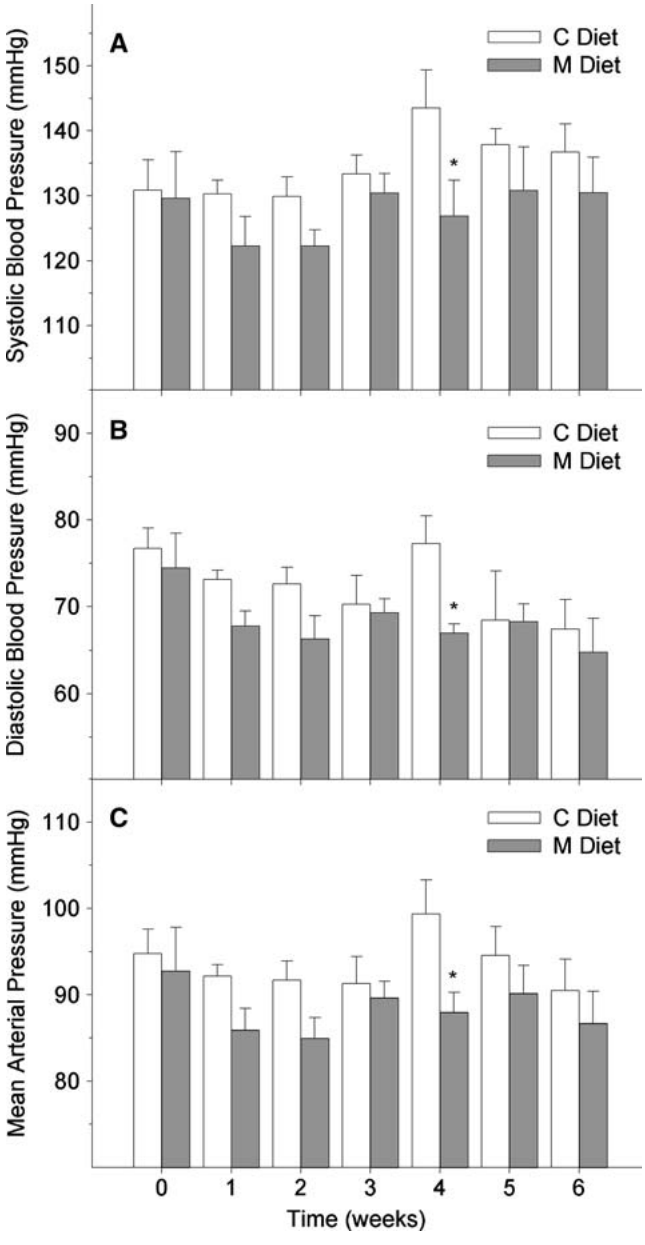

Fig. 2 Systolic blood pressure (SBP, panel A), diastolic blood pressure (DBP, panel B) and mean arterial pressure (MAP, panel C) values over 6 weeks in rats fed a control (C) or methionine-supplemented (M) diet. Values are means ± SEM. SBP tended to be lower ($0.05 < P < 0.10$), and DBP and MAP were significantly lower ($P < 0.05$) in the M group as compared to the C group. *: significantly different from C diet, $P < 0.05$

to be activated with a high methionine intake [11]. Furthermore, the fact that plasma cysteine was lower in the methionine-supplemented group as early as week 1 suggests that an early increase in the disposal of cysteine in the M group may have favored the onset of an increase in transsulfuration. Finally, adaptation resulted in similar plasma methionine concentrations in the supplemented and control groups, i.e., no accumulation of methionine following methionine supplementation. The rich protein content of the basal diet may have been implicated in these complex changes, presumably through a higher intake of serine [25, 26] that could have favored the disposal of Hcy in the context of adaptation by the liver to a high-methionine diet, as described by Finkelstein and Martin [34]. One major finding of the present work is that plasma ADMA was not affected by methionine supplementation, despite marked HHcy. Plasma ADMA significantly rose in both groups, but final level was still low as compared to other literature data on animals and humans [24, 30, 35, 36]. This present rise, however, suggests that ADMA may naturally and slowly accumulate during development and aging, although other longitudinal data are lacking to determine whether there is a general correlation between ADMA and age [35]. Despite the very marked degree of HHcy as evidenced at weeks 1 and 3, plasma ADMA concentrations were not higher in the methionine group. As pointed out in the introduction, in a non-acute dietary setting, an HHcy-induced rise in

Table 3 Hemodynamics, as assessed by the response to an acetylcholine challenge, left ventricular mass index and plasma nitrate + nitrite, after 5–6 weeks in rats fed a control (C) or methionine-supplemented (M) diet

	C diet	M diet
Effect of acetylcholine on		
Mean arterial pressure (mmHg)	−20.6 ± 6.6	−15.8 ± 5.5
Tail blood flow index (%)	−45.6 ± 10.2	−41.5 ± 6.8
Heart rate (%)	+13.9 ± 3.5	+12.3 ± 3.4
Left ventricular mass index (mg/g)	0.449 ± 0.008	0.409 ± 0.009*
Plasma NO*x* (μmol/l)	74.1 ± 13.3	59.6 ± 8.4

Values are means ± SEM. *: significantly different from C diet, $P < 0.05$

ADMA has only been reported in the context of a methionine-enriched and folic acid and choline-depleted diet in monkeys [24]. First, this naturally suggests that this discrepancy may be related to the true metabolic nature of the dietary model for HHcy. A concomitant depletion of folic acid and choline in the diet induces an important deficiency in methyl donors that markedly impairs remethylation and favors the demethylation of methionine into Hcy [37, 38]. Second, the rich protein content in the basal diet may also be implicated. The modulation of plasma ADMA during HHcy, as with other conditions, has mostly been documented as being related to an oxidative stress-mediated down-regulation of ADMA hydrolysis [39–41], while a protein-rich diet (26%, similar to that used during the present experiment) has been shown to reduce oxidative stress when compared to a standard (14%) protein diet in rats [42]. In conclusion, our study questions the general view that Hcy always has an impact upon plasma ADMA in experimental short/medium-term HHcy [39], as it has also been questioned in human (acute) experimental HHcy [21, 22] and in other situations [23, 43, 44].

The last important result in the present study is that although it induced marked HHcy, the methionine-enriched diet resulted in a slightly but significantly lower blood pressure (5.7 mmHg-lower MAP). According to the integration theory [i.e., the cardio phenotype captures the integrated effect of blood pressure over an individual's lifetime, 45], the significantly lower left ventricular mass at week 6 is a clear confirmation that enrichment of the diet with methionine did slightly improve hemodynamic control throughout the experiment. Some authors have reported no significant changes to SBP in Wistar-Hanover or Sprague-Dawley rats supplemented with methionine [46, 47], and paradoxical effects of methionine enrichment on SBP have been reported recently in some models of primary and secondary hypertension [46, 48]. Furthermore, at the end of the experiment, the methionine supplementation caused no alterations in vascular reactivity and no significant changes to plasma NO*x*. Therefore, the present data indicate that the global HHcy had not so far induced any alterations to the basal or stimulated production of NO. Although many mechanisms of Hcy-induced vascular dysfunction have been documented, the prevalent view is that Hcy alters NO production. However, this association is not always consistent because most experimental models of HHcy may mask different metabolic conditions. Recent data strongly suggest that the adverse effects of Hcy on health may only occur when other aspects of the nutrient/metabolic status are altered, particularly folate and methylation [27, 49]. Although we cannot rule out the possibility that the methionine, enriched diet used during this study could eventually increase the risk of atherosclerosis, the results clearly suggest that, in this favorable overall dietary context, there is rather a positive impact of methionine supplementation on cardiovascular risk. We suggest that the absence of adverse effects of HHcy on vascular reactivity, which unmasked positive effects of methionine supplementation on blood pressure, may have been linked to a lack of effect of HHcy on ADMA. This is in line with the emerging view that ADMA is crucial to the initiation of vascular dysfunction and is an independent factor of cardiovascular risk [18, 50].

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