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Determination of asymmetric and symmetric dimethylarginines in plasma of hyperhomocysteinemic subjects

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Summary. The aim of this study was to investigate the possible relationship among dimethylarginines (asymmetric, ADMA; symmetric, SDMA) and homocysteine (Hcy) levels in subjects affected by chronic, mild to intermediate, hyperhomocysteinemia.

ADMA and SDMA were assayed by an optimised HPLC method in 75 patients (Hcy = 20.8 μmol/L, 17.1–30.2; median and percentile range) and, for comparison, in 85 healthy subjects (Hcy = 8.0 μmol/L, 7.0–9.1). In controls, the cut-off values were set at 0.61 μmol/L for ADMA and 0.56 or 0.48 μmol/L for male and female SDMA, respectively. In patients, ADMA and SDMA levels were increased ($p < 0.001$) with respect to controls, but no correlation with Hcy was observed. Hyperhomocysteinemic subjects showed a different behaviour in respect to ADMA and SDMA levels and this allowed their stratification in 3 subgroups characterized by ADMA and SDMA in the normal range, only SDMA, or both ADMA and SDMA over the cut-off values. A lack of correlation with Hcy was again observed, thus minimizing the direct role of Hcy on ADMA and SDMA metabolism and suggesting the need for further studies on this issue.

Keywords: Asymmetric dimethylarginine – Symmetric dimethylarginine – Hyperhomocysteinemia – Plasma

Introduction

Nitric oxide (NO), synthesized from L-arginine (L-Arg) by nitric oxide synthases (NOSs: EC 1.14.13.39), is the major endothelium-derived mediator for the maintenance of vascular tone (Moncada and Higgs, 1993; Tomasian et al., 2000). Among its effects, there is the inhibition of platelet adhesion and aggregation, thus contributing to the antithrombotic properties of intact vascular wall (Radomski et al., 1990; Cooke et al., 1997). Several clinical studies substantiate the role of increased plasma homocysteine (Hcy) concentrations as a risk factor for occlusive vascular disease and thrombosis (D'Angelo and Selhub, 1997; Tawakol et al., 1997; Guba et al., 1999). Although still under discussion, one of the mechanisms proposed for the pathological activity of Hcy, is the capacity to induce the loss of NO-mediated inhibition of platelet aggregation (Leoncini et al., 2003; Stuhlinger et al., 2001).

Proteins containing L-Arg are methylated *in vivo* by different highly specific N-methyltransferases, generating N-monomethyl-L-arginine (MMA), N,N-dimethyl L-arginine (asymmetric dimethylarginine; ADMA) and N,N′-dimethyl L-arginine (symmetric dimethylarginine; SDMA) that are released in plasma as free methylarginines after proteolysis (Boger, 2003). ADMA can be excreted from the body through the kidneys, or catabolized by the dimethylarginine dimethylaminohydrolase enzyme (DDAH) to citrulline and dimethylamine; on the contrary SDMA can be only directly excreted (Boger, 2003). ADMA inhibits NOS and DDAH enzyme activity (Vallance et al., 1992a; Tran et al., 2003), while both ADMA and SDMA interfere with L-Arg transport across cell membranes via the transporter Y^+ system (Closs et al., 1997; Tsikas et al., 2000); also Hcy influences such transport in a concentration dependent manner (Leoncini et al., 2003). Therefore endogenous NO concentration can be regulated directly by inhibiting NOS activity and/or by indirectly decreasing the availability of L-Arg.

Elevated ADMA level has been reported in humans during experimental hyperhomocysteinemia (Boger et al., 2001), in elderly patients with stroke (Yoo and Lee, 2001), in people affected by atherosclerosis, heart failure

and hypertension (Boger, 2003; Kielstein et al., 2003). In some of these studies, SDMA levels were not modified in respect to control range, whereas in some others the dosage was lacking. Increased levels of both SDMA and ADMA have been observed in subjects affected by chronic renal failure (Vallance et al., 1992b; Petterson et al., 1997; Kielstein et al., 1999) or peripheral arterial occlusive disease with manifested atherosclerosis (Boger et al., 1997).

In the light of growing interest on the role for ADMA as a risk factor for endothelial dysfunction, several analytical assays based on the HPLC technique have been proposed for the evaluation of dimethylarginines. However these methods differ for the addition (Pi et al., 2003; Teerlink et al., 2002; Marra et al., 2003) or omission (Petterson et al., 1997; Chen et al., 1997; Unceta et al., 2002; Holven et al., 2003) of the internal standard, the sample clean-up and the analytical conditions (Unceta et al., 2002; Marra et al., 2003; Heresztyn et al., 2004). This gives rise to widely differing dimethylarginine ranges even in healthy subjects (Unceta et al., 2002; Kielstein et al., 2001) with the ADMA and SDMA ''reference range'' still to be standardized. The aim of this study was at first to optimise the analytical procedure for dimethylated amino acids determination in order to define our cut-off values for both ADMA and SDMA in healthy subjects. Secondly, we were prompted to search for a possible relationship among Hcy, ADMA and SDMA in subjects affected by chronic, mild to intermediate, hyperhomocysteinemia.

Materials and methods

Study population

The study cohort included 85 healthy controls (blood donors, 35 males, age 43.1 ± 14.9 yr and 50 females, age 37.9 ± 16.1 yr) (mean ± SD) and 75 hyperhomocysteinemic patients (42 males, age 56.3 ± 13.5 yr and 33 females, age 52.8 ± 18.7 yr). The enrolled patients were recruited, after informed consent, from in- and out-patients referring to the Dept. Laboratory Medicine of IRCCS H San Raffaele. Hcy was considered normal when $<$12.0 and $<$14.5 μmol/L for women and men, respectively (Fermo et al., 1998). All the patients had hyperhomocysteinemia associated with a normal (51/75) or moderate impaired renal function (24/75), due to T1 diabetes mellitus or glomerulopathies, and were on insulin therapy only and/or ACE inhibitors treatment (5/24). No patient received vitamin B12 or folate, nor was under methionine loading.

Clinical assays

Sodium citrate blood, drawn in the fasting state, was centrifuged and immediately frozen. Plasma tHcy was detected by HPLC according the method previously reported (Fermo et al., 1998). Serum creatinine was determined using an automated analyser (Modular, Roche Diagnostics, Basel, Switzerland).

ADMA and SDMA extraction and HPLC assay

Previously reported analytical procedures (Petterson et al., 1997; Pi et al., 2000) were optimised as follows: plasma (0.1 mL), added with L-homoarginine as internal standard (IS) (50 μL; 1 nmol) and 7 mol/L HCl (2 μL; 14 μmol), was applied to a cation-exchange SPE cartridge (Phenomenex STRATA SCX 100 mg/mL, Chemtek Analitica, Bologna, Italy) on a Visiprep DL (Supelco, Bellefonte, PA, USA) vacuum system. After activation with methanol (1 mL) and TCA 2% (2 mL), the column was washed with TCA 2% (1 mL), 150 mmol/L phosphate buffer pH 8.0 (1 mL) and methanol (1 mL) and eluted with 1 mL of triethylamine (TEA) solution (2 mL TEA in 100 mL methanol:water; 70:30; v:v). The eluate was dried under nitrogen and the residue dissolved in 0.1 mL bi-distilled water. The sample was mixed with the OPA reagent (1:1; v:v) and after 1 min, aliquot (10 μL) was injected into HPLC (Fermo et al., 1990). The HPLC was equipped with a fluorescent detector (λ_{ecc} 340 nm, λ_{em} 455 nm) and an Ultrasphere ODS Beckman column (Palo Alto, CA, USA) (250 × 4.6 mm, 5 μm) protected by a guard-column LiChrospher RP-18 from Merck (Darmstadt, Germany). The analysis was performed at room temperature, at a flow-rate of 1.1 mL/min with a mobile phase consisting of A (sodium citrate buffer, 50 mmol/L, pH 6.2) and B (distilled water: acetonitrile: methanol; 1:2:2; v:v:v). The gradient analysis started at 25% of phase B which, after 0.5 min, was increased linearly to 30% over 10 min and held for up to 23 min. Then phase B was increased to 80% in 1 min, the column was flushed for 8 min and reconditioned to 25% in 1 min (total run time: 38 min). Linearity was assessed by adding known amounts of ADMA and SDMA to the plasma samples in the final concentration range of 0.31–5.0 μmol/L. Analytical recovery was tested at the same concentration ranges as for linearity. The within-day CV% was determined on seven extractions of a control plasma sample, while the between-day CV% was determined by analysing the same sample for six consecutive days. L-Arg concentration was dosed within the same analytical run.

Creatinine clearance

Creatinine clearance (CrCl) was estimated from the Cockroft-Gault equation (Cockroft and Gault, 1976) which has been found to correlate with the measured CrCl and with the glomerular filtration rate. The cut-off value for impaired renal function was considered $<$ 70 mL/min as previously reported (Kanauchi et al., 2002).

Statistical analysis

All the analyses were performed using the Sigma Stat (Statistical Analysis System, version 2.03) statistical software package (Jandel Scientific GmbH, Herckrath, Germany). Data are reported as median (25^{th}–75^{th} percentile range). The significance of differences between groups was assessed by the Mann-Whitney Rank sum test. Spearman correlation was used to assess the strength of association between variables. The cut-off value was defined at the 90^{th} percentile of the distribution of ADMA and SDMA in controls. The significance level was set at p value $<$0.05.

Results

The chromatograms of a standard mixture (Fig. 1A) and of a patient plasma sample (Fig. 1B) show that ADMA and SDMA were separated with a good resolution both when dimethylarginines were present in approximately 1:1 ratio and when SDMA level was increased in comparison to ADMA. The MMA, well resolved in the standard

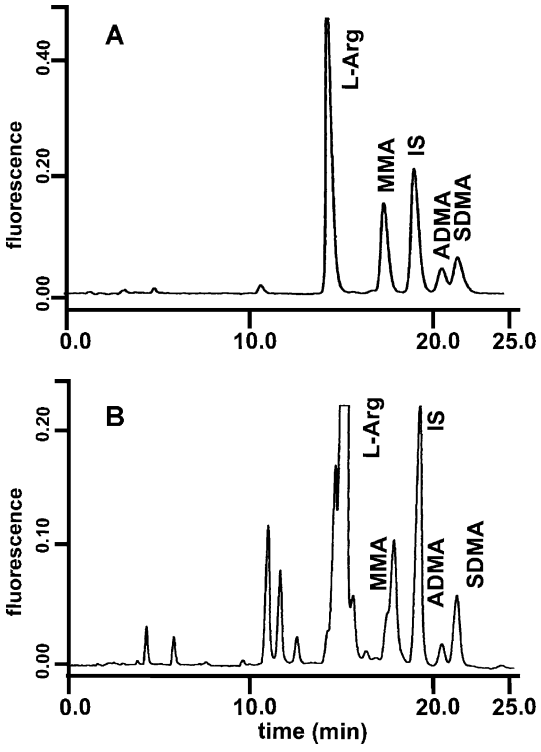

Fig. 1. HPLC chromatograms of **A** a standard mixture of authentic L-Arg, MMA, L-homoarginine (*IS*), ADMA and SDMA at final concentration of 13.6, 4.7, 20.0, 0.76 and 1.2 μmol/L, respectively. **B** Human plasma sample from a patient with impaired renal function containing endogenous ADMA and SDMA at concentrations of 0.90 and 2.6 μmol/L, respectively. The chromatographic conditions are described in the Materials and methods section

solution, was undetectable in biological samples due to the presence of a plasma component eluting close to it. Linear regression analysis, obtained by plotting the area ratio ADMA/IS and SDMA/IS against the known concentration of the two dimethylarginines, yielded $Y = 0.045\ (\pm 0.004) + 0.096\ (\pm 0.001)X\ (\pm SE)$, $r = 1.000$, $S_{x/y} = 0.006$ and $Y = 0.053\ (\pm 0.009) + 0.114\ (\pm 0.004)X\ (\pm SE)$, $r = 0.999$, $S_{x/y} = 0.014$, respectively. The detection limit of the assay was 1.5 pmol, injected at a signal-to-noise ratio of 3:1. The analytical recovery was $98 \pm 3.2\%$ for ADMA, $103 \pm 4.5\%$ for SDMA and $99 \pm 3.0\%$ for L-Arg. The within-day CV% and the between-day CV% were respectively <3.0% and <6.0% for all the analytes.

Healthy controls (Table 1) with normal tHcy and renal function showed similar amounts of ADMA and SDMA without gender differences for ADMA, whereas SDMA levels in males (0.44 μmol/L, 0.38–0.49) were significantly higher ($p < 0.003$) than in females (0.38 μmol/L, 0.31–0.42). The resulting cut-off value was 0.61 μmol/L for ADMA, and 0.56 μmol/L and 0.48 μmol/L for SDMA males and females, respectively. L-Arg concentration in control subjects was 75.0 (67–85) μmol/L. While the selected hyperhomocysteinemic population differed significantly ($p < 0.001$) from the controls for tHcy, ADMA, SDMA and CrCl (Table 1), the arginine levels did not vary (data not shown). In addition, the SDMA concentration was higher than the ADMA one ($p < 0.001$). No correlation between tHcy and ADMA, SDMA or CrCl was found neither in controls nor in patients. In the patients, both SDMA ($r = -0.60$, $p < 0.0001$) and ADMA ($r = -0.24$, $p < 0.04$) correlated with CrCl, and a correlation between ADMA and SDMA ($r = +0.57$, $p < 0.0001$) was also observed.

Based on the observation that the patient population had SDMA levels widely distributed (60% of values in the highest quartile), associated to a more homogeneous ADMA distribution (only 21% above the cut-off value) (Fig. 2), the patients were stratified into three subgroups showing ADMA and SDMA in the normal range (A), only SDMA outside the cut-off value (B), and both ADMA and SDMA above the cut-off value (C). The three groups (Table 2) differed in ADMA, SDMA and CrCl but had similar tHcy concentrations. According to this new distribution, patients with impaired renal function resulted

Table 1. Plasma tHCY, ADMA, SDMA and renal function in controls and patients

Groups	tHCY (μmol/L)	ADMA (μmol/L)	SDMA (μmol/L)	Creatinina (mg/dL)	CrCl (mL/min)
Controls (n = 85)	8.0 (7.0–9.1)	0.44 (0.34–0.56)	0.40 (0.33–0.47)	0.75 (0.5–0.85)	108.4 (97.7–123.5)
Patients (n = 75)	20.8* (17.1–30.2)	0.57* (0.40–0.69)	0.70*,§ (0.49–1.19)	0.91* (0.71–1.41)	83.3* (54.7–98.5)

Data are reported as median (25^{th}–75^{th} percentile range)
* $p < 0.001$ *vs* controls, § $p < 0.001$ *vs* ADMA in patients
Cut-off value for creatinine was 1.25 and 1.10 mg/dL for males and females, respectively. The CrCl cut-off value for impaired renal function was considered <70 mL/min

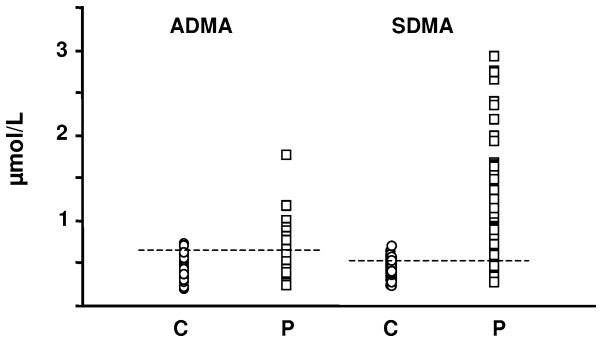

Fig. 2. Distribution of ADMA and SDMA plasma concentrations in healthy controls (C; n = 85) and hyperhomocysteinemic subjects (P; n = 75). Each point represents 1 subject of the two classes described in Table 1. Cut-off (- - -) values for ADMA and SDMA

mainly included in the C subgroup (12/15), while only 12/30 fell in the B group and none at all into the A group. Thus, creatinine and CrCl values were significantly higher in the B and C *vs* A, although the values of the B group did not fall in the pathological range (Kanauchi et al., 2002). In the A group, the only correlation found was between ADMA and SDMA ($r = 0.52$, $p < 0.003$). In the groups B and C, SDMA strongly correlated with CrCl ($r = -0.52$, $p < 0.003$; $r = -0.72$, $p < 0.002$, respectively); however, 18/30 patients of the B group had elevated SDMA levels associated to a normal renal function.

Of all the patients, only one could not be included in these subgroups having both SDMA (0.49 μmol/L) and renal function at normal levels, associated with elevated ADMA (0.94 μmol/L) and Hcy (124 μmol/L).

Discussion

The present study focussed on the interaction between tHcy, ADMA and SDMA levels in fasting chronic, mild to moderate, hyperhomocysteinemic patients. First it was necessary to optimise the analytical procedure for the quantification of the two dimethylarginines and to define their range and cut-off values in our healthy controls.

In respect to previous procedures (Petterson et al., 1997; Pi et al., 2000), the method used in the present study is characterized by the total recovery of ADMA and SDMA from the SPE extraction step, allowing analysis using only 0.1 mL plasma instead of 1.5 mL (Petterson et al., 1997) or 0.5 mL (Pi et al., 2000). For the HPLC separation, the pH value and the composition of the mobile phase were found to have a marked effect on the peak shape and on the resolution of the ADMA and SDMA derivatives. In our conditions, peaks overlapping never compromised the analysis of the dimethylarginines even in pathological plasma with 3-fold increased SDMA/ADMA ratio. In respect to a recently published procedure (Heresztyn et al., 2004), our method allowed the quantification of ADMA, SDMA and L-Arg within the same analytical run. This could be possible thanks to the quantitative recovery of the two dimethylarginines from the SPE extraction and to the very high sensitivity of the pre-column derivatization agent (OPA) that allowed to inject very small amount of the biological sample.

The ADMA and SDMA basal values reported in the literature (Petterson et al., 1997; Marra et al., 2003; Unceta et al., 2002; Kielstein et al., 2001) are widely distributed (0.30–1.30 μmol/L and 0.4–3.1 μmol/L for ADMA and SDMA, respectively), differing, in some cases, >10-fold from those evaluated by liquid chromatography-tandem mass spectrometry (Vishwanathan et al., 2000). ADMA and SDMA concentrations detected in our controls were in line with the lower values of the above-indicated literature range and, in agreement with other authors (Teerlink et al., 2002), only SDMA differed with gender, giving rise to a new hypothesis on hormonal control of SDMA levels.

Table 2. Plasma tHCY, ADMA, SDMA and renal function in patient subgroups

Groups	tHCY (μmol/L)	ADMA (μmol/L)	SDMA (μmol/L)	Creatinine (mg/dL)	CrCl (mL/min)
A (n = 29)	20.3 (15.0–27.0)	0.47 (0.40–0.50)	0.46 (0.40–0.50)	0.82 (0.64–0.91)	95.5 (83.4–112.2)
B (n = 30)	20.4 (15.0–28.8)	0.58 (0.40–0.60)	0.89$^{\#}$ (0.70–1.20)	0.99* (0.73–1.56)	79.11* (50.2–97.3)
C (n = 15)	22.1 (19.8–45.3)	0.89$^{\bullet}$ (0.82–0.96)	1.75$^{\#}$ (0.90–2.50)	1.71$^{\#,\S}$ (0.81–5.29)	43.8$^{\#,\S}$ (11.65–61.1)

Data are reported as median (25^{th}–75^{th} percentile range)
$^{\bullet}$ $p < 0.001$ *vs* A and B, $^{\#}$ $p < 0.001$ *vs* A, * $p < 0.003$ *vs* A, § $p < 0.01$ *vs* B
Cut-off value for creatinine was 1.25 and 1.10 mg/dL for males and females, respectively. The CrCl cut-off value for impaired renal function was considered <70 mL/min

With regard to the aim of this study concerning the relation between Hcy and dimethylarginine pathways, the results seem to diminish the role of Hcy in ADMA and SDMA metabolism as evidenced in Table 2. This finding is in contrast with the effect of Hcy on increased ADMA level shown in "*in vitro*" studies on cell cultures supplemented with non-physiological high Hcy concentration (Stuhlinger et al., 2001; Boger et al., 2000a), or in "*in vivo*" animal models with induced hyperhomocysteinemia (Boger et al., 2000b) or in subjects after acute treatments like oral methionine loading or folic acid supplementation (Boger et al., 2001; Holven et al., 2003). As recently suggested (Jonasson et al., 2003), the metabolic Hcy pathways could be modified after methionine loading, thus we might suppose that the chronic state of our hyperhomocysteinemic subjects (mild to moderate) precluded our finding evidence of a correlation between tHcy and one, or both, dimethylated amino acids.

In agreement with other studies (Vallance et al., 1992; Petterson et al., 1997; Kielstein et al., 1999), the SDMA level appears to be related to renal function as evidenced by its good correlation with CrCl in all patients and even more in the C subgroup where SDMA was 4-fold higher than in controls and 80% of the patients had pathological CrCl. On the contrary, Hcy never correlated with renal function, suggesting that, in our patients, hyperhomocysteinemia could be more likely caused by an impairment in extrarenal metabolism rather than by renal failure, as reported (van Guldener et al., 1998).

A peculiar behaviour was presented by the hyperhomocysteinemic population of the B group, with elevated SDMA but ADMA in the normal range. The higher amounts of SDMA respect to ADMA may reflect the fact that SDMA can only be disposed off by renal excretion whereas ADMA is also degraded by the DDAH enzyme that is present in all tissues (Boger, 2003). The normal ADMA level in this group could be explained for 5 patients receiving ACE inhibitors therapy for hyperthension, a treatment known to reduce ADMA levels (Chen et al., 2002). A correlation between SDMA and CrCl was found also in the B group, in which 40% of the subjects had impaired renal function. However, it is important to note that some patients of the C group (20%) and the main part of B group (60%) had normal renal function, suggesting that mechanism(s) other than reduced kidney functionality might contribute to plasma accumulation of one or both dimethylarginines. The homeostasis of these two amino acids is related to different metabolic pathways like the methylation of proteins containing L-Arg by *N*-methytransferases, the proteolytic enzyme release from methylated proteins, the transport into endothelial cells by the transporter Y^+ carrier, catabolism and excretion (Boger, 2003; Vallance et al., 1992; Closs et al., 1997). Consequently, the modulation of ADMA levels might be closely related to the up- or down-regulation of the activity of DDAH enzyme (Achan et al., 2002; Dayoub et al., 2003; Cooke, 2003; Ueda et al., 2003), while, excluding renal dysfunction, an increase only in SDMA levels could depend on its biosynthesis via the highly specific *N*-methytransferase (protein methylase II) (Boger, 2003), on the activation of the transporter Y^+ system and/or on a still unknown catabolic route.

In conclusion, our results on chronic mild to intermediate hyperhomocysteinemic patients minimize the direct role of tHcy on ADMA and SDMA plasma levels. This is of considerable clinical interest since elevation of plasma ADMA concentration by Hcy-induced impairment of DDAH enzyme activity (Stuhlinger et al., 2001) has been suggested to be a possible pathomechanism in homocysteine-associated vascular pathology. Moreover, the observed increase in SDMA associated with normal renal function and baseline ADMA values suggests the importance to include the determination of both dimethylarginines in the design of clinical trials in order to evidence also for SDMA any possible involvement in pathological conditions.

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References

Achan V, Tran CTL, Arrigoni F, Whitley GStJ, Leiper JM, Vallance P (2002) All-*trans*-retinoic acid increases nitric oxide synthesis by endothelial cells: a role for the induction of dimethylarginine dimethylaminohydrolase. Circ Res 90: 764–769

Boger RH (2003) The emerging role of asymmetric dimethylarginine as a novel cardiovascular risk factor. Cardiovasc Res 59: 824–833

Boger RH, Bode-Boger SM, Thiele W, Junker W, Alexander K, Frolich JC (1997) Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. Circulation 95: 2068–2074

Boger RH, Sydow K, Borlak J, Thum T, Lenzen H, Schubert B, Tsikas D, Bode-Boger SM (2000a) LDL cholesterol upregulates synthesis of asymmetrical dimethylarginine in human endothelial cells: involvement of S-adenosylmethionine-dependent methyltransferases. Circ Res 87: 99–105

Boger RH, Bode-Boger SM, Sydow K, Heistad DD, Lentz SR (2000b) Plasma concentration of asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide synthase, is elevated in monkeys with hyperhomocyst(e)inemia or hypercholesterolemia. Arterioscler Thromb Vasc Biol 20: 1557–1564

Boger RH, Lentz SR, Bode-Boger SM, Knapp HR, Haynes WG (2001) Elevation of asymmetrical dimethylarginine may mediate endothelial dysfunction during experimental hyperhomocyst(e)inaemia in humans. Clin Sci 100: 161–167

Chen BM, Xia LW, Zhao RQ (1997) Determination of N^G,N^G-dimethylarginine in human plasma by high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl 692: 467–471

Chen J-W, Hsu N-W, Wu T-C, Lin S-J, Chang M-S (2002) Long-term angiotensin-converting enzyme inhibition reduces plasma asymmetric dimethylarginine and improves endothelial nitric oxide bioavailability and coronary microvascular function in patients with syndrome X. Am J Cardiol 90: 974–982

Closs EI, Basha FZ, Habermeier A, Forstermann U (1997) Interference of L-arginine analogues with L-arginine transport mediated by the y^+ carrier hCAT-2B. Nitric Oxide 1: 65–73

Cockroft DW, Gault MH (1976) Prediction of creatinine clearance from serum creatinine. Nephron 16: 31–41

Cooke JP (2003) NO and angiogenesis. Atheroscler [Suppl 4]: 53–60

Cooke JP, Dzau VJ (1997) Nitric oxide synthase: role in the genesis of vascular disease. Annu Rev Med 48: 489–509

D'Angelo A, Selhub J (1997) Homocysteine and thrombotic disease. Blood 90: 1–11

Dayoub H, Achan V, Adimoolam S, Jacobi J, Stuhlinger MC, Wang B, Tsao PS, Kimoto M, Vallance P, Patterson AJ, Cooke JP (2003) Dimethylarginine dimethylaminohydrolase regulates nitric oxide synthesis. Genetic and physiological evidence. Circulation 108: 3042–3047

Fermo I, De Vecchi E, Diomede I, Paroni R (1990) Serum amino acid analysis with pre-column derivatization: comparison of the *o*-phtalaldehyde and n,n-diethyl-2,4-dinitro-5-fluoroaniline methods. J Chromatogr 534: 23–35

Fermo I, Arcelloni C, Mazzola G, D'Angelo A, Paroni R (1998) High-performance liquid chromatographic method for measuring total plasma homocysteine levels. J Chromatogr B Biomed Sci Appl 719: 31–36

Guba SC, Fonseca V, Fink LM (1999) Hyperhomocysteinemia and thrombosis. Semin Thromb Hemost 25: 291–299

Heresztyn T, Worthley MI, Horowitz JD (2004) Determination of L-arginine and N^G, $N^{G'}$-dimethyl-L-arginine in plasma by liquid chromatography as AccQ-Fluor^{TM} fluorescent derivatives. J Chromatogr B Biomed Sci Appl 805: 325–329

Holven KB, Haugstad TS, Holm T, Aukrust P, Ose L, Nenseter MS (2003) Folic acid treatment reduces elevated plasma levels of asymmetric dimethylarginine in hyperhomocysteinaemic subjects. Br J Nutr 89: 359–363

Jonasson TF, Hedner T, Hultberg B, Ohlin H (2003) Hyperhomocysteinaemia is not associated with increased levels of asymmetric dimethylarginine in patients with ischaemic heart disease. Eur J Clin Invest 33: 543–549

Kanauchi M, Akai Y, Hashimoto T (2002) Validation of simple indices to assess insulin sensitivity and pancreatic Beta-cell function in patients with renal dysfunction. Nephron 92: 713–715

Kielstein JT, Boger RH, Bode-Boger SM, Schaffer J, Barbey M, Koch KM, Frolich JC (1999) Asymmetric dimethylarginine plasma concentrations differ in patients with end-stage renal disease: relationship to treatment method and atherosclerotic disease. J Am Soc Nephrol 10: 594–600

Kielstein JT, Bode-Boger SM, Frolich JC, Haller H, Boger RH (2001) Relationship of asymmetric dimethylarginine to dialysis treatment and atherosclerotic disease. Kidney Int Suppl 78: S9–S13

Kielstein JT, Bode-Boger SM, Klein G, Graf S, Haller H, Fliser D (2003) Endogenous nitric oxide synthase inhibitors and renal perfusion in patients with heart failure. Eur J Clin Invest 33: 370–375

Leoncini G, Pascale R, Signorello MG (2003) Effects of homocysteine on L-arginine transport and nitric oxide formation in human platelets. Eur J Clin Invest 33: 713–719

Marra M, Bonfigli AR, Testa R, Testa I, Gambini A, Coppa G (2003) High-performance liquid chromatographic assay of asymmetric dimethylarginine, symmetric dimethylarginine, and arginine in human plasma by derivatization with naphthalene-2,3-dicarboxaldehyde. Anal Biochem 318: 13–17

Moncada S, Higgs A (1993) The L-arginine-nitric oxide pathway. N Engl J Med 329: 2002–2012

Petterson A, Uggla L, Backman V (1997) Determination of dimethylated arginines in human plasma by high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl 692: 257–262

Pi J, Kumagai Y, Sun G, Shimojo N (2000) Improved method for simultaneous determination of L-arginine and its mono- and dimethylated metabolites in biological samples by high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl 742: 199–203

Radomski MW, Palmer RM, Moncada S (1990) An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proc Natl Acad Sci USA 87: 5193–5197

Stuhlinger MC, Tsao PS, Her JH, Kimoto M, Balint RF, Cooke JP (2001) Homocysteine impairs the nitric oxide synthase pathway: role of asymmetric dimethylarginine. Circulation 104: 2569–2575

Tawakol A, Omland T, Gerhard M, Wu JT, Creager MA (1997) Hyperhomocyst(e)inemia is associated with impaired endothelium-dependent vasodilation in humans. Circulation 95: 1119–1121

Teerlink T, Nijveldt RJ, de Jong S, van Leeuwen PAM (2002) Determination of arginine, asymmetric dimethylarginine, and symmetric dimethylarginine in human plasma and other biological samples by high-performance liquid chromatography. Anal Biochem 303: 131–137

Tomasian D, Keaney JF, Vita JA (2000) Antioxidants and the bioactivity of endothelium-derived nitric oxide. Cardiovasc Res 47: 426–435

Tran CTL, Leiper JM, Vallance P (2003) The DDAH/ADMA/NOS pathway. Atherosclerosis [Suppl 4]: 33–40

Tsikas D, Boger RH, Sandmann J, Bode-Boger SM, Frolich JC (2000) Endogenous nitric oxide synthase inhibitors are responsible for the L-arginine paradox. FEBS Lett 478: 1–3

Ueda S, Kato S, Matsuoka H, Kimoto M, Okuda S, Morimatsu M, Imaizumi T (2003) Regulation of cytokine-induced nitric oxide synthesis by asymmetric dimethylarginine: role of dimethylarginine dimethylaminohydrolase. Circ Res 92: 226–233

Unceta N, San Vicente A, Goicolea MA, Salles J, Barrio RJ (2002) Determination of methylarginines in human plasma by HPLC with precolumn derivatization using naphthalenedicarboxaldehyde as fluorogenic agent. J Sep Sci 25: 665–670

Vallance P, Leone AM, Calver A, Collier J, Moncada S (1992a) Endogenous dimethylarginine as an inhibitor of nitric oxide synthesis. J Cardiovasc Pharmacol 20 [Suppl 12]: S60–S62

Vallance P, Leone A, Calver A, Collier J, Moncada S (1992b) Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet 339: 572–575

van Guldener C, Donker AJM, Jakobs C, Teerlink T, de Meer K, Stehouwer CDA (1998) No net renal extraction of homocysteine in fasting humans. Kidney Int 54: 166–169

Vishwanathan K, Tackett RL, Stewart JT, Bartlett MG (2000) Determination of arginine and methylated arginines in human plasma by liquid chromatography-tandem mass spectrometry. J Chromatogr B Biomed Sci Appl 748: 157–166

Yoo JH, Lee SC (2001) Elevated levels of plasma homocyst(e)ine and asymmetric dimethylarginine in elderly patients with stroke. Atherosclerosis 158: 425–430

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