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Asymmetric and symmetric dimethylarginines are of similar predictive value for cardiovascular risk in the general population

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ABSTRACT

Objectives: Asymmetric dimethylarginine (ADMA) has raised considerable interest, as it is an endogenous inhibitor of nitric oxide synthesis. While increased plasma levels of ADMA have been reported in different cardiovascular disease states, its association with symmetric dimethylarginine (SDMA) has not been evaluated in a prospective population-based study.
Methods and results: We performed a mass spectrometry-based analysis of ADMA and SDMA in the plasma of 572 participants of the Bruneck study. Levels of ADMA and SDMA were significantly correlated with each other ($r = 0.189$, $p < 0.001$). Age and parameters of renal function, however, showed a stronger influence on SDMA than on ADMA. Both ADMA and SDMA were predictive of cardiovascular disease in multivariate analysis and the associated hazard ratios over the 5-year observation period were of similar strength: 3.86 (1.36–10.9) and 7.91 (1.94–32.3) for ADMA and SDMA, respectively ($p = 0.011$ and 0.004). Separate analyses focused on quintile groups of SDMA revealed that the increase in cardiovascular risk was mainly confined to the top category (>0.80 μmol/L).
Conclusion: This study argues against an exclusive ADMA effect in mediating cardiovascular risk. Instead, SDMA, its supposedly inactive counterpart, has similar diagnostic value in this large prospective cohort.

1. Introduction

Three methylarginines, asymmetric dimethylarginine (ADMA), N-monomethylarginine (L-NMMA) and symmetric dimethylarginine (SDMA), are found in all human tissues and biological fluids. Methylarginines are generated by the post-translational methylation of arginine residues in proteins. Following proteolysis, free methylarginines are released into the cytosol where they accumulate before being removed to the plasma and cleared into the urine by the kidney. In addition to renal clearance, the asymmetrically methylated arginines, ADMA and L-NMMA, are mainly subject to hydrolysis catalysed by the enzyme dimethylarginine dimethylamino-hydrolase [1].

ADMA and L-NMMA are competitive inhibitors of all three isoforms of nitric oxide synthase (NOS) [2]. As the ADMA blood concentration is about 10-fold higher than that of L-NMMA, it is considered to be the predominant endogenous NOS inhibitor [2]. Elevated concentrations of ADMA have consistently been associated with cardiovascular disease [3–14]. Unlike ADMA, SDMA does not inhibit NOS [2]. Thus, little attention has been paid to this structural isomer and it has not been evaluated in most studies focused on cardiovascular disease (CVD) [4,5,7,9,13,14]. Here we use the gold standard of a mass spectrometric assay to determine dimethylarginines in a prospective cohort and to investigate whether ADMA, SDMA or both predict CVD risk in the general population.

2. Methods

2.1. Study population

Population recruitment was performed as part of the Bruneck Study. The survey area was located in the north of Italy (Bolzano

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Province). At the 1990 baseline evaluation, the study population was recruited as an age- and sex-stratified random sample of all inhabitants of Bruneck aged 40–79 years (125 women and 125 men in the fifth to eighth decades each). Assessments were carried out every 5 years and participation exceeded 90%. The current study focused on the 2000 evaluation and follow-up period between 2000 and 2005. Level of ADMA and SDMA was assessed in a random subsample of 572 (out of the 706 subjects participating in 2000). Special features of the study design and protocol have been described previously in detail [15,16]. The appropriate Ethics Committees approved the study protocol and all study subjects gave their written informed consent before entering the study. Blood samples were drawn after an overnight fast and 12 h abstinence from smoking. All laboratory parameters were assessed by standard methods and the clinical history was recorded as detailed previously [17–19].

2.2. Determination of ADMA and SDMA

A high-performance liquid chromatographic assay with mass spectrometric detection (LC/MS/MS) was used to measure ADMA and SDMA. Briefly, a 25 μL aliquot of sample was mixed with 25 μL of 500 ng/mL monoethylarginine (NMEA, internal standard) and 100 μL 3% trichloroacetic acid to precipitate the plasma proteins. A portion of the supernatant (50 μL) was diluted with 200 μL 0.1% trifluoroacetic acid in deionised water and analysed by LC/MS/MS. The analytes were separated using a Hypercarb column (Thermo Electron Corporation, 100 mm × 4.6 mm, 5 μm). The mobile phase, pumped at 1 mL/min, was a mixture of 95% deionised water and 5% acetonitrile, containing 1 mL/L of trifluoroacetic acid. An API4000 tandem mass spectrometer (MS) fitted with an APCI source operated at 600 °C was used to detect the three analytes. The MS was operated in positive multiple reaction mode (MRM). The precursor ion/product ions measured were: ADMA 203.0/46.3, SDMA 203.0/172.2 and NMEA 203.0/46.3. The assay was linear over the range 50–1000 ng/mL (0.25–5 μmol/L). Within-batch precision at 100 ng/mL was <10% ($n = 138$) and the between-batch precision was <8% ($n = 8$).

2.3. Other parameters

Measurements of osteoprotegerin, myeloperoxidase, stromal-derived factor 1 (SDF-1), receptor activator of nuclear factor kB ligand (RANKL) and matrix metallopeptidase 9 were performed as previously described [19,20]. Glomerular filtration rate was calculated by the abbreviated modification of diet in renal disease equation (GFR-MDRD).

2.4. Cardiovascular disease (CVD)

Myocardial infarction was deemed confirmed when World Health Organization Criteria for definite disease status were met. Ischemic stroke and transient ischemic attacks were classified according to the criteria of the National Survey of Stroke. Vascular mortality included deaths from ischemic stroke, myocardial infarction, rupture of aortic aneurysms and sudden cardiac deaths. The diagnosis of symptomatic peripheral artery disease and angina pectoris required a positive response to the Rose questionnaire with the vascular nature of complaints confirmed by standard diagnostic procedures (ankle-brachial pressure index or angiography and exercise electrocardiogram or coronary angiography). Revascularization procedures (angioplasty and surgery) were carefully recorded. Ascertainment of events or procedures did not rely on hospital discharge codes or the patient's self-report but on a careful review of medical records provided by the general practitioners, death certificates, and Bruneck Hospital files, and the extensive clinical and laboratory examinations performed as part of the study protocols. The primary composite CVD endpoint comprised all cardiovascular events, including ischemic stroke and TIA, myocardial infarction, vascular death and revascularization procedures. Extended composite endpoints considered new onset symptomatic peripheral artery disease and angina.

2.5. Statistical analysis

The data were analyzed using the SPSS 12.0 and BMDP software packages. Variables with a skewed distribution were $\log_e$-transformed to satisfy the assumption of normality and constant variance of the residuals. Partial correlation coefficients corrected for age and sex or age, sex and creatinine concentration were calculated to estimate the association between ADMA or SDMA and cardiovascular risk factors, life-style characteristics and other parameters. Predictive significance of ADMA or SDMA concentration for cardiovascular events was tested by means of Cox regression analysis. Proportional hazard assumptions were satisfied. Multivariate models included the variables age, sex, previous CVD, hypertension, smoking, social status, diabetes, heart failure, physical activity, waist circumference, GFR-MDRD and levels of HDL and LDL cholesterol, homocysteine, osteoprotegerin, soluble RANKL, urinary albumin and $\log_e$-transformed C-reactive protein. Separate analyses focused on quintile groups for ADMA and SDMA. A two-sided p-value <0.05 was considered significant.

3. Results

3.1. Population characteristics

Table 1 depicts selected demographic characteristics and risk factors of subjects ($n = 572$) with and without cardiovascular events over the 5-year observation period. Cardiovascular events were strongly associated with age and a history of previous CVD ($p < 0.001$). Social status, physical activity score and waist circumference were also correlated to cardiovascular risk. Among plasma parameters, high sensitivity C-reactive protein (CRP), creatinine, fibrinogen and homocysteine were positively associated with CVD. Additionally, the RANKL and osteoprotegerin emerged as a positive predictor for cardiovascular events.

3.2. Associations of ADMA and SDMA with clinical parameters

Fig. 1A and B shows the skewed distribution of ADMA and SDMA in the general population with a mean of 0.98 and 0.66 μmol/L and a median of 0.95 and 0.63 μmol/L, respectively. Concentrations of ADMA and SDMA were significantly correlated with each other ($r = 0.189$, $p < 0.001$). While ADMA tended to be higher in females (1.00 μmol/L in females vs. 0.96 μmol/L in males, $p = 0.104$), the opposite was the case for SDMA (0.64 μmol/L vs. 0.68 μmol/L in females and males, respectively, $p = 0.010$). Both were markedly elevated in patients with heart failure (New York Heart Association (NYHA) functional classification scale, $p = 0.005$ and $p = 0.003$). This correlation of ADMA and SDMA with the heart failure scale remained significant if subjects with incident CVD were excluded from the analysis ($p = 0.049$ and <0.001). Notably, ADMA showed no statistically significant interaction with most cardiovascular risk factors (Supplemental Table), except for an inverse correlation to social status ($p = 0.005$). Age and parameters of renal function had a much stronger influence on SDMA than ADMA. The correlation between SDMA and the renal parameters creatinine and cystatin C is depicted in the Supplemental Figure ($r_p = 0.452$ and $r_p = 505$

Table 1
Association of baseline demographic and life-style characteristics, vascular risk factors and laboratory parameters with incident cardiovascular disease (2000–2005) in the Bruneck cohort ($N = 572$).

Characteristic[a]	Cardiovascular disease (2000–2005)		p-Value[b]
	No ($n = 529$)	Yes ($n = 43$)	
Demographic variables			
Age (years)	65.7 ± 10.1	73.5 ± 9.7	**<0.001**
Male, sex (%)	46.9	58.1	0.155
Social status—low/medium/high (%)	56.9/23.8/19.3	76.7/11.6/11.6	**0.039**
Previous cardiovascular disease			
Ischemic stroke, myocardial infarction, angina, PAD, revascularizations (%)	14.7	44.2	**<0.001**
Heart failure NYHI scale 0/1/2/≥3 (%)	68.2/17.6/12.9/1.3	30.2/23.3/39.5/7.0	**<0.001**
Life-style and vascular risk variables			
Smoking (%)	15.9	16.3	0.945
Smoking (cigarettes per day)	1.8 ± 5.0	2.9 ± 7.1	0.208
Physical activity score (Baecke)	2.4 ± 0.8	2.0 ± 0.6	**0.011**
Diabetes mellitus (%)	11.9	14.0	0.692
Fasting glucose (mg/dL)	101.4 ± 25.2	104.4 ± 21.0	0.453
Body mass index (kg/m^2)	25.3 ± 3.9	26.3 ± 5.0	0.119
Waist (cm)	90.5 ± 11.5	94.4 ± 12.4	**0.035**
HDL cholesterol (mg/dL)	57.8 ± 14.7	55.3 ± 15.8	0.291
LDL cholesterol (mg/dL)	148.6 ± 35.4	154.5 ± 48.2	0.312
Triglycerides (mg/dL)	135.5 ± 73.0	149.6 ± 90.3	0.341
Homocysteine (μmol/L)	13.1 ± 8.5	15.7 ± 6.4	**0.049**
Hypertension (%)	53.9	62.8	0.259
Systolic blood pressure (mmHg)	138.9 ± 18.2	143.5 ± 22.4	0.122
Diastolic blood pressure (mmHg)	83.9 ± 8.1	82.9 ± 8.1	0.480
Creatinine (mg/dL)	0.88 ± 0.15	0.94 ± 0.22	**0.014**
GFR-MDRD (mL/(min 1.73 m^2))	81.8 ± 14.2	78.1 ± 17.4	0.184
Plasma proteins			
High-sensitivity CRP (mg/L)	3.4 ± 5.0	4.5 ± 5.0	**0.035**
Fibrinogen (mg/dL)	288.6 ± 57.2	317.8 ± 72.1	**0.002**
Soluble RANKL (pmol/L)	1.2 ± 0.9	1.7 ± 2.1	**0.002**
Osteoprotegerin (pmol/L)	4.0 ± 1.4	4.7 ± 1.3	**0.005**

PAD, peripheral artery disease; GFR-MDRD, glomerular filtration rate according to the MDRD formula; CRP, C-reactive protein; RANKL, receptor activator of nuclear factor kB ligand.

[a] Values presented are unadjusted mean ± S.D. or percentages.

[b] p-Values were calculated with the t-test (continuous variables) or the chi-square test (categorical variables). For these computations triglyceride and CRP levels were $\log_e$-transformed because of a markedly skewed distribution. Bold numbers highlight p-values <0.05.

for creatinine and cystatin C, respectively, both correlation coefficients were significant at a $p < 0.001$ level after adjustment for age and sex). Commonly prescribed drugs, including hormone replacement therapy, did not influence ADMA and SDMA. Only statins had a moderate effect in lowering ADMA. Interestingly, SDMA concentrations were positively associated with levels of osteoprotegerin, myeloperoxidase and the CXC chemokine SDF-1alpha. These associations, however, were of moderate strength and, apart from osteoprotegerin, lost statistical significance after adjustment for creatinine.

3.3. Associations of ADMA and SDMA with cardiovascular risk

Table 2 provides the hazard ratios (HR) for cardiovascular events. Notably, an increase in either ADMA or SDMA was associated with an elevated risk of CVD in the general population over the

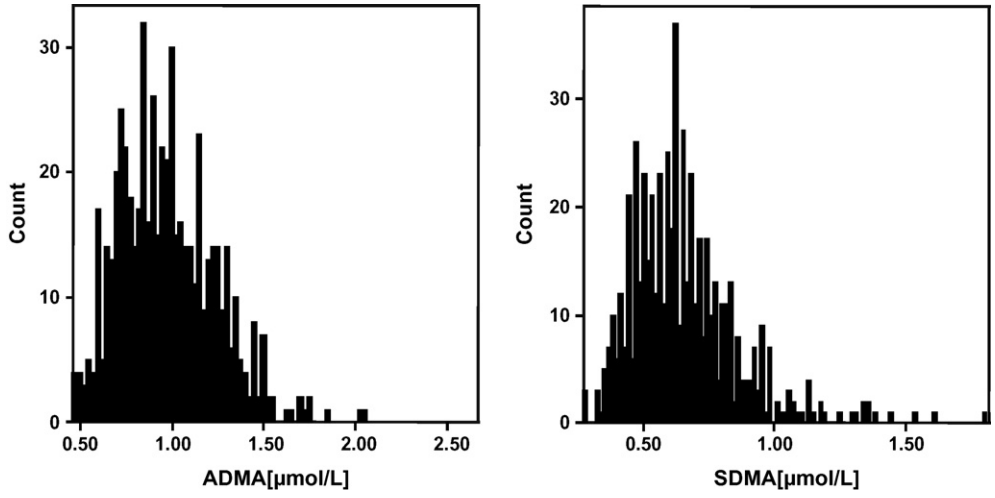

Fig. 1. Distribution of plasma concentrations of (A) ADMA and (B) SDMA in the Bruneck population ($n = 572$).

Table 2
Association of ADMA and SDMA levels with cardiovascular disease risk in the Bruneck study (2000–2005).

Model	Events	Incidence rate[a] per 1000 PY	ADMA		SDMA	
			Hazard ratio 95% CI	*p*-Value	Hazard ratio 95% CI	*p*-Value
Composite endpoint						
Adjusted for age, sex and GFR-MDRD level	43	16.2	4.35 (1.75–10.8)	**0.002**	6.95 (1.98–24.4)	**0.002**
Multivariate Cox model[b]	–	–	3.94 (1.40–11.1)	**0.009**	8.21 (2.19–30.8)	**0.002**
Extended composite endpoints						
Multivariate Cox model[c]	53	20.0	4.15 (1.59–10.8)	**0.001**	5.87 (1.73–20.0)	**0.005**
Multivariate Cox model[d]	57	21.6	3.54 (1.42–8.85)	**0.007**	5.20 (1.56–17.3)	**0.007**
Multivariate Cox model[e]	62	23.6	2.75 (1.13–6.70)	**0.026**	4.56 (1.43–14.6)	**0.011**

Hazard ratios and 95% confidence intervals were derived from Cox models and calculated for a one-unit increase in ADMA and SDMA. Composite endpoint: stroke, TIA, myocardial infarction and vascular death. Bold numbers highlight *p*-values <0.05.

[a] Incidence rates were calculated per 1000 person-years (PY) of follow-up.

[b] Multivariate adjustment: age (years), sex (0, 1), previous cardiovascular disease (0, 1), hypertension (0, 1), number of daily cigarettes, diabetes (0, 1), social status (0, 1, 2), heart failure (NYHI scale), physical activity (Baecke sports score), waist circumference (cm), and levels of HDL and LDL cholesterol (mg/dL), homocystein (mmol/L), osteoprotegerin (pmol/L), soluble RANKL (pmol/L), GF-MDRD (mL/(min 1.73 m^2)), urinary albumin (mg/dL) and $\log_e$-transformed C-reactive protein (mg/L).

[c] Composite endpoint: stroke, TIA, myocardial infarction, vascular death and any revascularization procedure.

[d] Symptomatic peripheral artery disease (intermittent claudication).

[e] De novo angina.

5-year observation period, and the associations were of comparable strength for both parameters. Findings remained significant after multivariate adjustment (see legend to Table 2). Results for composite disease endpoints were consistent with individual disease endpoints, i.e. myocardial infarction ($n = 18$, HR (95% CI): 4.0 (1.1–14.9) and 7.5 (1.5–38.1) for ADMA and SDMA, respectively, $p = 0.039$ and $p = 0.016$), stroke and TIA ($n = 27$, HR (95% CI): 4.6 (0.9–22.6) and 12.2 (1.7–87.9) for ADMA and SDMA, respectively, $p = 0.069$ and $p = 0.013$). Excluding subjects with baseline CVD revealed similar results ($n = 24$, HR (95% CI) for composite endpoint adjusted for age, sex and GFR-MDRD: 6.4 (2.2–18.7) and 9.4 (1.5–57.4) for ADMA and SDMA, respectively, $p = 0.001$ and $p = 0.016$). When SDMA levels were subdivided into quintiles, the increase in cardiovascular risk was mainly confined to the top category (fifth quintile, >0.80 μmol/L, Fig. 2).

Addition of ADMA or SDMA to equations including standard risk factors resulted in a modest increase in the area under the receiver-operating characteristic (ROC) curve (0.839 or 0.844 vs. 0.834) but a significant improvement in the likelihood function (Δ likelihood ratio chi-square 6.17 and 8.50; $p = 0.013$ and 0.004). *p*-Values

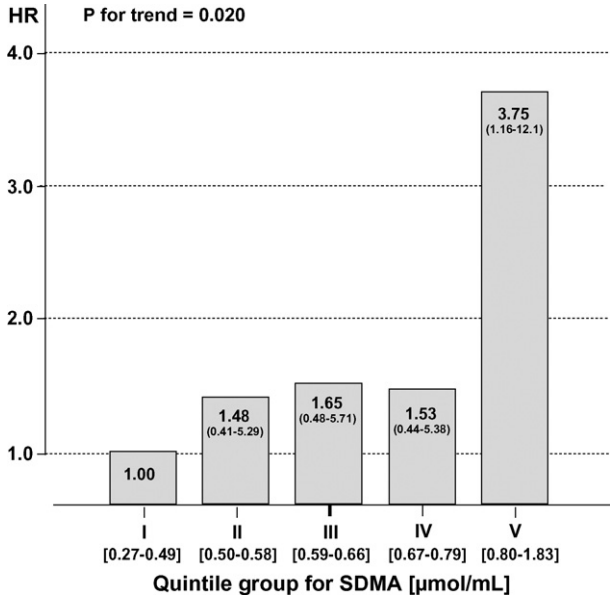

Fig. 2. Association of SDMA quintile groups with CVD risk. Hazard ratios (HR) were derived from Cox models adjusted for age, sex and GFR-MDRD.

derived from the modified Hosmer–Lemeshow calibration statistic (comparing observed and predicted risk using decile categories of predicted probabilities) amounted to 0.392 and 0.506/0.621 for the models not including and including ADMA/SDMA indicating a marginal gain in model calibration.

4. Discussion

The present study provides the first evidence that ADMA was not better than SDMA in predicting CVD risk in the general population. This has not been previously reported partially due to the limitations of the methodologies commonly employed to determine ADMA and SDMA concentrations [12].

4.1. Accurate quantification of ADMA and SDMA

The scientific interest in the L-arginine pathway created a particular need for reliable methods to determine ADMA and other related compounds. Although ADMA has consistently emerged as cardiovascular risk factor [4–7,13,21,22], interpretation of the available studies is complicated by the use of different bioanalytical methods that lack standardization. The majority of clinical studies are based on high-performance liquid chromatography (HPLC), which requires chemical derivatization and relies on fluorimetric detection of dimethylarginine derivatives. In addition, a competitive ELISA assay has been used as platform for ADMA and SDMA measurements. Unlike HPLC and ELISA, mass spectrometric detection, following chromatographic separation, provides both selectivity and sensitivity for the measurement of ADMA and SDMA, but it is costly and technically demanding. Thus, none of the previous studies [3–14] has used the gold standard of mass spectrometry to accurately measure both dimethylarginines in the general population. However, mass spectrometry is already an established technique in clinical chemistry laboratories for a number of analytes. Notably, its high capital costs are, relatively, falling and the lower running costs make it increasingly competitive to immunoassay techniques, especially if both analytes are to be determined.

4.2. SDMA increases cardiovascular risk

ADMA is supposedly the better predictor of cardiovascular risk because of its inhibitory effect on NOS. Our study now demonstrates for the first time that both ADMA and SDMA are associated with CVD in the general population and that their predictive value is of similar strength. Although elevating ADMA could potentially

be used therapeutically to limit excessive vascular NO production under pathophysiological conditions [22], the concentrations required to induce this inhibitory effect exceed the plasma levels measured in the general population, i.e. the IC_{50} for inhibition of NOS is about 2–20 μmol/L ADMA. It has been argued that plasma concentrations of ADMA are only the spill-over, and that intracellular concentrations in endothelial cells may be higher [22]. Nonetheless, our findings raise questions whether the pro-atherosclerotic effects of high ADMA can be exclusively explained by inhibition of NOS.

4.3. Association between SDMA and renal function

Recent evidence suggests that mild impairment of renal function may be the underlying factor in the associations identified between elevations of plasma dimethylarginines and CVD [12]. Although the correlation of ADMA and SDMA is only of moderate strength, which could suggest that the two compounds act in different ways and/or reflect different biological phenomena, ADMA is known to accumulate in patients with renal failure ("uraemic toxin"). Interestingly, attempts to normalize ADMA in these patients by frequent dialysis failed to restore vascular function and improve clinical outcome [23]. As expected, SDMA showed stronger associations to parameters of renal function than ADMA, but its predictive value for cardiovascular risk remained significant even after adjustment for creatinine and cystatin C. Hence, SDMA is either a more sensitive marker of renal dysfunction [24] or SDMA itself is biologically active, i.e. it has been suggested that high concentrations of SDMA might compete with cellular L-arginine for uptake [25,26]. This would be consistent with our observation that the greatest increase in the HR was observed for the fifth quintile of SDMA. Alternatively, raised SDMA may indicate higher rates of protein turnover or increased activity of type 2 protein arginine methyltransferases (PRMT2), which generate SDMA [2]. While further studies are needed to determine the precise mechanisms explaining the relation between SDMA and cardiovascular risk, investigators should, at the very least, report SDMA together with ADMA when assessing cardiovascular risk. Otherwise, the importance of NO bioavailability may be overestimated.

5. Conclusion

The novel finding in this study is that elevated plasma SDMA increases the risk for cardiovascular events in the general population and that the association is of similar strength as the one observed for ADMA, thus arguing against an exclusive ADMA effect in mediating cardiovascular risk.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2008.10.040.

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