Asymmetric Dimethylarginine Reference Intervals Determined with Liquid Chromatography–Tandem Mass Spectrometry: Results from the Framingham Offspring Cohort

Edzard Schwedhelm,1* Vanessa Xanthakis,2 Renke Maas,1,3 Lisa M. Sullivan,2,4 Friedrich Schulze,1 Ulrich Riederer,5 Ralf A. Benndorf,1 Rainer H. Böger,1 and Ramachandran S. Vasan4,6

BACKGROUND: Accumulating evidence links higher circulating asymmetric dimethylarginine (ADMA) to greater risk of cardiovascular disease (CVD). Relatively small differences in ADMA concentrations between healthy individuals and those with disease underscore the need to formulate reference intervals that may aid risk stratification of individuals.

METHODS: We formulated reference intervals for plasma ADMA concentrations using a community-based reference sample from the Framingham Offspring Study consisting of 1126 nonsmoking individuals [mean (SD) age 56 (9) years; 60% women] who were free of clinical CVD, hypertension, diabetes, and obesity and who attended a routine examination at which ADMA was assayed. ADMA concentrations were determined using a validated tandem mass spectrometry–liquid chromatography assay.

RESULTS: In the study sample, the mean ADMA concentration was 0.52 (0.11) μmol/L, and the reference limits were 0.311 and 0.732 (2.5th and 97.5th percentile). The sex-specific reference limits were 0.310 and 0.745 in men and 0.313 and 0.721 μmol/L in women. In multivariable regression analysis, ADMA plasma concentrations were positively correlated with age and total plasma homocysteine (both \( P < 0.001 \)).

CONCLUSIONS: Reference limits calculated for circulating ADMA in our large community-based healthy reference sample confirm the previous observation of a relatively narrow distribution of concentrations. This suggests a tight physiological control of ADMA plasma concentrations, presumably by dimethylarginine dimethylaminohydrolase (DDAH) metabolism of ADMA. © 2009 American Association for Clinical Chemistry

Increased plasma concentrations of the endogenous inhibitor of nitric oxide synthase (NOS)1 asymmetric dimethylarginine (ADMA) are associated with endothelial dysfunction and subclinical atherosclerosis (1–3), which are key precursors of overt cardiovascular disease (CVD) (4). Evidence for ADMA being causally related to CVD comes from both case-control (5) and prospective cohort (6–9) studies. These associations of ADMA with CVD risk have been observed in community-dwelling individuals free of prior CVD (6, 7), as well as in people with preexisting overt CVD (8, 9).

It is worth noting that whereas the relative risk for CVD associated with the top tertile of ADMA relative to the lowest is 2.4 in the MONICA/KORA (Monitoring of Cardiovascular Disease/Cooperative Health Research in the Region of Augsburg) sample of 9796 apparently healthy men and women ages 25–74 years (7), the highest tertile included people with ADMA >0.85 μmol/L compared to the lowest tertile, which included people with ADMA <0.70 μmol/L. This exemplifies the narrowness of the distribution of plasma ADMA concentrations in selected populations. Therefore, formulation of reference limits for ADMA in the general population is indicated.

1 Clinical Pharmacology Unit, Institute of Experimental and Clinical Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; 2 Department of Biostatistics, Boston University School of Public Health, Boston, MA; 3 Institute of Experimental and Clinical Pharmacology and Toxicology, University of Erlangen, Erlangen, Germany; 4 Framingham Heart Study, Framingham, MA; 5 Institute of Pharmacy, University of Hamburg, Hamburg, Germany; 6 Preventive Medicine and Cardiology Sections, Department of Medicine, Boston University School of Medicine, Boston, MA.

* Address correspondence to this author at: Institute of Experimental and Clinical Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf, Martinistr. 52, D-20246 Hamburg, Germany. Fax 49-40-42803-9757; e-mail schwedhelm@uke.uni-hamburg.de.

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1 Nonstandard abbreviations: NOS, nitric oxide synthase; ADMA, asymmetric dimethylarginine; CVD, cardiovascular disease; MONICA/KORA, Monitoring of Cardiovascular Disease/Cooperative Health Research in the Region of Augsburg; LC-MS/MS, liquid chromatography–tandem mass spectrometry; IQR, interquartile range; BP, blood pressure; SBP, systolic BP; DBP, diastolic BP; BMI, body mass index; MDRD, Modification of Diet in Renal Disease; eGFR, estimated glomerular filtration rate; hsCRP, high-sensitivity C-reactive protein; DDAH, dimethylarginine dimethylaminohydrolase.
population may facilitate the definition of increased ADMA concentrations.

The Framingham Heart Study is one of the largest population-based studies to identify risk predictors for cardiovascular morbidity and mortality. Within the Offspring Cohort of this study, we were able to show that ADMA is a risk predictor for total mortality (10). In this context, we now investigate the distribution of ADMA in a reference sample consisting of nonsmoking Framingham Offspring Cohort participants who were free of overt CVD, hypertension, diabetes, and obesity. These individuals underwent measurements of plasma ADMA using a validated liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay (11). This LC-MS/MS–based assay was chosen because it has previously demonstrated high accuracy and precision (11, 12), factors that are a prerequisite for the determination of reliable reference limits. In addition, we evaluated the cross-sectional correlates of ADMA concentrations in this large community-based sample.

Materials and Methods

STUDY SAMPLE
The design and selection criteria of the original Framingham Heart Study have been described (13). Of the 3532 participants who attended the sixth examination cycle (1995 through 1998), we excluded 212 participants from the present investigation for the following reasons: 15 had a serum creatinine concentration >176 \( \mu \text{mol/l} \) (2.0 mg/dL), 79 had missing ADMA, and 118 had missing covariate data. From eligible participants, we excluded all nonhealthy individuals in a stepwise exclusion procedure (Fig. 1). After exclusion of 39 outliers [ADMA outliers were defined as values \(<Q_1 - 1.5*IQR\) or \(>Q_3 + 1.5*IQR\) (IQR, interquartile range), according to Solberg and Lahti (14)], 1126 participants made up the reference sample. The ADMA distribution of the reference population is depicted in Fig. 2. Alternatively, we used a more relaxed definition of outlier identification to generate a second expanded range reference population (ADMA outliers were defined as values \(<Q_1 - 3*IQR\) or \(>Q_3 + 3*IQR\)). This expanded range reference population included 1162 participants after exclusion of 3 outliers (see Supplemental Fig. 1, which accompanies the online version of this article at www.clinchem.org/content/vol55/issue8).

At the sixth examination cycle, all attendees underwent standardized measurements of blood pressure (BP), anthropometry, medical history, physical examination, and assessment of laboratory parameters (15, 16). Hypertension was defined as increased BP [systolic blood pressure (SBP) >140 mmHg or diastolic blood pressure (DBP) >90 mmHg] or use of antihypertensive medication. Obesity was defined as body mass index (BMI) \(\geq 30\) kg/m\(^2\). Preexisting CVD was defined as prior coronary heart disease (myocardial infarction, stable or unstable angina), cerebrovascular disease (stroke or transient ischemic attack), peripheral vascular disease (intermittent claudication), or heart failure. Diabetes was defined as fasting glucose \(\geq 7\) mmol/L (126 mg/dL) or antidiabetic medication.

We used the Modification of Diet in Renal Disease (MDRD) equation to calculate an estimated glomerular filtration rate (eGFR): \(186.3 \times (\text{serum creatinine})^{-1.154} \times \text{age}^{-0.203} \times (0.742\) for women). The study protocol was approved by the Institutional Review Board of the Boston University Medical Center and the Ethics Committee of the Hamburg Board of Physicians. All participants provided written informed consent.

BLOOD SAMPLE COLLECTION AND ANALYSIS
At the sixth examination cycle, phlebotomy was performed (typically between 0800 and 0900) on fasting participants, who were supine for approximately 5–10 min. Blood was immediately centrifuged, and plasma/serum were separated and stored at \(-80^\circ\)C until assayed. Blood lipids were analyzed in the Framingham Heart Study laboratory with automated enzymatic assays. High-sensitivity C-reactive protein (hsCRP) and total homocysteine were measured as described (17, 18).

MEASUREMENT OF PLASMA ADMA CONCENTRATION
We performed mass spectrometric measurement of ADMA using a validated high-throughput LC-MS/MS assay that is commercially available (DLD Diagnostika) as described (11, 12). In brief, proteins were precipitated with methanol using 96-well 0.20-\(\mu\)m microfiltration plates precoated with 40 pmol [\(^2\)H\(_6\)]ADMA (internal standard). After centrifugation, the microfiltrates were dried, and analytes were converted to their butyl ester derivatives. We performed subsequent analyses using a Chirobiotic T, 20\(\times\)1.0 mm i.d., microbore guard column (Astec) connected to a Varian 1200L Triple Quadrupole MS (Varian) in the positive electrospray ionization (ESI+) mode. The sample run time was 1.6 min with intra- and interassay precision of 3.2% and 4.4%, respectively.

STATISTICAL ANALYSES
We used the SAS statistical program (SAS Institute) for statistical analyses. A 2-sided \(P\) value of \(<0.05\) indicated statistical significance. Data are presented as mean (SD) or mean (SE) unless otherwise indicated.

We calculated reference limits according to the recommendations of the IFCC as described (19, 20). ADMA concentrations were normally distributed in our sample (assessed by histograms and normal prob-
ability plots), and we analyzed ADMA as a continuous variable (without transformation) and as categorical variable (quartiles). We constructed multivariable linear regression models with stepwise forward selection (significance criterion for entry into the model, $P < 0.1$) of variables using the following candidate covariates: age, sex, BMI, SBP, DBP, total/HDL cholesterol ratio, triglycerides, alcohol consumption, hsCRP, eGFR, and total homocysteine.

**Results**

The characteristics of our reference sample are displayed in Table 1. The mean age of the participants was 56 years; 60% were women. Women had lower values for DBP, total/HDL cholesterol ratio, total homocysteine, fasting glucose, SBP, BMI (all $P < 0.001$), LDL cholesterol ($P < 0.01$), and triglycerides ($P < 0.01$). The mean ADMA concentration in the overall reference sample was 0.52 (0.11) μmol/L [median (IQR) 0.51 (0.45–0.59), reference limits 0.311 and 0.732 (2.5th and 97.5th percentile, and 95% CI 0.302–0.319 and 0.724–0.741, respectively). Plasma ADMA concentrations were similar in men [0.53 (0.11) μmol/L, median (IQR) 0.51 (0.45–0.60) μmol/L] and women [0.52 (0.10) μmol/L, median (IQR) 0.50 (0.45–0.58) μmol/L]. The sex-specific 2.5th and 97.5th percentile reference limits were 0.310 and 0.745 in men and 0.313 and 0.721 μmol/L in women. The histogram of the ADMA distribution in the reference sample is depicted in Fig. 2. In the subgroup of women before menopause [mean age 47 (5) years], ADMA was 0.49 (0.08)

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**Fig. 1. Study design of The Framingham Heart Study, 6th examination cycle (1995–1998), offspring cohort.** Nonhealthy participants were excluded from the reference population by a stepwise exclusion procedure. *Not eligible for the following reasons: serum creatinine concentration $>176$ mmol/L ($n = 15$), missing ADMA ($n = 79$), and missing covariate data ($n = 118$).
μmol/L compared with postmenopausal women, 0.53 (0.11) μmol/L [mean age 61 (8) years, P < 0.001]. A correlation between age and ADMA was found in women (unadjusted Pearson r = 0.14, P < 0.001) as well as in men (r = 0.15, P = 0.0017) (Fig. 3). Also, a correlation between ADMA and homocysteine existed in the reference sample (unadjusted Pearson r = 0.12, P < 0.001) (Fig. 4).

The results of the multivariable regression model are shown in Table 2. We included all candidate variables in a stepwise selection procedure (cutoff for model entry P < 0.1) and created a final model. Data for men and women were combined, since ADMA concentrations were similar. In the final regression model, age, total homocysteine, and eGFR were positively associated with ADMA, whereas DBP was inversely related to ADMA. Sex, alcohol intake, SBP, blood lipids, BMI, and hsCRP were not associated with plasma ADMA concentration in our sample. We also calculated stepwise selection and regression procedures forcing sex into the model. These models produced the same set of significant variables (age, homocysteine, eGFR, and DBP) for the final model and also a very similar magnitude of each β-coefficient and P value (data not shown). Thus, neither stepwise selection nor inclusion of sex had an impact on the multivariable regression procedure output.

Discussion

Experimental and clinical data suggest that increased ADMA concentrations are associated with greater CVD risk (5–9). For individual risk stratification, it is a prerequisite to define reliable reference intervals (21). In the present analysis of a large community-based reference sample, we defined the 95% ADMA reference interval from 0.311–0.732 μmol/L (2.5th and 97.5th percentiles) in a reference sample without CVD, diabetes, obesity, smoking, or hypertension. The relatively narrow distribution of ADMA concentration has been observed in other cohorts (22) and suggests a tight physiological control of ADMA in vivo, presumably by dimethylarginine dimethylaminohydrolase (DDAH) metabolism of ADMA (23–25).

ADMA plasma concentrations increased by 0.0017 μmol/L for each year increase in age (β, Table 2), after adjusting for other variables in the model. This is consistent with a similar increase reported in a smaller sample of men (26). This association between age and ADMA was evident in women and men (Fig. 3, unadjusted relations). In line with this finding, premenopausal women had lower ADMA concentrations than postmenopausal women. This confirms previous reports (27, 28) and may reflect the influence of estrogens on ADMA concentrations (29, 30). Estrogens enhance DDAH gene expression by promoter activation (31). When menopausal status is considered as a candidate variable in the stepwise procedure, however, this variable does not meet the P < 0.1 entry selection criterion. Thus, the most likely explanation for the observed difference in ADMA in pre- and postmenopausal women is the different age in these 2 subgroups. This observation is substantiated by previous data from a smaller cohort where younger women (mean age 31 years) had as much as 23% lower ADMA plasma concentrations compared with older women (mean age 62 years) (27).

![Fig. 2. Histogram of plasma ADMA with reference interval. ADMA was determined from EDTA plasma samples using LC-MS/MS and stable isotope dilution with [2H6]ADMA.](image-url)
The finding of a positive association between plasma ADMA and total homocysteine is consistent with recent clinical and experimental studies (26, 32, 33). In our analyses, we observed an increase of ADMA plasma concentrations of 0.0038 μmol/L per 1 μmol/L increase of homocysteine (Table 2). Homocysteine is known to interact with DDAH activity by modification of enzyme redox status and protein stability (34). Despite this biochemical interaction, clinical studies of homocysteine lowering have noted only minor effects on plasma ADMA concentrations (35). Nevertheless, both substances (homocysteine and ADMA) were found to be associated with intimal-medial thickness in other cohorts (36–38). This suggests that both biomolecules act hand in hand in impairing endothelial function.

Besides age and total homocysteine, ADMA was influenced by eGFR and DBP in our cohort of healthy individuals (Table 2). It is well known that ADMA is increased in patients with renal function impairment, as well as in patients with increased blood pressure (39–41). In contrast to previous reports from various patient populations, renal function was neither impaired nor was blood pressure increased in our refer-

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**Table 2. Multivariable regression of ADMA plasma concentrations in the reference sample.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>β (SE)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, per 1 year increase</td>
<td>0.0017 (0.0003)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Homocysteine, per 1 μmol/L</td>
<td>0.0038 (0.0010)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GFR, per 1 mL/min increase</td>
<td>0.0003 (0.0001)</td>
<td>0.017</td>
</tr>
<tr>
<td>DBP, per 1 mmHg increase</td>
<td>−0.0008 (0.0004)</td>
<td>0.036</td>
</tr>
</tbody>
</table>

* Candidate variables that were considered for model entry were age, sex, BMI, SBP, DBP, total/HDL cholesterol ratio, alcohol consumption, triglycerides, GFR, homocysteine, and CRP. All regression coefficients (β estimates) represent the estimated mean change in ADMA (μmol/L) per 1-unit increase in the corresponding covariate.

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**Fig. 3. Association between age and ADMA in men and women.**

Data represent mean (SE). The unadjusted relationship between age and ADMA was significant for both men and women (Pearson r = 0.15 and r = 0.14 in men and women, respectively; P < 0.002 for both).

**Fig. 4. Association between quartiles (Q) of plasma ADMA and total homocysteine.**

Data represent mean (SE). The unadjusted relationship between ADMA and homocysteine was significant for pooled sex (Pearson r = 0.12, P < 0.001).
ence sample. The weak positive association between ADMA plasma concentrations and eGFR may thus point to a physiological regulation mechanism of renal filtration, or it may be a chance finding due to the comparatively narrow range of renal function levels included in our study. In line with this interpretation, the positive association observed between ADMA and eGFR is further weakened for the expanded-range reference population (online Supplemental Tables 1 and 2). Under pathophysiologic conditions, however, impaired renal excretory function or impaired renal DDAH activity may dominate and explain the inverse associations between ADMA and eGFR found in most previous studies.

Also, the unexpected weak negative association between ADMA and DBP in our healthy reference cohort was further weakened when the relation was examined in the expanded-range reference population (online Supplemental Table 1) with the stepwise multivariable regression model (online Supplemental Table 2). Again, under pathophysiologic conditions, impaired DDAH activity may become predominant. In consequence, ADMA degradation is impaired, ADMA accumulates, and blood pressure increases. This association is clearly evidenced by gene polymorphism studies and genetically modified animal models (23–25, 42).

STRENGTHS AND LIMITATIONS

The large community-based reference sample and the quantification of ADMA plasma concentrations by a validated LC-MS/MS method are strengths of our investigation. We acknowledge several limitations, however. We evaluated a community-based reference sample of white, middle-aged individuals of European descent. Caution must be exercised in extrapolating these results to other populations of different ethnicity or with a different age range. Also, from our epidemiological data, no functional conclusions of the observed associations can be made.

Conclusions

We have formulated reference limits for plasma ADMA concentrations using a large community-based reference sample. The relatively narrow distribution of ADMA concentration suggests a tight physiological control of ADMA plasma concentrations, presumably by DDAH metabolism of ADMA.

References


Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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