

Reference Intervals for Plasma L-Arginine and the L-Arginine:Asymmetric Dimethylarginine Ratio in the Framingham Offspring Cohort^{1–3}

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Abstract

L-Arginine, as a precursor of NO synthesis, has attracted much scientific attention in recent years. Experimental mouse models suggest that L-arginine supplementation can retard, halt, or even reverse atherogenesis. In human studies, supplementation with L-arginine improved endothelium-dependent vasodilation. However, L-arginine levels are best interpreted in the context of levels of asymmetric dimethylarginine (ADMA), a competitive inhibitor of NO synthase. Thus, reference limits for circulating L-arginine and the L-arginine:ADMA ratio may help to determine the nutritional state of individuals at high cardiovascular risk in light of increased ADMA levels. We defined reference limits for plasma L-arginine in 1141 people and for the L-arginine:ADMA ratio in 1138 relatively healthy individuals from the Framingham Offspring Cohort. Plasma L-arginine and ADMA concentrations were determined by using a stable isotope-based LC-MS/MS method. The reference limits (2.5th and 97.5th percentiles) for plasma L-arginine were 41.0 $\mu\text{mol/L}$ (95% CI = 39.5–42.5 $\mu\text{mol/L}$) and 114 $\mu\text{mol/L}$ (95% CI = 112–115 $\mu\text{mol/L}$), whereas corresponding reference limits (2.5th and 97.5th percentiles) for the L-arginine:ADMA ratio were 74.3 $\mu\text{mol/L}$ (95% CI = 71.1–77.3 $\mu\text{mol/L}$) and 225 $\mu\text{mol/L}$ (95% CI = 222–228 $\mu\text{mol/L}$). Plasma L-arginine was positively associated with the estimated glomerular filtration rate (eGFR) and blood glucose levels, whereas the L-arginine:ADMA ratio was positively associated with eGFR and diastolic blood pressure but inversely associated with homocysteine and (log)C-reactive protein. We report reference levels for plasma L-arginine and for the L-arginine:ADMA ratio that may be helpful for evaluation of the effects of L-arginine supplementation in participants with an impaired L-arginine/NO pathway. *J. Nutr.* 141: 2186–2190, 2011.

Introduction

L-Arginine (2-amino-5-guanidino-pentanoic acid) is a conditionally essential amino acid that is a natural constituent of dietary proteins, with the relative amount of L-arginine ranging from 3 to 15% in various proteins. In addition to its role in protein anabolism, L-arginine is involved in various metabolic pathways, such as the synthesis of creatine, L-ornithine, L-

glutamate, and polyamines (1), and it is the substrate for synthesis of NO, one of the most potent endogenous vasodilators (2). NO is mainly released by the endothelium to regulate vascular tone and modulate the interaction of circulating blood cells with the vascular wall. The synthesis of NO by the endothelial NO synthase results in multiple vasoprotective effects that have been summarized as antiatherogenic (3).

Intracellular L-arginine levels have been demonstrated to be considerably higher than those in the extracellular fluid or in plasma (3,4). However, plasma L-arginine can be rapidly taken up by endothelial cells via the cellular y⁺ transporter for cationic amino acids and can directly contribute to NO production (5), suggesting that plasma L-arginine concentrations may influence endothelial NO production. Although a lack of L-arginine-derived NO formation may result in endothelial dysfunction, a pathophysiological finding that is common in patients with cardiovascular risk factors, reduced L-arginine concentrations in plasma have rarely been described (6). Mouse and human

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³ Supplemental Figure 1 is available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.

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studies that used ^{15}N -labeled L-arginine as a precursor demonstrate that the major part of dietary L-arginine is metabolized in the liver and utilized in the hepatic urea cycle (7). Only a small portion of dietary L-arginine is converted to NO (8).

Additionally, besides its function as a substrate for NO synthase, L-arginine plays an important role in glucose metabolism. In adipocytes, L-arginine enhances glycogen synthesis in response to insulin, an effect that is independent of NO generation (9). Oral L-arginine supplementation during 6 mo in a referral sample has been reported to improve glucose tolerance and enhance insulin sensitivity (10).

One common cause of impaired L-arginine/NO metabolism is the presence of elevated levels of ADMA¹¹. ADMA displaces L-arginine from the substrate binding site of the NO synthase and thereby competitively inhibits this enzyme. ADMA is elevated in patients with renal failure and CVD and in advanced stages of diabetes mellitus (11–14).

In the present study, we determined reference limits for plasma L-arginine and the L-arginine:ADMA ratio, because these data may be of value for determining the nutritional state of participants at high cardiovascular risk due to their elevated ADMA levels. Such reference limits may also be useful for guiding nutraceutical interventions with L-arginine supplements. Accordingly, we measured plasma L-arginine and plasma ADMA concentrations in the Framingham Offspring Cohort and subsequently calculated the L-arginine:ADMA ratio. Plasma L-arginine and ADMA measurements were performed with a LC-MS/MS method that is characterized by high accuracy and precision (15,16). Plasma ADMA reference levels from this cohort have been reported elsewhere (17).

Methods

Study sample. The design and sampling strategy of the Framingham Offspring Study were previously described (18). In brief, in 1971 the Framingham Offspring Study began with the enrolment of 5124 participants who were the children of the original cohort or the spouses of these children. Of 3532 participants who attended the 6th examination cycle (1995 through 1998), we excluded participants with a serum creatinine level $> 1.8 \times 10^{-4}$ mol/L, those with missing L-arginine, and attendees with missing covariate data. From this sample consisting of 3320 participants, smokers and individuals with CVD, obesity, hypertension, and diabetes were excluded in a hierarchical fashion (Supplemental Fig. 1). After the exclusions, 1165 participants comprised the reference sample for the present investigation. To create reference limits, outliers [defined as values $< Q1 - 1.5 \times IQR$ or $> Q3 + 1.5 \times IQR$ according to Solberg and Lahti (19)] were removed so that the final reference sample size comprised 1141 participants for analysis of plasma L-arginine and 1138 for analysis of the L-arginine:ADMA ratio, respectively. Hypertension was defined as increased BP (SBP > 140 mm Hg or DBP > 90 mm Hg) or use of antihypertensive medication. All participants provided written informed consent and the study protocol was approved by the Institutional Review Board of the Boston University Medical Center and by the Ethics Committee at the Hamburg Board of Physicians.

Blood sample collection and analysis. Laboratory assessment of several biomarkers was conducted on samples from fasting participants drawn at the 6th examination cycle; plasma/serum samples used for the present investigation were stored for ~ 8 y at -80°C without freeze-thaw cycles.

Phlebotomy was performed (typically between 0800 and 0900 h) on fasting participants. All participants were supine for ~ 5 –10 min. The blood was immediately centrifuged and plasma/serum was separated and stored at -80°C until analysis. Serum high-sensitivity CRP was measured with an immunoprecipitation assay (20). Plasma homocysteine was analyzed by using HPLC with fluorometric detection (21). Serum blood lipids and creatinine were analyzed in the Framingham Heart Study laboratory with automated enzymatic assays (22).

Mass spectrometric determination of plasma L-arginine and ADMA was performed by using a fully validated, high throughput LC-MS/MS assay. The method details and stability of L-arginine and ADMA were previously described (15,16,23). In brief, plasma samples were analyzed using 96-well, $0.20\text{-}\mu\text{m}$ microfiltration plates precoated with 40 pmol of [$^2\text{H}_6$]-ADMA and 800 pmol of L-[$^2\text{H}_7$]-L-arginine (internal standards). After conversion to their butyl ester derivatives, analytes were analyzed on a Varian 1200L Triple Quadrupole MS (Varian) in the positive electrospray ionization mode. The sample run time was 1.6 min, with an intra- and inter-assay precision of 2.2 and 4.8% for L-arginine and 3.2% and 4.4% for ADMA, respectively.

Statistical analysis. The SAS statistical software (SAS Institute) was used for statistical analyses. A 2-sided P value of < 0.05 was considered significant. Data in the text are mean \pm SD unless otherwise indicated. Associations between variables were assessed by Pearson correlation coefficients.

The calculation of the reference limits was done according to the recommendations of the International Federation of Clinical Chemistry as described elsewhere (24). Plasma L-arginine and ADMA levels were normally distributed in our sample and we analyzed these values as continuous dependent variables (without transformation). We constructed multivariable linear regression models with stepwise forward selection (significance criterion for entry of independent variables into the model, $P < 0.1$) using the following eligible covariates: age, sex, BMI, SBP, DBP total:HDL cholesterol ratio, TG, eGFR, homocysteine, alcohol consumption, glucose, and (log)CRP. To calculate the eGFR, we used the Modification of Diet in Renal Disease equation [$186.3 \times (\text{serum creatinine})^{-1.154} \times \text{age}^{-0.203} \times (0.742 \text{ for women})$] (25).

Results

At baseline, the plasma L-arginine concentrations in the reference sample used for L-arginine analysis were $77.4 \pm 18.2 \mu\text{mol/L}$ and the plasma ADMA concentrations were $0.53 \pm 0.12 \mu\text{mol/L}$ (Table 1). The L-arginine:ADMA ratio in the reference sample used for the L-arginine:ADMA ratio analysis was 150 ± 38 . The reference limits (2.5th and 97.5th percentiles) for plasma L-arginine were $41.0 \mu\text{mol/L}$ (95% CI = 39.5 – $42.5 \mu\text{mol/L}$) and $114 \mu\text{mol/L}$ (95% CI = 112 – $115 \mu\text{mol/L}$) (Fig. 1A), whereas corresponding reference limits (2.5th and 97.5th percentiles) for the L-arginine:ADMA ratio were $74.3 \mu\text{mol/L}$ (95% CI = 71.1 – $77.3 \mu\text{mol/L}$) and $225 \mu\text{mol/L}$ (95% CI = 222 – $228 \mu\text{mol/L}$) (Fig. 1B).

The sex-specific 2.5th and 97.5th percentile reference limits for L-arginine were $42.0 \mu\text{mol/L}$ (95% CI = 39.5 – $44.2 \mu\text{mol/L}$) and $113 \mu\text{mol/L}$ (95% CI = 111 – $116 \mu\text{mol/L}$) in men and $40.8 \mu\text{mol/L}$ (95% CI = 38.8 – $42.6 \mu\text{mol/L}$) and $113 \mu\text{mol/L}$ (95% CI = 111 – $115 \mu\text{mol/L}$) in women. The sex-specific 2.5th and 97.5th percentile reference limits for the L-arginine:ADMA ratio were 72.0 (95% CI = 66.6 – $76.7 \mu\text{mol/L}$) and $228 \mu\text{mol/L}$ (95% CI = 224 – $234 \mu\text{mol/L}$) in men and $75.6 \mu\text{mol/L}$ (95% CI = 71.5 – $79.4 \mu\text{mol/L}$) and $224 \mu\text{mol/L}$ (95% CI = 220 – $228 \mu\text{mol/L}$) in women.

ADMA plasma concentrations were positively correlated with L-arginine (age- and sex-adjusted $r = 0.31$; $P < 0.0001$). A correlation between L-arginine and blood glucose was observed in the reference sample (unadjusted Pearson correlation $r = 0.09$; $P = 0.002$). Also, a correlation between L-arginine and eGFR

¹¹ Abbreviations used: ADMA, asymmetric dimethylarginine; BP, blood pressure; CRP, C-reactive protein; CVD, cardiovascular disease; DBP, diastolic blood pressure; DDAH, dimethylarginine dimethylaminohydrolase; eGFR, estimated glomerular filtration rate; SBP, systolic blood pressure.

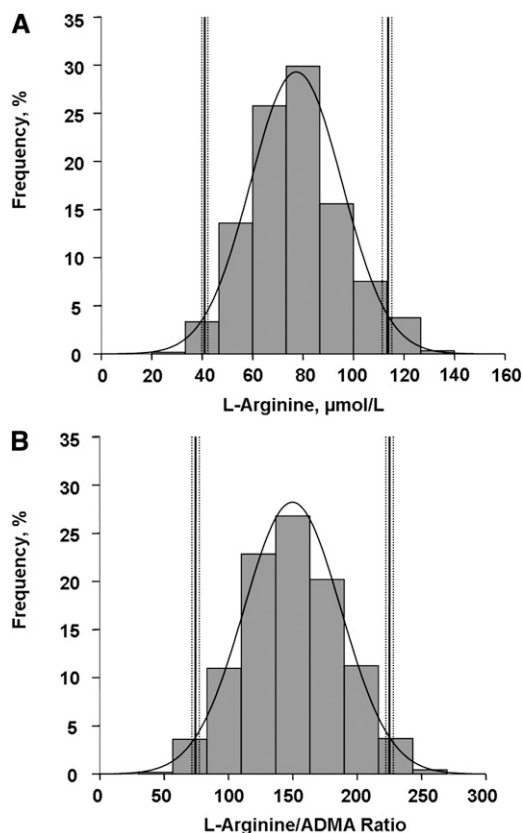


FIGURE 1 Distribution of plasma L-arginine concentration (A; $n = 1141$) and the L-arginine:ADMA ratio (B; $n = 1138$) from participants of the Framingham Offspring Cohort with reference intervals and integrated normal distribution lines. ADMA, asymmetric dimethylarginine.

was observed in the reference sample (unadjusted Pearson correlation $r = 0.06$; $P < 0.05$).

In the final multivariable regression model for L-arginine, glucose and eGFR were positively associated with L-arginine (Table 2), with other variables not meeting the P value of 0.05 required for denoting significance. The L-arginine:ADMA ratio was positively associated with eGFR and diastolic BP and inversely associated with homocysteine and (log)CRP (Table 3).

Discussion

The amino acid L-arginine has gained major scientific interest in recent years due to its multi-faceted roles in metabolism, among which the most prominent is its role as a precursor of NO synthesis. In this respect, not only L-arginine plasma concentrations per se but also its levels relative to its competitive antagonist, ADMA, are important; thus, the L-arginine:ADMA ratio has been inversely associated with all-cause mortality in a prospective cohort study (26). We report here reference limits for plasma L-arginine and for the L-arginine:ADMA ratio from a healthy reference sample of participants from the Framingham Offspring cohort. These reference limits were similar for men and women.

In most clinical conditions associated with endothelial dysfunction, circulating L-arginine concentrations have been observed to be within the usual normal range. Yet, it is intriguing that short-term dietary supplementation with oral L-arginine restores vascular function and improves clinical symptoms of diseases associated with vascular dysfunction [examples include angina pectoris (27), hypertension (28) erectile dysfunction (29,30), congestive heart failure (31), and pulmonary arterial

TABLE 1 Clinical characteristics of the reference samples used for calculating the reference levels of L-arginine and L-arginine:ADMA ratio in the Framingham Offspring Cohort¹

	Reference sample for L-arginine	Reference sample for L-arginine:ADMA ratio
n	1141	1138
Age, y	56 ± 9	56 ± 9
BMI, kg/m^2	25.1 ± 2.7	25.1 ± 2.7
SBP, $mm\ Hg$	118 ± 12	118 ± 12
DBP, $mm\ Hg$	72 ± 8	72 ± 8
Fasting glucose, $mmol/L$	5.2 ± 0.4	5.2 ± 0.4
LDL cholesterol, $mmol/L$	3.3 ± 0.9	3.3 ± 0.9
Total-HDL cholesterol	3.92 ± 1.29	3.93 ± 1.30
TG, $mmol/L$	1.3 ± 0.7	1.3 ± 0.7
eGFR, mL/min	88.3 ± 21.4	88.6 ± 25.4
High sensitive CRP, mg/L	1.20 (0.59–2.79)	1.20 (0.60–2.80)
Total homocysteine, $\mu mol/L$	8.96 ± 3.05	8.98 ± 3.06

¹ Values are means \pm SD or median (Q1–Q3). All analytes were measured in serum. ADMA, asymmetric dimethylarginine; CRP, C-reactive protein; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; SBP, systolic blood pressure.

hypertension (32)]. Many of these conditions are associated with elevated circulating ADMA levels and also a lower L-arginine:ADMA ratio (28,33). Thus, an imbalance between L-arginine and ADMA can result in relative L-arginine deficiency. Our data therefore extend previous data on circulating L-arginine reference levels (34), because we also report reference intervals for the L-arginine:ADMA ratio in a healthy, community-based sample. Our data may help to better understand the roles of L-arginine and ADMA in the pathophysiology of various cardiovascular and metabolic conditions and also facilitate the interpretation of some of the inconsistent results of recent studies evaluating dietary supplementation with L-arginine.

Our reference sample was chosen to be free of CVD, hypertension, diabetes, obesity, and smoking, and participants with higher serum creatinine concentrations were also excluded. Therefore, we were unable to assess the influence of variables such as BP, serum lipids, BMI, and renal function on plasma L-arginine levels across their broad range of values.

We observed a mean decrease in the L-arginine plasma concentration of $0.05\ \mu mol/L$ per 1 mL/min decline of eGFR. This may not necessarily point toward an association of L-arginine plasma concentration with glomerular filtration per se, because it is well known from experimental studies that the kidney releases more L-arginine into plasma than it extracts (35,36), suggesting that renal L-arginine synthesis outweighs L-arginine consumption. Thus, renal diseases leading to reductions

TABLE 2 Multivariable regression analysis of plasma L-arginine concentrations in 1141 healthy individuals from the Framingham Offspring Cohort

Variable ¹	$\beta \pm SE^2$	P value
Glomerular filtration rate	0.05 ± 0.03	0.044
Fasting glucose	0.20 ± 0.06	0.002

¹ Candidate variables that were considered for model entry were age, sex, BMI, SBP, DBP, the total-HDL cholesterol ratio, alcohol consumption, TG, glomerular filtration rate, homocysteine, fasting glucose, and CRP. CRP, C-reactive protein; DBP, diastolic blood pressure; SBP, systolic blood pressure.

² All regression coefficients (β estimates) represent the estimated mean change in L-arginine ($\mu mol/L$) per 1-unit increase in the corresponding covariate.

TABLE 3 Multivariable regression analysis of the L-arginine:ADMA ratio in 1138 healthy individuals of the Framingham Offspring Cohort¹

Variable ²	$\beta \pm SE^3$	P value
Homocysteine	-1.21 \pm 0.37	0.001
log CRP	-2.35 \pm 1.02	0.022
Glomerular filtration rate	0.09 \pm 0.04	0.035
DBP	0.31 \pm 0.15	0.037

¹ ADMA, asymmetric dimethylarginine; CRP, C-reactive protein; DBP, diastolic blood pressure; SBP, systolic blood pressure.

² Candidate variables that were considered for model entry were age, sex, BMI, SBP, DBP, the total:HDL ratio, alcohol consumption, TG, glomerular filtration rate, homocysteine, fasting glucose, and CRP.

³ All regression coefficients (β estimates) represent the estimated mean change in L-arginine:ADMA ratio per 1-unit increase in the corresponding covariate.

in eGFR may result in diminished L-arginine production, independent of the age-related decline in eGFR itself. These data are in line with the observation in clinical studies that patients with end-stage renal disease undergoing chronic hemodialysis treatment have significantly lower plasma L-arginine concentrations than participants without renal disease (37).

We also observed a mean increase of L-arginine plasma concentration of 0.20 μ mol/L per 0.6 mmol/L increase of fasting blood glucose. There is growing evidence that dietary supplementation with L-arginine reduces plasma levels of glucose, fatty acids, and TG and improves insulin sensitivity in various mouse and rat models of diabetes and obesity (38). Similar results have been reported for obese humans with type II diabetes receiving L-arginine supplementation (10,39).

The findings of a positive association of the L-arginine:ADMA ratio with DBP and the inverse association with total homocysteine are consistent with our findings for plasma ADMA in the same study population and may thus be mainly driven by the associations of ADMA with these variables (17).

The inverse relation of the L-arginine:ADMA ratio and plasma homocysteine may be partly explained by a redox-mediated inhibition of DDAH, the enzyme degrading ADMA, by homocysteine (40). DDAH is known to be redox sensitive and homocysteine might modify redox status and the protein stability of DDAH, resulting in decreased DDAH activity (41). Böger et al. (41) also reported a metabolic link between the homocysteine and ADMA pathways at the level of protein arginine methylation, which might also contribute to this inverse association.

L-Arginine has been studied in numerous clinical trials involving various patient groups with respect to its ability to improve endothelium-dependent, NO-mediated vasodilation. Whereas some of the early, smaller studies showed improved endothelial function after L-arginine supplementation (42,43), 2 larger and more recent studies showed presumed deleterious effects of L-arginine on vascular function (44,45). It is noteworthy, however, that in both of these studies patients were not selected for relative or absolute L-arginine deficiency. The results of these studies have generated considerable debate. Whether L-arginine may have more pronounced beneficial effects in patients with a low L-arginine:ADMA ratio is the focus of ongoing clinical trials. The normal ranges presented here may help to define patient selection in future clinical trials and may facilitate interpretation of data from such trials.

Strengths and limitations. The large, community-based reference sample and the quantification of L-arginine plasma levels

by a validated LC-MS/MS method are strengths of our investigation. However, we acknowledge several limitations. 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 a different ethnicity or with a different age range. Also, no causal inferences can be drawn from the associations observed in our cross-sectional epidemiological investigation.

In conclusion, we report reference ranges for both plasma L-arginine and the L-arginine:ADMA ratio, which may reflect NOS substrate availability more closely than plasma L-arginine concentrations alone. These reference limits may facilitate the design of better clinical trials of L-arginine supplementation in those with relative deficiency of this amino acid as reflected by the L-arginine:ADMA ratio.

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