

Genome-Wide Association Study of L-Arginine and Dimethylarginines Reveals Novel Metabolic Pathway for Symmetric Dimethylarginine

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Background—Dimethylarginines (DMA) interfere with nitric oxide formation by inhibiting nitric oxide synthase (asymmetrical DMA [ADMA]) and L-arginine uptake into the cell (ADMA and symmetrical DMA [SDMA]). In prospective clinical studies, ADMA has been characterized as a cardiovascular risk marker, whereas SDMA is a novel marker for renal function and associated with all-cause mortality after ischemic stroke. The aim of the current study was to characterize the environmental and genetic contributions to interindividual variability of these biomarkers.

Methods and Results—This study comprised a genome-wide association analysis of 3 well-characterized population-based cohorts (Framingham Heart Study [FHS; n=2992], Gutenberg Health Study [GHS; n=4354], and Multinational Monitoring of Trends and Determinants in Cardiovascular Disease Study [MONICA]/Cooperative Health Research in the Augsburg Area, Augsburg, Bavaria, Germany [KORA] F3 [n=581]) and identified replicated loci (*DDAH1*, *MED23*, *Arg1*, and *AGXT2*) associated with the interindividual variability in ADMA, L-arginine, and SDMA. Experimental in silico and in vitro studies confirmed functional significance of the identified *AGXT2* variants. Clinical outcome analysis in 384 patients of the Leeds stroke study demonstrated an association between increased plasma levels of SDMA, *AGXT2* variants, and various cardiometabolic risk factors. *AGXT2* variants were not associated with poststroke survival in the Leeds study or were they associated with incident stroke in the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium.

Conclusions—These genome-wide association study support the importance of *DDAH1* and *MED23/Arg1* in regulating ADMA and L-arginine metabolism, respectively, and identify a novel regulatory renal pathway for SDMA by *AGXT2*. *AGXT2* variants might explain part of the pathogenic link between SDMA, renal function, and outcome. An association between *AGXT2* variants and stroke is unclear and warrants further investigation. (*Circ Cardiovasc Genet.* 2014;7:864-872.)

Key Words: biological markers ■ genome wide association study ■ nitric oxide

Dimethylarginines are endogenous analogues of the amino acid L-arginine which contain 2 methyl groups. Asymmetrical dimethylarginine (ADMA) and symmetrical dimethylarginine (SDMA) both interfere with the L-arginine/

nitric oxide (NO) pathway. In a large number of prospective clinical studies, ADMA has been characterized as a predictor of major cardiovascular events and mortality in patients with low, medium, and high cardiovascular risk.^{1,2} SDMA, in contrast,

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has not been studied to a similar extent. Some recent studies suggest that SDMA is associated with cardiovascular events,^{3,4} and we have shown that SDMA, but not ADMA, is predictive of all-cause mortality after ischemic stroke.^{5,6} This finding pointed to potential differences in the prognostic impact of ADMA and SDMA in cardiovascular disease. In a targeted metabolomic approach, this hypothesis of differences in predicting cardiovascular disease and a combined end point (myocardial infarction, stroke, and death) by ADMA and SDMA was confirmed.⁷

Clinical Perspective on p 872

Both dimethylarginines inhibit cellular L-arginine uptake by inhibiting the accordant transport system⁸ while only ADMA acts as an endogenous competitive inhibitor of NO synthases.⁹ In addition, experimental data suggest that SDMA may affect vascular homeostasis by NO-independent mechanisms.¹⁰ Regulation of plasma and tissue ADMA largely depends on the enzymatic activity of dimethylarginine dimethylaminohydrolase (DDAH) while ADMA excretion by the kidneys plays only a minor role.¹¹ DDAH is expressed in 2 isoforms, *DDAH1* and *DDAH2*, which are characterized by distinct tissue distribution and may exert distinct functional roles.^{12,13}

In contrast, SDMA seems to be eliminated almost exclusively through the kidneys¹⁴ and shows a closer association with renal function than ADMA.^{14,15} The varying prognostic significance of dimethylarginines for cardiovascular events and mortality in different patient populations and differences in their metabolism render it important to understand the environmental and genetic factors contributing to interindividual variability of circulating L-arginine and dimethylarginine concentrations. In this study, we hypothesized that circulating levels of ADMA, L-arginine, and SDMA are (1) heritable traits, (2) associated with common genetic variants, and (3) associated with poststroke mortality.

Methods

Study Populations

All participants provided written informed consent (including consent for genetic analyses), and the study protocols were approved by local institutional review boards and ethical committees. Blood samples of Framingham Heart Study (FHS), Gutenberg Health Study (GHS), and Multinational Monitoring of Trends and Determinants in Cardiovascular Disease Study (MONICA)/Cooperative Health Research in the Augsburg Area, Augsburg, Bavaria, Germany (KORA) were fasting samples, and plasma was immediately separated, frozen, and stored at -80°C .

Framingham Heart Study

The FHS is a longitudinal observational, community-based cohort initiated in 1948 in Framingham, MA, to investigate risk factors prospectively for cardiovascular diseases.¹⁶ After exclusions, 2992 participants of the Framingham Offspring Cohort had complete genotypic information and plasma levels for L-arginine, SDMA, and ADMA available.

Gutenberg Health Study

The GHS was initiated in 2007 as a community-based, prospective cohort study including participants aged 35 to 74 years.¹⁷ For GHS I 3175 and for GHS II 1179 individuals, respectively, had genome-wide data available, of whom 3166, 3164, and 3161 (GHS I) and 1159, 1152, and 1151 (GHS II) had measured plasma concentrations of L-arginine, SDMA, and ADMA, respectively.

MONICA/KORA F3 Cohort

The individuals of the MONICA/KORA sample participated in the third survey (S3) of the MONICA) Augsburg study, which is now continued in the framework of KORA.¹⁸ Overall, 581 participants had plasma concentrations of L-arginine, SDMA, and ADMA and complete genotypic information available.

Leeds Stroke Cohort

White European patients (n=609) with a clinical diagnosis of acute ischemic stroke (classified after the Oxfordshire Community Stroke Project) were consecutively recruited from 4 hospitals in Leeds.¹⁹ Patients who survived for >30 days after the acute event with sufficient plasma available for analysis of L-arginine, SDMA and ADMA were included in this study (n=394). For case-control analysis, genotype distributions between patients with ischemic stroke (n=394), hemorrhagic stroke (n=57), and age-matched healthy controls (n=430) were evaluated.²⁰

CHARGE Consortium

The design of the stroke population of the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium includes 4 prospective cohorts from the United States and Europe: the Atherosclerosis Risk in Communities Study (ARIC), the Cardiovascular Health Study (CHS), the FHS, and the Rotterdam Study. The consortium was formed to facilitate genome-wide association study (GWAS) meta-analysis and replication opportunities.²¹ In this study, the CHARGE consortium was used to evaluate the association between incident stroke, longevity, and mortality in general and SDMA-related genome-wide significant single nucleotide polymorphisms (SNP).

Heart and Vascular Health Study

The setting for this study was Group Health, a large integrated health-care system in western Washington State. Data were used from an ongoing case-control study of incident myocardial infarction and stroke cases with a shared common control group.²² Further details of all study samples are available in the Section I in the Data Supplement.

Genotyping and Imputation

Genome-wide genotyping in the Framingham Offspring cohort was performed on the Affymetrix GeneChip Human Mapping 500k Array Set and the 50K Human Gene Focused Panel. The genotyping of the KORA sample was performed with the Affymetrix Human Mapping 500k Array 2 chip set (Sty I and NSP I). Genotypes were imputed in both samples to the HapMap-CEU panel using MACH algorithm. Genotyping in the GHS I and GHS II was performed using the Affymetrix Genome-Wide Human SNP Array 6.0 (<http://www.affymetrix.com>), as described by the Affymetrix user manual.

The individual studies included in the stroke population of the CHARGE consortium had finalized their genome-wide association scans before forming the consortium. In the ARIC study, genotyping was performed with the GeneChip SNP Array 6.0 (Affymetrix); in the CHS, the HumanCNV370-Duo (Illumina) was used and in the Rotterdam Study, version 3.0 of the Infinium HumanHap550 chip (Illumina) was used.

In the Leeds stroke cohort, we used a fluorescence-based assay (Applied Biosystems) to genotype directly the SNPs reaching genome-wide significance in the discovery analysis for SDMA. Genotyping in the Heart and Vascular Health Study (HVH) study was performed using the Illumina 370CNV BeadChip system. Filtering and imputation methods are detailed in the Section II in the Data Supplement.

Heritability Analysis

Within the family-based FHS sample, heritability estimates for each biomarker (ADMA, SDMA, L-Arginine) were performed using

variance component analyses as implemented in the software package in SOLAR.

Statistical Methods for Discovery, Replication, and Meta-Analysis

Within each cohort, each biomarker and the L-arginine/ADMA ratio was tested for association as outcome variable, adjusted for sex, age, diabetes mellitus, systolic and diastolic blood pressure, smoking, body mass index, and serum creatinine. GWAS results were combined by using inverse-variance weighted meta-analysis. Loci that reached genome-wide significance in the discovery analysis were replicated in the GHS II cohort. To guarantee independency of both GHS cohorts, the genetic analyses of GHS II were performed 6 months after analysis of GHS I and with a different batch of assays. At the final stage, inverse-variance weighted meta-analysis was used to combine discovery and replication cohorts. For the analysis, a minor allele frequency (MAF) filter <0.01 and a genome-wide significance level of $P<5\times 10^{-8}$ was applied.

Statistical Methods for Clinical Outcome Analysis

Non-normally distributed variables (including SDMA, as assessed by Kolmogorov–Smirnov test) were log transformed to achieve a normal distribution, and data presented as mean or geometric mean and 95% confidence intervals. Associations between AGXT2 variants and plasma SDMA levels in the Leeds stroke study were evaluated by 1-way ANOVA. Associations between AGXT2 variants and subtypes of ischemic stroke were evaluated by pairwise χ^2 analysis and Bonferroni adjustment for multiple comparisons. Cox regression analyses in the Leeds study were performed in a multivariable analysis adjusting for the demographic and clinical determinants previously shown to predict poststroke mortality in this cohort (age, atrial fibrillation, previous stroke, and stroke subtype) and in a second model additionally adjusting for renal function, expressed as estimated glomerular filtration rate. Log minus log plots were evaluated to test the validity of the proportionality of hazards assumption over time; all variables met this assumption. Longevity and mortality analysis in the CHARGE consortium was performed as described elsewhere.^{23–25} For more details of statistical analyses, see Sections III to V in the Data Supplement.

Measurement of Dimethylarginines

Plasma concentrations of L-arginine, ADMA, and SDMA were analyzed using a fully validated high throughput mass spectrometric method.²⁶ A detailed description of the methods is in the Section VI in the Data Supplement.

Computational Modeling and Structure Analysis

Methods for the computational modeling and structure analysis of AGXT2 are detailed in the Section VII in the Data Supplement.

Cloning and Expression of Human AGXT2 and Activity Assay in HEK Cells

Human expression analysis, cloning strategy and activity assay for experimental studies in human embryonic kidney (HEK) cells over-expressing AGXT2 wild type and mutant are detailed in the Section VIII to X in the Data Supplement.

Results

Study Samples

In total, 7927 individuals (6748 in stage 1 discovery and 1179 in stage 2 replication) contributed information to the genome-wide association analysis of plasma levels of ADMA, L-arginine, and SDMA. The baseline characteristics of the discovery study sample (FHS, KORA, and GHS I) and of the replication sample (GHS II) are displayed in Table 1. A

correlation matrix of biomarkers is presented in the Table I in the Data Supplement.

Table 2 illustrates the primary findings from the genome-wide association analyses and is detailed in the Table II in the Data Supplement. Multivariable-adjusted heritability estimates in the FHS cohort were as follows: 15% ($SE=0.051$; $P=1.26\times 10^{-3}$) for ADMA; 42% ($SE=0.056$; $P=4.5\times 10^{-16}$) for L-arginine; and 18% ($SE=0.059$; $P=6.7\times 10^{-4}$) for SDMA plasma levels.

Genetic Loci Associated With Plasma ADMA Levels

The SNPs reaching genome-wide significance for ADMA were all within the same chromosomal locus on 1p22 and were located in the *DDAH1* gene (Figure IA in the Data Supplement). The most significant SNP (rs18582) was located in intron 1 within the *DDAH1* gene (Table 2) and was in linkage disequilibrium (LD) ($R^2<0.88$) with the other genome-wide significant SNPs associated with ADMA. According to HapMap-CEU, rs18582 was in strong LD ($R^2>0.8$) with 20 SNPs of which 17 were located in intronic regions. This whole region was covered by 2 tagSNPs (rs233109, rs233113) located in the 3' untranslated region of *DDAH1* which were both in LD with rs18582 ($R^2<0.89$). Plasma levels of ADMA were higher in individuals with more minor alleles of rs18582 (Table 3). *DDAH1* is 1 of 2 known subtypes of hydrolases regulating the metabolism of ADMA.

Genetic Loci Associated With L-Arginine Plasma Levels

The most significant association signals for L-arginine were found in the chromosomal region 6q22 (Figure IB in the Data Supplement) including *MED23* (mediator complex subunit 23), which is a cofactor required for specificity protein 1 transcriptional activation. Four SNPs reached genome wide significance of which 3 could be replicated (rs2248551, P [meta]= 3.78×10^{-19} ; rs2608953, P [meta]= 5.64×10^{-19} ; and rs3756785, P [meta]= 1.11×10^{-13} ; Table II in the Data Supplement). From the SNPs associated with L-arginine plasma levels, rs2608953, rs3843995, and rs3756785 were in strong LD with the top SNP rs2248551 ($r^2=0.96$). The genetic locus *MED23* overlaps with that of *ARG1*, a hydrolase known to be involved in L-arginine degradation. The *MED23* variant rs2248551 is in strong LD ($R^2>0.9$) with a total of 11 SNPs (according to HapMap-CEU) of which 2 are within the *ARG1* locus.

The higher the number of minor alleles of rs2248551 was the higher was the plasma concentration of L-arginine (Table 3). Only MONICA/KORA showed opposite effects which might be because of the low MAF of 0.0037 for rs2248551 in this cohort. The SNP rs3843995 is located in the ectonucleotide pyrophosphatase/phosphodiesterase 3 (*ENPP3*) gene. *ENPP3* belongs to a group of enzymes that are involved in the hydrolysis of extracellular nucleotides.

We also looked for SNPs associated with the L-arginine/ADMA ratio, but there was no genome-wide hit for the L-arginine/ADMA ratio in the meta-analysis (Figure II in the Data Supplement). The mean L-arginine/ADMA ratios ($\pm SD$) for FHS, GHS I, and KORA were 149.37 (± 44.38), 149.86 (± 47.44), and 146.91 (± 55.42), respectively.

Table 1. Baseline Characteristics of the Discovery Samples FHS, GHS I, and KORA and From the Replication Sample GHS II

	FHS	GHS I	KORA	GHS II
Clinical characteristics				
Sample size	2992	3175	581	1179
Sex (male proportion), %	46	51	48	50
Age, y	59.0 (9.7)	56.0 (10.9)	53.2 (9.9)	55.10 (10.9)
Systolic blood pressure, mm Hg	128 (19)	134 (18)	134 (20)	131 (17)
Diastolic blood pressure, mm Hg	75 (9)	83 (9)	82 (11)	82 (9)
BMI, kg/m ²	27.89 (5.14)	27.20 (4.75)	26.99 (3.77)	27.32 (4.99)
Diabetes mellitus, %	11	7	4	8
Current cigarette smoking, %	15	18	16	21
Biochemical characteristics				
Serum creatinine, mg/dL	1.02 (0.18)	0.90 (0.19)	0.75 (0.16)	0.91 (0.31)
Plasma ADMA, μ mol/L	0.55 (0.13)	0.52 (0.11)	0.64 (0.20)	0.52 (0.08)
Plasma L-arginine, μ mol/L	78.9 (27.9)	75.9 (20.9)	89.0 (31.6)	74.3 (17.8)
Plasma SDMA, μ mol/L	0.40 (0.10)	0.48 (0.11)	0.50 (0.20)	0.37 (0.09)

There were no significant differences in any of the baseline variables between the cohorts. Data are given as mean (SD). ADMA indicates asymmetrical dimethylarginine; BMI, body mass index; FHS, Framingham Heart Study; GHS, Gutenberg Health Study; KORA, Cooperative Health Research in the Augsburg Area, Augsburg, Bavaria, Germany; and SDMA, symmetrical dimethylarginine.

Genetic Loci Associated With SDMA Plasma Concentrations

SDMA plasma levels were associated with various SNPs located at chromosome 5p13 (Figure 1C in the Data Supplement) including the *AGXT2* gene. *AGXT2* is 1 of 2 alanine-glyoxylate-aminotransferases which catalyze the conversion of glyoxylate to glycine using L-alanine as the amino group donor, as shown in rats.²⁷ Plasma levels of SDMA increase with each minor allele of rs37369 (Table 3).

The SNP rs37369 is located in the coding region of *AGXT2*. The *AGXT2* variant rs37369 in exon 4 is characterized by a C>T exchange, resulting in an amino acid exchange from valine to isoleucine at position 140 (Val140Ile). These characteristics suggest that the *AGXT2* variant rs37369 might modulate the activity of *AGXT2*.

LD analysis based on HapMap-CEU (HapMap genome browser phase 2) revealed overall 14 SNPs in strong LD with rs37369 ($R^2 > 0.8$), whereas one of these SNPs (rs2279651) is also located downstream in the coding region of *AGXT2*

but resulted in a synonymous exchange of histidine at position 118.

Structural Modeling of AGXT2

To evaluate whether the polymorphism rs37369 could affect *AGXT2* activity, we performed computer-based 3-dimensional structure modeling and analysis of *AGXT2* with and without the respective allele of the SNP and with SDMA as a possible substrate (Figure 1A). Valine 140 is located in a loop buried in the interior of the protein and forms tight interactions with the second subunit of the enzyme. In addition, this residue is located close to the substrate binding site (Figure 1B). Replacement of V140 by isoleucine (as coded by SNP rs37369) leads to clashes with a spatially adjacent glutamine (Q83) of the second subunit and with one of the methyl groups of SDMA (Figure 1C). The clash with Q83 at the subunit interface is reminiscent of that observed for a pathogenic G41R mutation in the isoform *AGXT1*, which disrupts the dimer interface and leads to peroxisomal aggregation.²⁸ The

Table 2. Association Data for the Meta-Analysis Studies for ADMA, L-Arginine, and SDMA Plasma Levels

Trait	SNP	Locus	SNP Type	Nearest Gene	Allele	Allele Frequency	Effect Size (SE)*	P Value Discovery	P Value			P Value			Het P Val
						Coded/ Noncoded of Coded Allele									
ADMA	rs18582	1p22	Intronic	<i>DDAH1</i>	A/G	0.656	−0.022 (0.002)	6.28×10^{-26}	−0.015	−0.026	−0.026	4.95×10^{-7}	−0.017	7.63×10^{-31}	0.042
L-Arginine	rs2248551	6q23	Intronic	<i>MED23</i>	A/G	0.146	4.022 (0.535)	5.66×10^{-14}	3.843	4.024	4.176	4.74×10^{-7}	5.054	3.78×10^{-19}	0.827
SDMA	rs37369	5p13	CDS	<i>AGXT2</i>	T/C	0.080	0.034 (0.003)	2.21×10^{-31}	0.034	0.078	0.032	7.62×10^{-5}	0.018	8.16×10^{-33}	0.009

Detailed information about all genome wide significant SNPs including *P* values and corrected *P* values are shown in the Table I in the Data Supplement. ADMA indicates asymmetrical dimethylarginine; CDS, coding sequence; FHS, Framingham Heart Study; GHS, Gutenberg Health Study; Het, Heterogeneity; KORA, Cooperative Health Research in the Augsburg Area, Augsburg, Bavaria, Germany; and SDMA, symmetrical dimethylarginine.

*Effect size was estimated from discovery analysis.

Table 3. Distribution of ADMA, L-Arginine, and SDMA Plasma Levels Stratified by Genotype of the Most Genome-Wide Significant Variants in the Discovery Samples FHS, GHS I, KORA, and the Replication Sample GHS II

Trait; SNP	Cohort	Mean	Median	IQR, $\mu\text{mol/L}$	Mean	Median	IQR, $\mu\text{mol/L}$	Mean	Median	IQR, $\mu\text{mol/L}$
		at Genotype 0 Minor Alleles			at Genotype 1 Minor Allele			at Genotype 2 Minor Alleles		
ADMA; rs18582	FHS	0.544	0.530	0.154	0.554	0.540	0.153	0.550	0.540	0.168
	GHS I	0.502	0.500	0.120	0.523	0.520	0.132	0.559	0.550	0.130
	KORA	0.620	0.630	0.260	0.646	0.630	0.295	0.699	0.710	0.235
	GHS II	0.508	0.504	0.100	0.515	0.510	0.100	0.547	0.541	0.120
L-Arginine; rs2248551	FHS	77.83	75.81	23.84	81.68	79.87	25.76	87.32	83.97	28.88
	GHS I	74.84	72.80	26.82	78.05	77.70	27.84	87.92	87.19	23.08
	KORA	88.83	85.75	38.22	90.44	83.28	43.43	76.09	76.09	2.67
	GHS II	72.63	72.20	23.38	78.33	76.55	25.00	81.73	81.31	23.70
SDMA; rs37369	FHS	0.390	0.378	0.122	0.425	0.410	0.131	0.469	0.495	0.166
	GHS I	0.471	0.460	0.125	0.501	0.484	0.130	0.597	0.520	0.202
	KORA	0.488	0.460	0.250	0.571	0.560	0.270	0.503	0.490	0.318
	GHS II	0.367	0.360	0.081	0.378	0.370	0.080	0.460	0.435	0.113

ADMA indicates asymmetrical dimethylarginine; FHS, Framingham Heart Study; GHS, Gutenberg Health Study; IQR, interquartile range; KORA, Cooperative Health Research in the Augsburg Area, Augsburg, Bavaria, Germany; and SDMA, symmetrical dimethylarginine.

accordant pathogenic mutation in *AGXT1* was identified as the molecular cause of primary hyperoxaluria type I, which is associated with diminished *AGXT1* activity.²⁹ Therefore, although the I140-Q83 clash is less pronounced in *AGXT2*, the variant was predicted to have an effect on loop conformation and substrate access to the active site. In addition, a large clash of I140 is observed with 1 methyl group of SDMA, which was predicted to dramatically reduce the affinity for this substrate.

Location of *AGXT2* Expression

The mRNA expression profile of human *AGXT2* is shown in Figure III in the Data Supplement. Amplification of the *AGXT2* fragment indicated the strongest mRNA expression

in kidney and liver, followed by tissues from placenta, heart, pancreas, skeletal muscle, and lung.

Effect of *AGXT2* rs37369 Variants on SDMA-Metabolizing Activity

Overexpression of *AGXT2* containing the rs37369 C-allele (Val140) and the mutated *AGXT2* rs37369 T-allele (Ile140) was performed in HEK 293 cells to confirm the results of the computer-based structure analysis. Transfection efficiency was examined by Western blot analysis and showed no significant difference between HEK cells expressing the *AGXT2* rs37369 C-allele in comparison with the *AGXT2* rs37369 T-allele (Figure IV in the Data Supplement). Overexpression of the *AGXT2* rs37369 C-allele resulted in a significantly enhanced d6-SDMA-metabolizing activity, which was

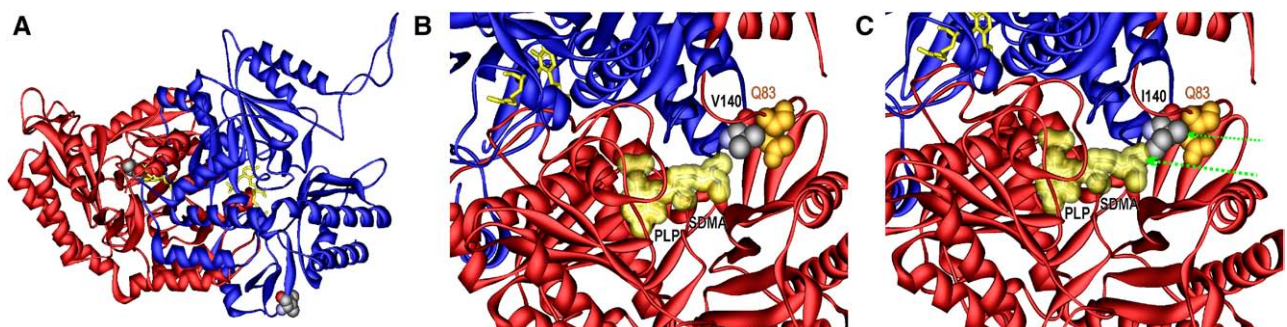


Figure 1. Results of the computerized 3-dimensional structure analysis of *AGXT2*. **A**, Three-dimensional model of human *AGXT2*, which in the absence of a known crystal structure of this enzyme itself was based on the crystal structure of dialkylglycine decarboxylase (protein data bank code; 1D7R), which shares 50% sequence homology to human *AGXT2*. The subunits A and B of the homodimeric protein are colored in blue and red, respectively, and are shown in a backbone ribbon presentation. The substrate symmetrical dimethylarginine (SDMA) and the cofactor pyridoxal phosphate (PLP) are highlighted as yellow sticks. The sites of mutation detected in this study are shown in space-filled presentation for subunit A. Note that V140 from subunit A deeply penetrates into subunit B and is located close to the active site of subunit B. Location of the residue 140 in wild-type (**B**) and V140I-mutant (**C**) *AGXT2*. Residue 140 of subunit B and the adjacent Q83 of subunit A are shown in space-filled presentation and colored by atom-type and in orange, respectively. The SDMA substrate and the PLP cofactor are shown in stick presentation, and their spatial requirement is indicated by a yellow translucent surface. The green arrows in (**C**) mark clashes of I140 with Q83 and with one N-methyl group of SDMA, which are not present in the wild type. The thickness of the arrows reflects the relative magnitude of the clashes.

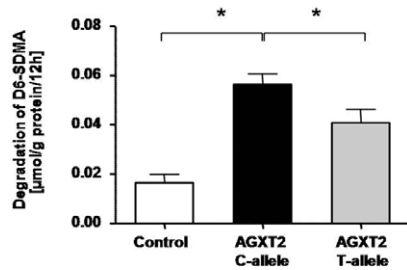


Figure 2. AGXT2 overexpression in human embryonic kidney (HEK) cells. Degradation of stable isotope-labeled d6-SDMA by HEK cells overexpressing wild-type AGXT2 in comparison with HEK cells overexpressing the AGXT2 rs37369 variant and control cells transfected with empty pcDNA3.1 vector. $n=10$ in each group; (2-tailed t test; $*P=0.006$).

significantly reduced when the AGXT2 rs37369 T-allele was overexpressed (Figure 2).

Phenotypic Associations of AGXT2 Variants With Stroke and Poststroke Mortality

Having established a functional relationship between SDMA and AGXT2 variants, we assessed the relationships between AGXT2 variants, plasma SDMA levels, and long-term all-cause mortality after acute ischemic stroke in the Leeds Stroke Study. Because of the low minor allele frequency (8%) of rs37369, we chose 2 additional SNPs with a minor allele frequency $>8\%$ which are in strong linkage disequilibrium with rs37369 (rs28305 MAF=10%, $R^2=0.80$; rs40200 MAF=9%, $R^2=0.80$). In 394 individuals of the Leeds Stroke Study, rs28305, rs40200, and rs37369 were significantly associated with plasma SDMA levels, with the lowest levels of SDMA in individuals homozygous for the minor allele of each variant in a manner suggestive of a recessive effect, indicated from post hoc analysis and Bonferroni correction for multiple comparisons (Table III in the Data Supplement). Univariate Cox regression analysis revealed a trend toward worse cumulative survival in individuals homozygous for the minor alleles, with significantly poorer survival in individuals homozygous for the A allele of rs40200 compared with individuals possessing the G allele (hazard ratio 3.05 [1.13, 8.22], $P=0.022$). After adjustment for age, atrial fibrillation, previous stroke, stroke subtype, and renal function, this association became nonsignificant (Table IV in the Data Supplement). We also observed significant and borderline significant associations between AGXT2 variants and subtypes of ischemic stroke (Table V in the Data Supplement). Analysis of the relationships between AGXT2 variants and cardiometabolic risk factors in the Leeds Stroke Study (Table VI in the Data Supplement) was performed assuming a recessive effect based on associations between SNPs and plasma SDMA and revealed significant associations between the minor alleles of AGXT2 variants and measures of renal function (plasma creatinine, estimated glomerular filtration rate), markers of inflammation (C-reactive protein), and hemostatic factors (fibrinogen, factor VIII, and von Willebrand Factor), suggesting a potential role in the pathogenesis of stroke. In further analyses of the Leeds Stroke Study, we did not identify significant associations between AGXT2 variant distributions of patients with ischemic stroke

and age-matched controls or were there significant differences in the AGXT2 genotype distributions of patients with ischemic and hemorrhagic stroke (data not shown).

The association between incident stroke and SDMA-related genotypes was investigated in 19602 individuals of the CHARGE consortium.²¹ No associations between AGXT2 variants and incidence of overall stroke ($P=0.045$ – 0.451) or ischemic stroke ($P=0.211$ – 0.895) were identified. In addition AGXT2 variants were not associated with longevity and time-to-death in the CHARGE consortium. Finally, the AGXT2 genotype frequencies were analyzed in participants of the HVH study (case–control study of 502 patients with prevalent ischemic stroke and 1314 controls),^{21,25,26} and no differences in AGXT2 genotype distributions were identified ($P=0.223$ – 0.626).

Discussion

The major findings of our study are (1) confirmation that circulating levels of ADMA, SDMA, and L-arginine are heritable traits; (2) identification of functional and known genetic loci for each of the 3 biomarkers: (*DDAH1* for ADMA, *MED23/Arg1* for L-arginine, and *AGXT2* for SDMA); (3) experimental confirmation of the role of a functional variant for AGXT2 in SDMA metabolism; and (4) no associations between AGXT2 variants and poststroke mortality.

Associations Between ADMA and DDAH1

The genetic association of ADMA and *DDAH1* for ADMA levels in this GWAS confirms in man data from animal models that indicated a role for DDAH1 in the regulation of ADMA.¹² Two subtypes of DDAH, *DDAH1*, and *DDAH2* have been described which differ in their tissue expression profiles.³⁰ The isoform mainly responsible for ADMA degradation remains uncertain. Mice overexpressing either *DDAH1* or *DDAH2* show equally reduced ADMA levels and enhanced NO synthesis.^{29,30} However, we recently reported that *DDAH1* is the major isoform involved in ADMA degradation based on studies in tissue-selective endothelial *DDAH1* knockout mice.¹¹ One of the SNPs most strongly associated with ADMA in the present GWAS was rs1554597 located in intron 1 of *DDAH1*. Caplin and coworkers identified a regulatory sequence within intron 1 in the *DDAH1* gene associated with the rate of decline of glomerular filtration rate in subjects with chronic kidney disease,³¹ along with decreased *DDAH1* mRNA expression and elevated ADMA plasma concentration. Taken together, these findings indicate that the genomic area tagged by rs1554597 might be a specific regulatory sequence within *DDAH1* and support the importance of *DDAH1* in regulating ADMA metabolism in man

Associations Between SDMA and AGXT2

Despite the observation that both ADMA and SDMA emerge from the same source of methylated proteins, the global *DDAH1* knockout mice showed no differences in SDMA tissue concentration²⁷ to indicate differential regulation of these 2 dimethylarginines. The strong association of SDMA with biomarkers of renal function and calculated glomerular filtration rate³¹ led to the suggestion that SDMA might be involved in another pathway with previously unknown function located

in the kidney. Our finding that AGXT2 expressed in kidney cells metabolizes SDMA in a manner regulated by gene variants of AGXT2 rs37369 supports this hypothesis and may hint to a pathophysiological link between SDMA, renal function, and cardiovascular outcome. The AGXT2 rs37369 variant is located in the coding region of the gene, and in silico modeling showed distinct similarity of this SNP to a coding variant in the AGXT1 gene which disrupts activity of the encoded enzyme and causes type 1 hyperoxaluria.²⁸ Overexpression of the AGXT2 rs37369 T-allele (Ile140) resulted in significantly reduced metabolism of stable isotope-labeled SDMA in HEK 293 cells compared with the AGXT2 rs37369 C-allele (Val140). Our finding of the association between circulating SDMA and AGXT2 is in line with a recent GWAS performed in the Young Finns Study (YFS) and in the Ludwigshafen Risk and CHS (LURIC). In this study the same coding AGXT2 rs37369 was identified to be associated with higher heart rate variability, pointing to a possible effect on autonomic balance by modulating vagal tone.³² However, no in vitro experiments verifying the functional relevance of the coding variants in respect of their SDMA-metabolizing capacity were shown in these studies.

Rodionov et al³³ reported that adenoviral overexpression of AGXT2 in mice was linked to significantly lower hepatic and plasma ADMA concentrations, a finding we were unable to replicate here. However, our identification of AGXT2 as the SDMA-metabolizing enzyme may have therapeutic implications because several studies have identified SDMA as an independent predictive marker of cardiovascular events and mortality^{4,6,34,35}

Associations Between L-Arginine, MED23, and Arg1

The locus on chromosome 6q22 found to be associated with plasma L-arginine levels included the gene *MED23*, a cofactor required for the transcriptional activation of various RNA polymerase II-dependent genes, for example *Elk1*. Recent data suggest that *MED23* serves as a critical link transducing insulin signaling to the transcriptional cascade during adipocyte differentiation.³⁴ A gene overlapping *MED23* is *ARG1*, which codes for 1 of 2 arginase subtypes that regulate L-arginine bioavailability.³⁵ The *ARG1* SNPs (rs2248551) identified in this analysis is in LD with 1 *ARG1* SNP (rs2781168) which is part of a haplotype previously found to be associated with an increased risk for myocardial infarction in humans.³⁶ One of the biological functions of arginase may lie in the regulation of NO synthesis by competing with NO synthase for the common substrate, L-arginine. For example, in activated macrophages, use of L-arginine by the inducible isoform of NO synthase is limited by arginase activity, resulting in a suppressed cytotoxic response of these cells.^{37,38} Dysregulation of arginase is also associated with endothelial dysfunction because of decreased NO formation.³⁹ These data further underline the delicate balance between NOS and arginase activities in the control of NO formation and the pathogenesis of cardiovascular disease.⁴⁰

Our finding that the L-arginine/ADMA ratio lacks any genetic association is reasonable, given the fact that L-arginine

and ADMA are not involved in one common metabolic pathway. Calculation of the L-arginine/ADMA ratio is generally performed to address substrate availability of the NO synthase while in many cases metabolite ratios have been applied to better characterize one common metabolic pathway.

Associations Between SDMA, AGXT2, and Stroke

Because 2 studies pointed to a potential role for SDMA as a predictor of short-term and long-term outcome after ischemic stroke, we investigated the association between AGXT2 and long-term mortality after stroke. Individuals homozygous for the minor allele showed significantly higher SDMA plasma levels, higher plasma levels of various cardiometabolic risk factors, and a trend toward poorer survival in unadjusted analyses. This trend was lost, however, after adjusting for previously identified determinants of poststroke mortality including renal function. In addition, no differences in the genotype distributions of AGXT2 variants between patients with stroke and healthy controls were identified in the Leeds Stroke Study. Nor were associations with incident stroke identified in the CHARGE consortium or in the HVH study, pointing to a more indirect role for AGXT2 in explaining the association of SDMA, renal function, and outcome. This hypothesis is supported by the lack of association between AGXT2 variants and total mortality in the CHARGE consortium after exclusion of stroke as a cause of death. Our finding is also in line with a recent analysis showing that none of the AGXT2 variants were associated with cardiovascular and overall mortality in the Ludwigshafen Risk and CHS study.³² However, in that study, a possible link between AGXT2 variants, renal function, and outcome was not considered. Our data may explain a relationship between AGXT2 genotype and long-term mortality after acute ischemic stroke, potentially mediated through the indirect link between AGXT2, SDMA, and renal function. However, it must be remembered that only 18% of interindividual variance of SDMA is hereditary, so AGXT2 variants may explain only part of the association between SDMA, renal function, and outcome. Further studies will be needed to investigate this hypothesis further.

Conclusions

In conclusion, this collaborative study comprising GWAS of large, population-based cohorts has revealed conclusive data that identifies the genes for the critical enzymes involved in the regulation of ADMA, L-arginine, and SDMA. We identified AGXT2 regulation of SDMA as a novel renal pathway that might be a pathophysiological link between SDMA, renal function, and cardiovascular disorders. Further prospective studies in man are warranted to establish the precise nature of this relationship and the potential for translational approaches to modulate cardiorenal disease in man.

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Disclosures

None.

Appendix

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CLINICAL PERSPECTIVE

Asymmetrical dimethylarginine and symmetrical dimethylarginine are known to be cardiovascular risk factors. Both biomarkers interfere with the L-arginine/nitric oxide pathway and are often associated with an endothelial dysfunction. High plasma and tissue concentrations of these biomarkers are associated with poor outcome in various clinical conditions. Understanding the environmental and genetic contributions to the interindividual variability of these biomarkers is one important question. We identified genetic associations of dimethylarginine dimethylaminohydrolase I and arginase 1 with asymmetrical dimethylarginine and L-arginine plasma concentrations, respectively. In addition, we identified alanine glyoxylate aminotransferase 2 as a new pathway genetically as well as functionally associated with symmetrical dimethylarginine plasma concentrations. This genome-wide association study of asymmetrical dimethylarginine, symmetrical dimethylarginine, and L-arginine provides new insights into the pathways associated with these biomarkers and gives the opportunity of potential new pharmaceutical treatment strategies for cardiovascular diseases like atherosclerosis, hypertension, stroke, and diabetes mellitus.

SUPPLEMENTAL MATERIAL

A. Supporting Methods

1. Cohorts

All participants provided written informed consent (including consent for genetic analyses), and the study protocols were approved by local institutional review boards and ethical committees.

Framingham Offspring Cohort

The present analyses were performed within the Offspring Cohort of the Framingham Heart Study ¹. In brief, the Framingham Heart Study is a longitudinal observational, community-based cohort initiated in 1948 in Framingham, MA, to prospectively investigate risk factors for cardiovascular diseases. The Framingham Offspring Cohort consists of the children (and children's spouses) of the original Framingham cohort participants ¹. After exclusions, 2992 participants had complete genotypic information from the 550k gene array (as detailed below) and plasma levels for L-Arginine, SDMA and ADMA available.

MONICA/KORA F3 Cohort

The individuals of the MONICA/KORA sample participated in the third survey (S3) of the Multinational Monitoring of Trends and Determinants in Cardiovascular Disease Study (MONICA) Augsburg study, which is now continued in the framework of KORA (Cooperative Health Research in the Augsburg Area, Augsburg, Bavaria, Germany ². Blood samples for biomarker measurement were drawn at the examination cycle in 1994/95. Overall, 4856 subjects participated in the study, of which 3006 individuals had a follow-up examination between 2004 and 2005 (KORA F3). Out of this population, 1644 participants (age range 35-79 years) had a genome-wide association study conducted of which 581 had plasma concentrations of L-Arginine, SDMA and ADMA available.

Gutenberg Health Study

The Gutenberg Health Study (GHS) was initiated in 2007 as a community-based, prospective cohort study including participants, aged 35 to 74 years³. All participants were drawn randomly from the local registry offices in the city of Mainz and the district of Mainz-Bingen. The sample is stratified according to sex (50% women), decade of age and residence (urban and rural). A large variety of non-invasive cardiovascular phenotypes have been assessed. Blood samples have been drawn for biomarker measurements and genetic analyses. For GHS I 3,175 and GHS II 1,179 individuals, respectively, had genome-wide data available of whom 3166, 3164 and 3161 (GHS I) and 1159, 1152 and 1151 (GHS II) had plasma concentrations of L-Arginine, SDMA and ADMA respectively available.

LEEDS stroke cohort

The recruitment and characteristics of patients included in the Leeds stroke study have been described elsewhere ⁴. Briefly, consecutive white European patients (**n** = 609) with a clinical diagnosis of acute ischaemic stroke were recruited from four hospitals in Leeds. Only patients who survived for longer than 30 days after the acute event with sufficient plasma available for analysis of ADMA, SDMA and L-arginine were included in the present analysis of post-stroke mortality (**n** = 394). According to the Oxfordshire Community Stroke Project ischaemic stroke was classified as lacunar infarction (LACI), total and partial anterior circulation infarction (TACI, PACI) ⁵. All subjects provided informed consent according to a protocol approved by the Leeds Teaching Hospitals Research Ethics Committee. For case-control analysis, genotype distributions between patients with ischemic stroke (**n**=394), haemorrhagic stroke (**n**=57) and age-matched healthy controls (**n**=430) were evaluated ⁶.

CHARGE consortium (Cohorts for Heart and Aging Research in Genomic Epidemiology)

The Cohorts for Heart and Aging Research in Genomic Epidemiology Consortium was formed to facilitate genome-wide association study meta-analysis and replication opportunities among multiple large population-based cohort studies, which collect data in a standardized fashion and represent the preferred method for estimating disease incidence. For each harmonized trait, within-cohort genome-wide association study analyses are combined by meta-analysis.

The design of the stroke population of the CHARGE consortium includes 4 prospective cohorts from the United States and Europe: the Atherosclerosis Risk in Communities Study (ARIC), the Cardiovascular Health Study (CHS), the Framingham Heart Study (FHS) and the Rotterdam Study. Details of the cohort selection and surveillance, and criteria for diagnosis and subtyping of stroke are described elsewhere ⁷. In brief, in the four cohorts composing the CHARGE consortium 19,602 white persons (mean age 63±8 years) were included. During a mean follow-up period of 11 years 1544 incident strokes developed of which 1164 were classified as ischemic strokes.

Heart and Vascular Health Study (HVH)

The setting for this study was Group Health (GH), a large integrated health care system in western Washington State. Data were utilized from an ongoing case-control study of incident myocardial infarction (MI) and stroke cases with a shared common control group. Methods for the study have been described previously and are briefly summarized below ⁸⁻¹⁰. The study was approved by the human subjects committee at GH, and written informed consent was provided by all study participants. All study participants were GH members and aged 30-79 years. MI and stroke cases were identified from hospital discharge diagnosis codes and were validated by medical record review. Controls were a random sample of GH members frequency matched to stroke cases on age (within decade), sex, treated hypertension, and calendar year of identification. The index date for controls was a computer-generated random date within the calendar year for which they had been selected. Participants were excluded if they were recent enrollees at GH, had a history of prior MI or stroke, or if the incident event was a complication of a procedure or surgery. Eligibility and risk factor information were collected by trained medical record abstractors from a review of the GH medical record using only data available prior to the index date and through a telephone interview. Medication use was ascertained using computerized GH pharmacy records. A venous blood sample was collected from all consenting subjects, and DNA was extracted from white blood cells using standard procedures.

2. Genotyping, quality controls and imputations

For the Framingham Offspring cohort, the Human Mapping 500k Array Set and the 50K Humane Gene Focused Panel genotyping platform were used. From a total of 534982 genotyped autosomal SNPs in FHS, 378163 SNPs were used in the imputation after filtering out 15586 SNPs (Hardy-Weinberg $p < 1e-6$), 64511 SNPs (call rate $< 97\%$), 45361 SNPs (mishap $p < 1e-9$, PLINK, <http://pngu.mgh.harvard.edu/purcell/plink/>), 4857 SNPs (> 100 Mendel errors), 67269 SNPs (frequency < 0.01), 2 SNPs (strandedness issues upon merging data with HapMap), and a further 13394 SNPs (as they were not present on HapMap). MACH (version 1.0.15, <http://www.sph.umich.edu/csg/abecasis/MACH/>) software was used to impute all autosomal SNPs on HapMap, using the publicly available phased haplotypes from HapMap (release 22, build 26, CEU population) as a reference panel. From a total number of 10,886 genotyped Chromosome X SNPs, 7,795 SNPs were used as input to the IMPUTE program (v0.5.0, <https://mathgen.stats.ox.ac.uk/impute/impute.html>). A total of 3,091 genotyped SNPs were removed based on the following conditions: 1) 159 SNPs that had a Hardy-Weinberg $p < 1e-6$; 2) 450 SNPs with call rate $< 97\%$; 3) 1,851 SNPs with MAF less than 0.01; 4) 12 SNPs with a male heterozygote count greater than or equal to 45; and 5) 619 SNPs not included in the HapMap legend files. The phased haplotype and legend files were based on Build 35 release 21 from the Hapmap project. The KORA sample was

genotyped with the Human Mapping 500k Array 2 chip set (Sty I and NSP I). The mean and median call rates for KORA were 98.3% and 98.5%, respectively, as detailed at http://epi.helmholtz-muenchen.de/kora-gen/index_e.php. Genotypes were imputed in both samples to the HapMap CEU panel using the MACH algorithm.

Genotyping in the GHS I and GHS II was performed using the Affymetrix Genome-Wide Human SNP Array 6.0 (<http://www.affymetrix.com>), as described by the Affymetrix user manual. Genotypes were called using the Affymetrix Birdseed-V2 calling algorithm and quality control was performed using PLINK¹¹.

Individuals with a call rate below 97% or a too high autosomal heterozygosity were excluded. After applying standard quality criteria (minor allele frequency <1%, genotype call rate <98% and P-value of deviation from Hardy-Weinberg equilibrium >10⁻⁴), 650,010 autosomal SNPs remained for analysis in GHS I.

The stroke population of the CHARGE consortium was formed after the individual studies had finalized their genomewide association study platforms. In the ARIC study, genotyping was performed with the GeneChip SNP Array 6.0 (Affymetrix); in the Cardiovascular Health Study, the HumanCNV370-Duo (Illumina) was used; in the Framingham Heart Study, the Gene-Chip Human Mapping 500K Array Set and 50K Human Gene Focused Panel (Affymetrix) were used; and in the Rotterdam Study, version 3.0 of the Infinium HumanHap550 chip (Illumina) was used. The detailed imputation methods and quality control measure are described elsewhere⁷. Logistic regression analysis was used to investigate the association of **AGXT2** variants and incidence of all cause and ischemic stroke, respectively.

In the LEEDS cohort we used a fluorescence-based assay (Applied Biosystems) to directly genotype the SNPs reaching genome-wide significance in the discovery analysis for SDMA. Pairwise association analysis between **AGXT2** variants and stroke subtypes was corrected after bonferroni.

Genotyping in the HVH study was performed at the General Clinical Research Center's Phenotyping/Genotyping Laboratory at Cedars-Sinai using the Illumina 370CNV BeadChip system. Genotypes were called using the Illumina BeadStudio software. Samples were excluded from analysis for sex mismatch or call rate < 95%. The following exclusions were applied to identify a final set of 301,321 autosomal SNPs: call rate < 97%, HWE P < 10⁻⁵, > 2 duplicate errors or Mendelian inconsistencies (for reference CEPH trios), heterozygote frequency = 0, SNP not found in HapMap, inconsistencies across genotyping batches. Imputation was performed using BAMB with reference to HapMap CEU using release 22, build 36 using one round of imputations and the default expectation-maximization warm-ups and runs. Logistic regression was used to investigate the association of **AGXT2** SNPs with the risk of stroke, adjusting for the matching factors of age, sex, hypertension status and index year. We used linear additive models with robust standard errors and estimated risk for each additional copy of the variant allele, using R. SNPs were excluded from analysis for variance on the allele dosage < 0.01. The stroke analysis included 501 ischemic stroke cases and 1314 controls. The genomic control lambda was 1.07 for stroke.

3. Heritability analyses

Within the family-based FHS sample, heritability estimates for each biomarker (ADMA, SDMA, L-Arginine) were performed using variance component analyses as implemented in the software package in SOLAR¹². Due to the lack of normal distribution normal quantile transformation to the three traits was done to comply with the null hypothesis of normality. We also performed adjustments for age, sex, diabetes systolic blood pressure, diastolic blood pressure, smoking, body mass index, and serum creatinine. The 3199 phenotyped participants belonged to 1065 different pedigrees (494 of them are singletons); the number of individuals per family range from 1 to 138 (median: 2).

4. Statistical methods for discovery, replication and metaanalysis

Within each cohort (FHS, KORA, GHS I), each biomarker (ADMA, SDMA, L-Arginine) and the L-arginine/ADMA ratio was tested for association using 2.2 million SNPs (FHS and KORA, imputed to the HapMap CEU sample) or 650,010 SNPs (GHS I, genotyped) as exposure variables, assuming an additive genetic model, and adjusting for sex, age, diabetes, systolic and diastolic blood pressure, smoking, BMI and serum creatinine. In the FHS sample, additional adjustments were performed to account for latent population substructure within the dataset using principal component analyses and a linear mixed effects model was used to account for within pedigree familial correlations. Cohort GWAS results were combined by using inverse-variance (fixed effects model) weighted meta-analysis. A MAF<0.01 filter was applied to each cohort GWAS to focus on common variants. After meta-analysis, genomic control corrected p-values were computed.

Loci that reached genome-wide significance (p-value < 5×10^{-8}) in the discovery meta-analyses) were assessed for replication in the GHS II cohort. At the final stage, inverse-variance (fixed effects model) weighted meta-analysis with the same MAF filter was used to combine results from discovery and replication cohorts. As for MONICA/KORA, the with L-arginine associated SNP rs2248551 has low imputation ratio (0.29) and low imputed allele frequency (0.0037), so rs2248551 was excluded in meta-analysis (filter MAF 0.01).

5. Statistical methods for clinical outcome analysis

In the Leeds stroke study, associations between AGXT2 variants and plasma SDMA levels were evaluated by one-way analysis of variance. Associations between AGXT2 variants and subtypes of ischemic stroke were evaluated by pairwise χ^2 analysis and Bonferroni adjustment for multiple comparisons. Cox regression analyses were carried out in a multivariable analysis adjusting for the demographic and clinical determinants previously shown to predict post-stroke mortality in this cohort (age, atrial fibrillation, previous stroke and stroke subtype) and in a second model additionally adjusting for renal function, expressed as eGFR. Associations between AGXT2 variants and cardiometabolic risk factors were assessed by independent samples t-test assuming a recessive model. Data were analyzed using SPSS v18.0.

Assuming a risk allele frequency of 0.08 (rs37369) in the control population, the HVH study was powered to detect a minimum odds ratio of 1.66 with 80% power at p=0.05.

Longevity and mortality analysis in the CHARGE consortium was performed as described elsewhere¹³⁻¹⁴. In brief, longevity was defined as reaching age 90 years or older in comparison to participants who died between the ages of 55 and 80 years. Only Caucasian participants were included. Mortality analysis was conducted in participants older than 55 years at baseline and free of any of the events: myocardial infarction, heart failure, stroke, dementia, hip fracture, cancer or death. Outcome was time to first event from baseline. Mean follow-up time was 10.6 (SD 5.4) years.

6. Measurement of dimethylarginines

Plasma samples for determination of SDMA were stored frozen at -80 °C without freeze thaw cycles until mass spectrometric determination was performed as described elsewhere by using a fully validated high throughput LC-MS/MS assay¹⁵⁻¹⁶. In brief, samples were processed by stable-isotope dilution and protein precipitation with methanol using 96-well 0.20- μ m microfiltration plates (Millipore, Schwalbach, Germany). After centrifugation, the microfiltrates were dried and analytes were converted to their butyl ester derivatives. Subsequent analyses were performed using a Chirobiotic T, 20 x 1.0 mm i.d., microbore guard column (Astec, Whippany, NJ, USA) connected to a Varian 1200L Triple Quadrupole MS (Varian, Walnut Creek, CA, USA) in the positive electrospray ionisation (ESI+) mode. The sample run time was 1.6 min with an intra-assay precision of 3.2% and an inter-assay precision of < 5% (CV).

7. Computational modelling and structure analysis

The three-dimensional structure of AGXT2 was modeled based on the crystal structure of the homologous dimeric dialkylglycine decarboxylase (PDB code; 1D7R) using the program Modeler 6.2¹⁷⁻¹⁸. The resulting model of the structure of wildtype AGXT2 exhibited a good local geometry and no steric hindrance. The SDMA and ADMA substrates were modeled based on the methylalanine ligand present in the 1D7V crystal structure by replacing the alanine side chain with N-methylated arginine. The I102S/T/N and V140I mutations were introduced into the structure using either the programs Sybyl 7.3 (Tripos Inc.) or DeepView¹⁹ by selecting the lowest-energy side chain rotamer. Clashes in the protein were analyzed with WhatCheck²⁰, and DS Visualizer v2.5 (Accelrys Software Inc.) was used graphical presentation.

8. Cloning and expression of human AGXT2

Human **AGXT2** cDNA was amplified in a RT-PCR approach using total RNA from the human kidney panel (ClonTech) as template.

For PCR the primer pair 5' TGAGTGGGAGAAATGACTCTAAT-3' (forward) and 5' CTGACAATGTTACTTAGCTCTTC-3' (reverse) were used and the amplified fragment was cloned into the pcR2.1-TOPO via TOPO® TA PCR Cloning Kit (Invitrogen) according manufacture instructions. The sequence was verified (Agowa GmbH, Germany) and base-pair exchanges to the reference sequence (NM_031900) resulting in amino acid exchanges were corrected using the QuickChange® Multi Site Directed Mutagenesis Kit (Agilent Technologies, Germany).

The resulting cDNA encoded for a protein identical to the one encoded by the reference sequence and was subcloned into the expression vector pcDNA3.1(+) leading to the plasmid pAGXT2-WT.31. This plasmid was used for site-directed mutagenesis (Multi Site Directed Mutagenesis Kit from Stratagene) with the primer 5' GTGGCATAACAAGCACCGTCTTCTTCCACCCTCC-3'. The resulting plasmid contains the single nucleotide polymorphism rs37369 with the genetic variation AGXT2c.418G>A (NM_031900.2) and leads to the amino acid exchange AGXT2p.Val140Ile (NP_114106.1).

Correctness of mutagenesis was verified by sequencing and both plasmids were used for stable transfection into HEK 293 cells.

9. AGXT2 activity assay in HEK 293 cells

Stably transfected HEK 293 cells with pcDNA3.1(+) as a control, pcDNA3.1(+)/AGXT2 WT expressing the wildtype protein and pcDNA3.1(+)/AGXT2 MUT expressing the mutant protein were grown in DMEM for 24 hours at 37°C (approx. 3 million cells per plate). Subsequently, the cells were washed with PBS and incubated for 24 hours with DMEM containing 1 mM sodium-pyruvate, 1mM sodium-glyoxylate, 0.8 mM pyridoxal-phosphate (cofactor) and 10 mM sodium-butyrate for induction (enhancement of AGXT2 over-expression). Cells were washed again with PBS and resuspended in 1ml PBS containing 1 mM sodium-pyruvate, 1 mM sodium-glyoxylate and 0,8mM pyridoxal-phosphate (cofactor). After cell lysis by sonification 100µl TBS (pH 9.0) and 0,5µmol [D6]deuterium-labeled SDMA (TRC, Canada) were added to 400µl of cell lysate. Samples from each assay tube were taken at baseline and after an incubation period of 12h. Reaction was stopped by shortly boiling the samples. [D6]-SDMA was determined by LC-MS/MS¹⁰. Protein content in the lysates was determined using a commercial assay (Pierce BCA protein assay kit, Thermo Fisher Scientific, Germany). Western blot analysis was performed according to standard procedures with an anti-AGXT2 antibody (1:500 dilution) purchased from Sigma-Aldrich (HPA037382).

10. Expression analysis of human AGXT2

The distribution of the AGXT2 mRNA in humans was determined by RT-PCR. Therefore standardized cDNA of multiple tissue cDNA panel (Human MTC Panel, Clontech) was used.

B. Supplemental Tables

Table S1: Correlation matrix of L-arginine, ADMA and SDMA within each in the GWAS participating cohort

	L-arginine	ADMA	SDMA
FHS			
L-arginine	1.000		
ADMA	0.300	1.000	
SDMA	0.188	0.463	1.000
KORA			
L-arginine	1.000		
ADMA	0.454	1.000	
SDMA	0.257	0.496	1.000
GHS I			
L-arginine	1.000		
ADMA	0.244	1.000	
SDMA	0.082	0.350	1.000
GHS II			
L-arginine	1.000		
ADMA	0.334	1.000	
SDMA	0.185	0.337	1.000

Data is presented as Pearson correlation coefficient;

Table S2: Association data for the discovery, replication and meta-analysis studies for ADMA, L-arginine and SDMA plasma levels

Trait	SNP	Locus	SNP type	Nearest gene	Coded/noncoded allele	Allele frequency of coded allele	Effect size (SE)	p-value discovery	Beta FHS	Beta KORA	Beta GHS I	p-value replication	Beta GHS II	p-value meta-analysis	Het Pval
ADMA	rs18582	1p22	Intronic	DDAH1	A/G	0.656	-0.022 (0.002)	6.28x10 ⁻²⁶	-0.015	-0.026	-0.026	4.95x10 ⁻⁷	-0.017	7.63x10 ⁻³¹	0.042
	rs2268667	1p22	Intronic	DDAH1	A/G	0.343	0.022 (0.002)	9.94x10 ⁻²⁶	0.015	0.033	0.026	1.95x10 ⁻⁷	0.017	3.94x10 ⁻³¹	0.045
	rs1554597	1p22	Intronic	DDAH1	T/C	0.651	-0.022 (0.002)	1.14x10 ⁻²⁵	-0.015	-0.003	-0.026	5.21x10 ⁻⁷	-0.017	1.42x10 ⁻³⁰	0.044
	rs10489513	1p22	Intronic	DDAH1	A/G	0.632	-0.020 (0.002)	1.33x10 ⁻²¹	-0.013	-0.033	-0.024	4.43x10 ⁻⁷	-0.017	8.62x10 ⁻²⁷	0.046
	rs6576765	1p22	Intronic	DDAH1	A/T	0.367	0.020 (0.002)	1.37x10 ⁻²¹	0.013	0.033	0.024	4.56x10 ⁻⁷	0.017	9.16x10 ⁻²⁷	0.051
	rs233124	1p22	Intronic	DDAH1	T/C	0.637	-0.020 (0.002)	3.16x10 ⁻²¹	-0.013	-0.031	-0.024	2.76x10 ⁻⁷	-0.017	1.14x10 ⁻²⁶	0.048
	rs233112	1p22	Intronic	DDAH1	T/C	0.637	-0.020 (0.002)	4.26x10 ⁻²¹	-0.013	-0.031	-0.024	3.12x10 ⁻⁷	-0.017	1.77x10 ⁻²⁶	0.051
	rs233118	1p22	Intronic	DDAH1	T/C	0.637	-0.020 (0.002)	5.05x10 ⁻²¹	-0.013	-0.031	-0.024	2.44x10 ⁻⁷	-0.017	1.55x10 ⁻²⁶	0.054
	rs12717763	1p22	Intronic	DDAH1	A/T	0.192	0.023 (0.002)	4.57x10 ⁻²⁰	0.018	0.031	0.026	6.80x10 ⁻⁷	0.020	3.65x10 ⁻²⁵	0.377
	rs17384381	1p22	Intronic	DDAH1	C/G	0.807	-0.023 (0.003)	5.79x10 ⁻²⁰	-0.018	-0.034	-0.026	1.35x10 ⁻⁶	-0.020	9.68x10 ⁻²⁵	0.347
	rs17388521	1p22	Intronic	DDAH1	A/G	0.195	-0.023 (0.003)	9.29x10 ⁻²⁰	-0.018	-0.033	-0.026	6.72x10 ⁻⁷	-0.020	7.21x10 ⁻²⁵	0.390
	rs12130591	1p22	Intronic	DDAH1	A/G	0.203	0.021 (0.003)	1.31x10 ⁻¹⁷	0.016	0.018	0.025	4.63x10 ⁻⁵	0.016	8.95x10 ⁻²¹	0.284
	rs11161614	1p22	Intronic	DDAH1	T/G	0.786	-0.019 (0.002)	3.62x10 ⁻¹⁵	-0.014	-0.027	-0.022	1.60x10 ⁻⁶	-0.019	5.52x10 ⁻²⁰	0.344
	rs1403955	1p22	Intronic	DDAH1	T/G	0.601	-0.016 (0.002)	1.10x10 ⁻¹⁴	-0.013	-0.001	-0.018	6.66x10 ⁻⁴	-0.011	1.38x10 ⁻¹⁶	0.226
	rs11161617	1p22	Intronic	DDAH1	T/C	0.776	-0.018 (0.002)	5.38x10 ⁻¹⁴	-0.012	-0.014	-0.022	6.38x10 ⁻⁵	-0.015	3.54x10 ⁻¹⁷	0.226
	rs11161616	1p22	Intronic	DDAH1	T/C	0.223	0.018 (0.002)	5.64x10 ⁻¹⁴	0.012	0.014	0.022	3.84x10 ⁻⁵	0.016	2.13x10 ⁻¹⁷	0.259
	rs10493763	1p22	Intronic	DDAH1	T/C	0.801	-0.019 (0.003)	9.57x10 ⁻¹⁴	-0.012	-0.014	-0.023	9.79x10 ⁻⁴	-0.013	1.68x10 ⁻¹⁵	0.074
	rs12129013	1p22	Intronic	DDAH1	A/C	0.734	-0.017 (0.002)	2.21x10 ⁻¹³	-0.014	-0.003	-0.019	6.45x10 ⁻⁵	-0.014	1.39x10 ⁻¹⁶	0.447
	rs11161615	1p22	Intronic	DDAH1	A/G	0.265	0.016 (0.002)	2.36x10 ⁻¹²	0.013	0.003	0.019	1.01x10 ⁻³	0.012	2.79x10 ⁻¹⁴	0.225

L-Arginine	rs2248551	6q23	Intronic	MED23	A/G	0.146	4.022 (0.535)	5.66x10 ⁻¹⁴	3.843	4.024	4.176	4.74x10 ⁻⁷	5.054	3.78x10 ⁻¹⁹	0.827
	rs2608953	6q23	Intronic	MED23	C/T	0.146	3.987 (0.534)	8.23x10 ⁻¹⁴	3.845	4.009	4.108	4.74x10 ⁻⁷	5.054	5.64x10 ⁻¹⁹	0.822
	rs3843995	6q23	Intronic	ENPP3	A/G	0.855	-3.988 (0.535)	9.39x10 ⁻¹⁴	-3.869	-3.991	-4.088	8.90x10 ⁻⁷	-4.928	1.09x10 ⁻¹⁸	0.873
	rs2608912	6q23	Intronic	MED23	T/C	0.796	-2.537 (0.455)	2.46x10 ⁻⁸	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
	rs3756785	6q23	Intronic	MED23	T/C	0.852	-4.028 (0.725)	2.75x10 ⁻⁸	n.r.	n.r.	-4.028	3.22x10 ⁻⁷	-5.123	1.11x10 ⁻¹³	0.380
SDMA	rs28305	5p13	Intronic	AGXT2	C/G	0.095	0.031 (0.003)	2.87x10 ⁻³⁰	0.030	0.076	0.030	4.29x10 ⁻⁸	0.023	4.17x10 ⁻³⁶	0.051
	rs37369	5p13	CDS	AGXT2	T/C	0.080	0.034 (0.003)	2.21x10 ⁻³¹	0.034	0.078	0.032	7.62x10 ⁻⁵	0.018	8.16x10 ⁻³³	0.009
	rs1428281	5p13	Intronic	AGXT2	T/C	0.754	-0.017 (0.002)	1.23x10 ⁻¹⁸	-0.019	-0.013	-0.015	1.79x10 ⁻³	-0.008	3.29x10 ⁻¹⁹	0.072
	rs11957653	5p13	Intronic	AGXT2	T/C	0.250	0.014 (0.002)	6.07x10 ⁻¹⁵	0.011	0.017	0.017	3.39x10 ⁻⁵	0.012	2.33x10 ⁻¹⁸	0.331
	rs40200	5p13	Intronic	AGXT2	G/A	0.093	0.031 (0.041)	1.71x10 ⁻¹⁴	0.030	0.076	-	2.77x10 ⁻⁸	0.024	9.16x10 ⁻²¹	0.043
	rs163594	5p13	Intronic	AGXT2	A/C	0.749	-0.014 (0.002)	9.54x10 ⁻¹⁴	-0.011	-0.018	-0.016	4.79x10 ⁻⁴	-0.010	6.57x10 ⁻¹⁶	0.404
	rs460053	5p13	Intronic	AGXT2	A/G	0.664	-0.013 (0.002)	1.08x10 ⁻¹³	-0.011	-0.013	-0.014	5.27x10 ⁻³	-0.007	1.77x10 ⁻¹⁴	0.334
	rs153009	5p13	Intronic	AGXT2	A/G	0.242	0.013 (0.002)	4.08x10 ⁻¹³	0.010	0.017	0.016	2.81x10 ⁻⁴	0.010	1.34x10 ⁻¹⁵	0.404
	rs163585	5p13	Intronic	AGXT2	T/C	0.345	0.012 (0.002)	1.73x10 ⁻¹²	0.010	0.016	0.013	4.66x10 ⁻³	0.007	1.73x10 ⁻¹³	0.458
	rs163588	5p13	Intronic	AGXT2	A/G	0.336	0.012 (0.002)	3.45x10 ⁻¹²	0.010	0.017	0.013	3.84x10 ⁻³	0.007	2.57x10 ⁻¹³	0.395
	rs10941225	5p13	Intronic	AGXT2	A/T	0.453	-0.010 (0.002)	1.57x10 ⁻¹⁰	-0.010	-0.012	-0.011	3.65x10 ⁻⁴	-0.008	5.53x10 ⁻¹³	0.832

Table S3: Associations between AGXT2 genotypes and SDMA plasma concentrations in the Leeds Stroke Study.

AGXT2 SNP	Genotype	SDMA [$\mu\text{mol/L}$]	P value
rs28305	CC (n=287)	0.50 (0.48. 0.52)	0.00015
	CG (n=62)	0.53 (0.49. 0.57)	
	GG (n=5)	0.99 (0.56. 1.74)	
rs40200	GG (n=303)	0.50 (0.48. 0.52)	8.43×10^{-6}
	GA (n=66)	0.53 (0.49. 0.57)	
	AA (n=4)	1.18 (0.67. 2.09)	
rs37369	CC (n=302)	0.50 (0.48. 0.52)	0.010
	CT (n=54)	0.53 (0.49. 0.58)	
	TT (n=7)	0.76 (0.48. 1.21)	

Data presented as geometric mean (95% CI). Linkage disequilibrium (D') between SNPs evaluated was >0.80 ($P < 0.0001$)

Table S4: Cox regression analysis of associations between AGXT2 SNPs and post-stroke mortality

	unadjusted	P-value	Model 1*	P-value	Model 2**	P-value
rs28305						
GG vs. CC+CG	1.97 (0.73, 5.29)	0.18	2.34 (0.84, 6.51)	0.10	1.94 (0.69, 5.44)	0.21
rs40200						
AA vs GG+GA	3.05 (1.13, 8.22)	0.027	2.39 (0.83, 6.34)	0.11	2.15 (0.76, 6.11)	0.15
rs37369						
TT vs CC+CT	1.37 (0.61, 3.08)	0.44	1.08 (0.44, 2.67)	0.87	2.97 (0.90, 9.76)	0.18

Data presented as hazard ratio (95% confidence intervals)

*Model 1: adjusting for age. atrial fibrillation. previous stroke. stroke subtype

**Model 2: adjusting for Model 1 + eGFR

Table S5: Associations between AGXT2 genotypes and stroke subtypes according to the Oxfordshire Community Stroke Project criteria in the Leeds Stroke Study

AGXT2 SNP	Genotype	LACI	PACI	TACI	P value *	
rs28305	CC	123 (0.76)	124 (0.81)	81 (0.85)	TACI vs LACI	0.069
	CG	38 (0.24)	30 (0.19)	11 (0.12)	TACI vs PACI	0.071
	GG	1 (<0.01)	0	3 (0.03)	LACI vs PACI	n.s.
rs40200	GG	133 (0.77)	130 (0.81)	85 (0.84)	TACI vs LACI	0.033
	GA	40 (0.23)	31 (0.19)	13 (0.13)	TACI vs PACI	n.s.
	AA	0	0	3 (0.03)	LACI vs PACI	n.s.
rs37369	CC	136 (0.79)	128 (0.82)	86 (0.89)	TACI vs LACI	n.s.
	CT	32 (0.24)	26 (0.17)	9 (0.09)	TACI vs PACI	n.s.
	TT	3 (0.02)	2 (0.01)	2 (0.02)	LACI vs PACI	n.s.

Data are displayed as number of events (percent).

*G² pairwise comparisons with Bonferroni correction for multiple comparisons;

LACI = lacunar infarction; PACI = partial anterior circulation infarction; TACI = total anterior circulation infarction

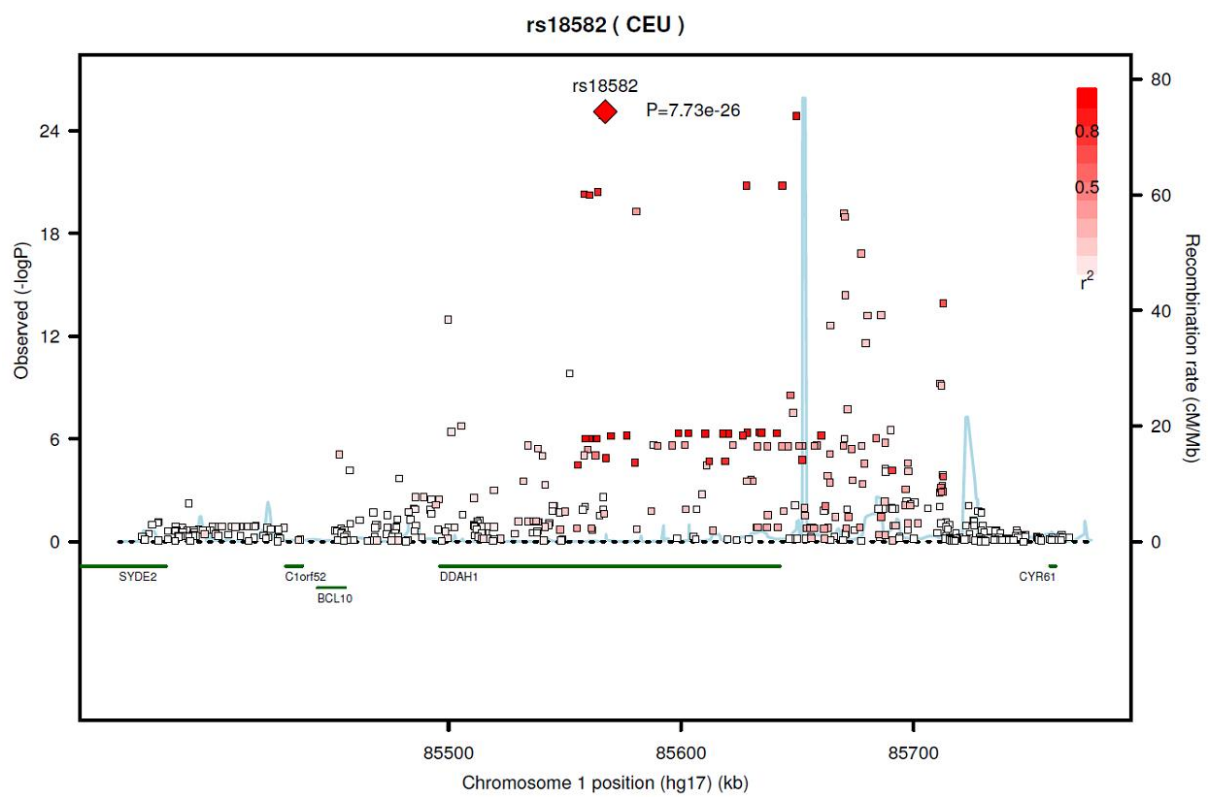
Table S6: Association between AGXT2 genotypes, renal function and hemostasis in the Leeds stoke study

	rs28305			rs40200			rs37369		
	CC+CG	GG	P	GG+GA	AA	P	CC+CT	TT	P
Serum Creatinine [μmol/l]	100.5 (97.4. 103.7)	151 (117.1. 196.0)	0.008	100.7 (97.7. 103.8)	170.6 (146.2. 199.1)	0.003	100.4 (97.4. 103.6)	126.0 (94.0. 168.8)	0.072
CRP [mg/l]	7.14 (6.13. 8.32)	30.39 (13.14. 70.3)	0.027	6.89 (5.95. 7.98)	44.86 (28.65. 28.65)	0.002	6.90 (5.93. 8.02)	25.46 (5.11. 46.77)	0.15
Albumin [g/l]	40.0 (39.5. 40.5)	39.3 (36.0. 42.5)	0.77	39.9 (39.4. 40.4)	40.0 (35.9. 44.1)	0.98	39.9 (39.4. 40.4)	40.8 (37.0. 44.7)	0.65
eGFR	55.1 (52.7. 57.6)	41.1 (10.8. 71.5)	0.25	55.2 (52.8. 57.6)	25.7 (21.8. 29.5)	0.043	55.2 (52.7. 57.7)	47.4 (28.0. 66.7)	0.45
f-thromboglobulin [ng/ml]	48.1 (44.5. 51.9)	77.9 (52.3. 116.0)	0.16	48.0 (44.6. 51.8)	77.9 (52.4. 116.0)	0.16	48.0 (44.5. 51.8)	52.6 (34.9. 79.3)	0.76
Fibrinogen [g/l]	4.30 (4.16. 4.44)	5.80 (5.46. 6.15)	<0.001	4.29 (4.16. 4.42)	5.83 (5.41. 6.29)	0.002	4.30 (4.18. 4.44)	5.20 (4.21. 6.41)	0.087
tPA [ng/ml]	12.0 (11.4. 12.7)	14.7 (12.1. 17.8)	0.40	12.0 (11.4. 12.7)	15.7 (12.9. 19.1)	0.32	12.0 (11.4. 12.7)	11.4 (8.7. 14.9)	0.80
vWF [IU/ml]	1.81 (1.72. 1.90)	3.21 (2.38. 4.31)	0.018	1.81 (1.72. 1.90)	3.21 (2.38. 4.31)	0.019	1.80 (1.71. 1.89)	2.20 (1.62. 2.97)	0.30
Factor VIII [U/ml]	-	-	-	2.16 (2.06. 2.26)	3.55 (3.22. 3.90)	0.026	-	-	-

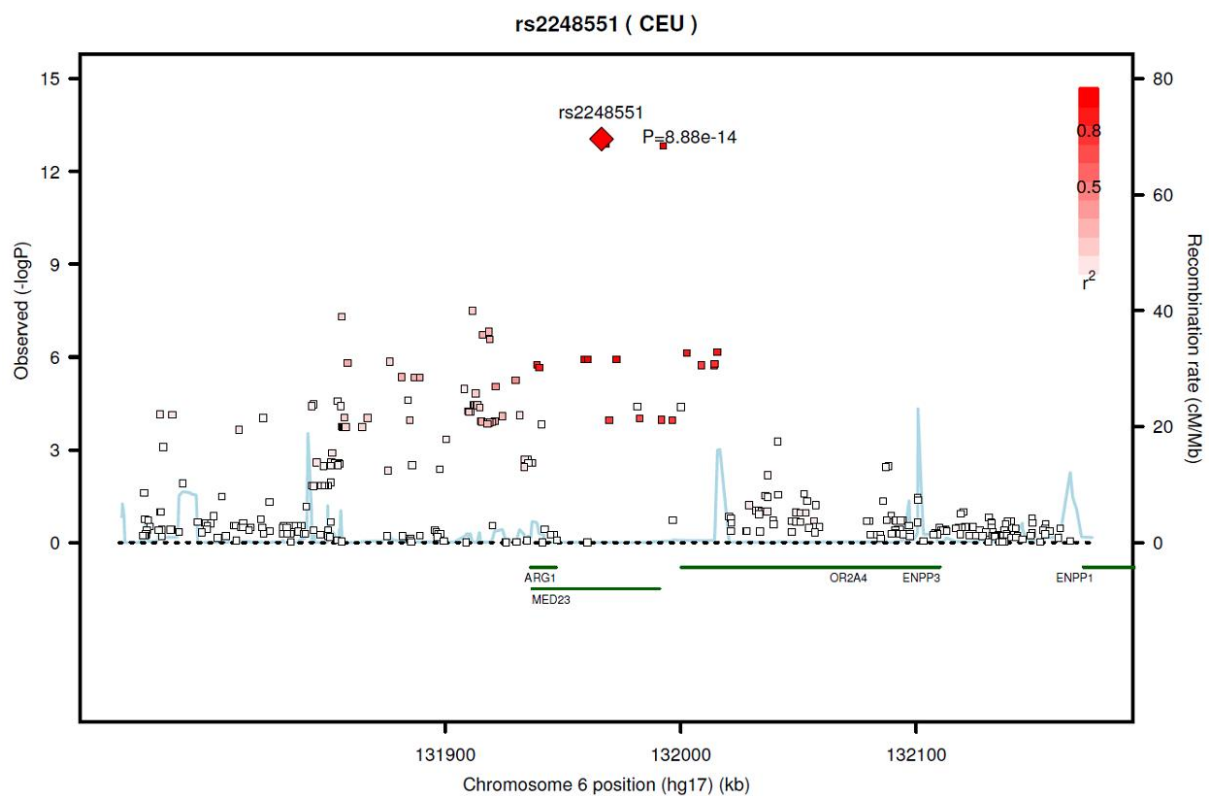
Data presented as mean or geometric mean (95% CI)

C. Supplemental Figures

a)



b)



c)

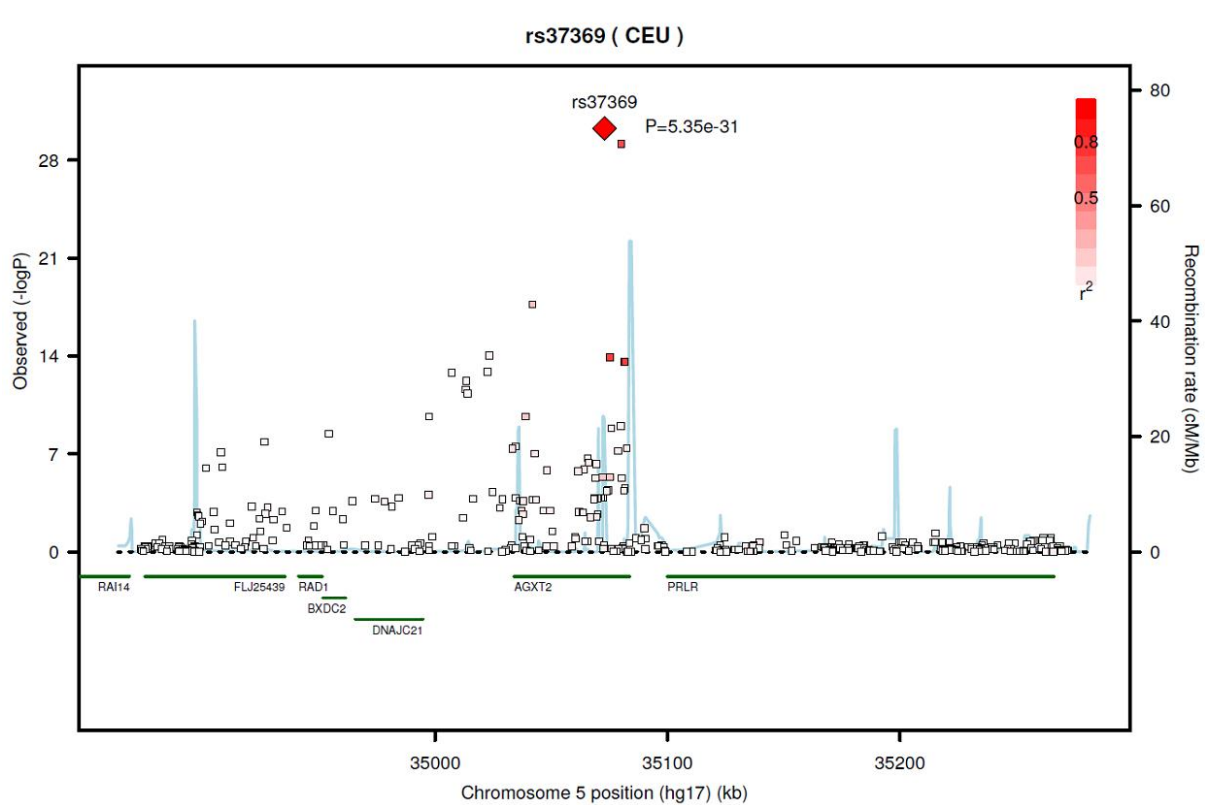


Figure S1: Associations in the regions located in a) 1p22 centered around rs18582 associated with plasma ADMA levels containing DDAH1. b) 6q22 centered around rs2248551 associated with plasma L-arginine levels containing Arg1 and c) 5p13 centered around rs37369 associated with plasma SDMA levels containing AGXT2 P values of all SNPs are plotted against their genomic positions. The estimated recombination rate is represented by the light blue line. The green lines indicate gene annotations.

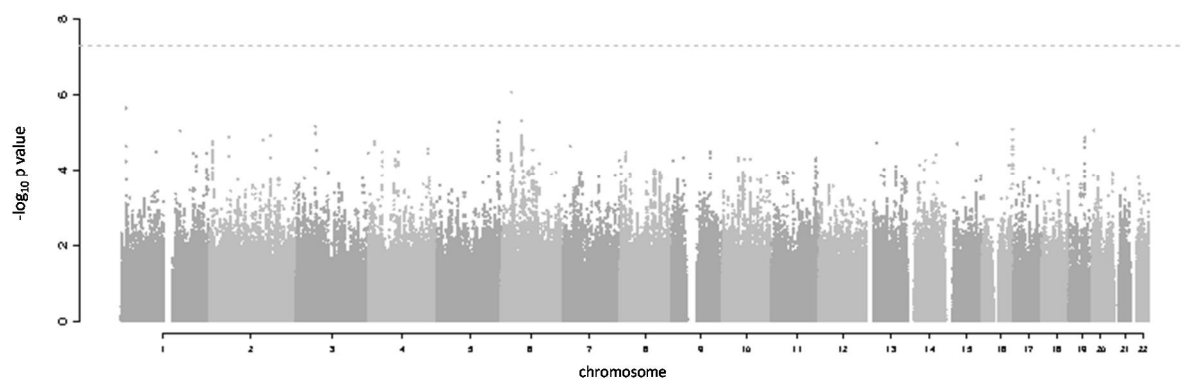


Figure S2: Results for the test of associations between the L-arginine/ADMA ratio and each SNP measured in the Genome-Wide Association Analysis

P values are shown in signal-intensity plots relative to their genomic positions for the L-arginine/ADMA ratio. The results are plotted left to right from the p-terminal end within each chromosome. The chosen threshold for genome-wide significance was set to 5×10^{-8} indicated by the dotted, horizontal line.

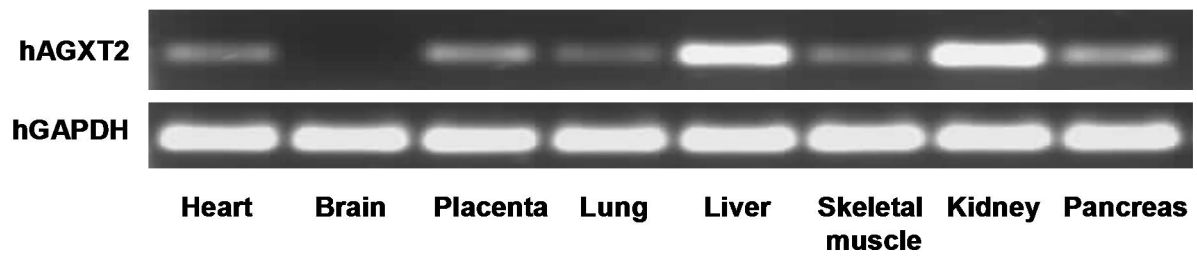


Figure S3: Human mRNA expression profile of AGXT2. GAPDH was used as internal expression control.

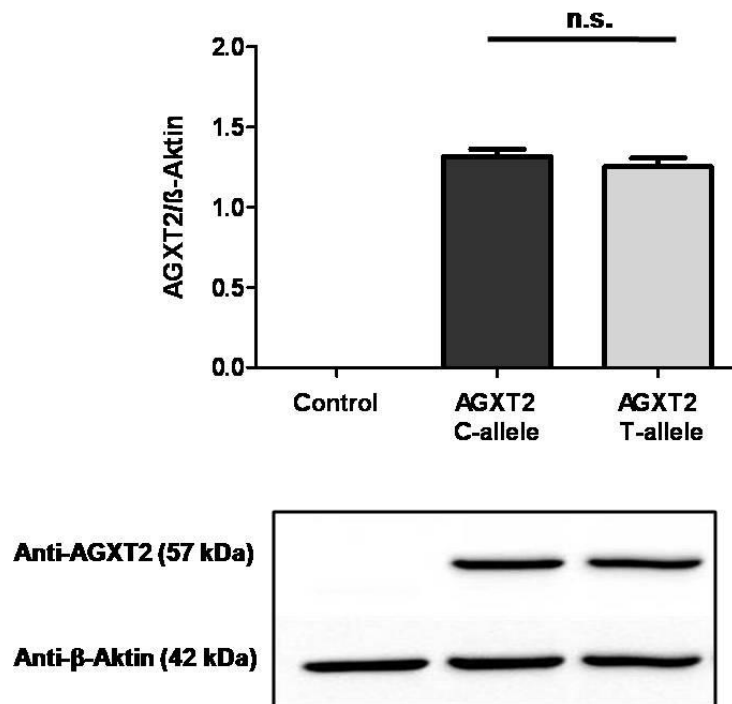


Figure S4: Representative protein expression of HEK cells overexpressing the rs37369 G-allele and the rs37369 A-allele in comparison to an empty control vector; western blot showing one representative example out of n=10

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Genome-Wide Association Study of L-Arginine and Dimethylarginines Reveals Novel Metabolic Pathway for Symmetric Dimethylarginine

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