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

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Abstract

Background—Dimethylarginines (DMA) interfere with nitric oxide (NO) formation by inhibiting NO synthase (asymmetric dimethylarginine, ADMA) and L-arginine uptake into the cell (ADMA and symmetric dimethylarginine, 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 characterise the environmental and genetic contributions to inter-individual variability of these biomarkers.

Methods and Results—This study comprised a genome-wide association analysis of 3 well-characterized population-based cohorts (FHS (n=2992), GHS (n=4354) and MONICA/KORA F3 (n=581)) and identified replicated loci (*DDAH1, MED23, Arg1 and AGXT2*) associated with the inter-individual 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 post-stroke survival in the Leeds study, nor were they associated with incident stroke in the CHARGE consortium.

Conclusion—These GWAS 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.

Keywords

biomarker; endothelial function; nitric oxide; Genome Wide Association Study

Introduction

Dimethylarginines are endogenous analogues of the amino acid L-arginine which contain two methyl groups. Asymmetric dimethylarginine (ADMA) and symmetric 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, 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 CVD and a combined endpoint (myocardial infarction, stroke and death) by ADMA and SDMA was confirmed[7].

Both dimethylarginines inhibit cellular L-arginine uptake by inhibiting the accordant transport system[8], whilst only ADMA acts as an endogenous competitive inhibitor of NO synthases[9]. Additionally, 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), whilst ADMA excretion by the kidneys plays only a minor role[11]. DDAH is expressed in two isoforms, *DDAH1* and *DDAH2*, which are characterized by distinct tissue distribution and may exert distinct functional roles[12–13].

In contrast, SDMA appears to be eliminated almost exclusively through the kidneys[14] 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 inter-individual variability of circulating L-arginine and dimethylarginine concentrations. In the present 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 post-stroke 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 FHS, GHS and MONICA/KORA were fasting samples and plasma was immediately separated, frozen and stored at −80 °C.

The Framingham Heart Study—The Framingham Heart Study (FHS) is a longitudinal observational, community-based cohort initiated in 1948 in Framingham, MA, to prospectively investigate risk factors 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 Gutenberg Health Study (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 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 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 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[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 four hospitals in Leeds[19]. Patients who survived for longer than 30 days after the acute event with sufficient plasma available for analysis of L-arginine, SDMA and ADMA were included in the present 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 (Cohorts for Heart and Aging Research in Genomic Epidemiology)—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. The consortium was formed to facilitate 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 SNPs.

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[22].

Further details of all study samples are available in the Supplemental Information File Section 1.

Genotyping and imputation

Genome-wide genotyping in the Framingham Offspring cohort was performed on the AffymetrixGeneChip 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 Cardiovascular Health Study, 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 directly genotype the SNPs reaching genome-wide significance in the discovery analysis for SDMA. Genotyping in the HVH study was performed using the Illumina 370CNV BeadChip system.

Filtering and imputation methods are detailed in the Supplemental Information File Section 2.

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, systolic and diastolic blood pressure, smoking, BMI 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 MAF filter <0.01 and a genome-wide significance level of $P<5\times10^{-8}$ 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 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 in the Leeds study 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. 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 Supplemental Information File Sections 3–5.

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 Supplemental Information File Section 6.

Computational modeling and structure analysis—Methods for the computational modeling and structure analysis of AGXT2 are detailed in the Supplemental Information File Section 7.

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 overexpressing AGXT2 wildtype and mutant are detailed in the Supplemental Information File Section 8–10.

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 Supplemental Information File Table S1.

Table 2 illustrates the primary findings from the genome-wide association analyses and is detailed in the Supplemental Information File Table S2. Multivariable-adjusted heritability estimates in the FHS cohort were as follows: 15% (SE=0.051; $P=1.26\times10^{-3}$) for ADMA; 42% (SE=0.056; $P=4.5\times10^{-16}$) for L-arginine; and 18% (SE=0.059; $p=6.7\times10^{-4}$) 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 (Supplemental Figure S1a). The most significant SNP (rs18582) was located in intron 1 within the *DDAH1* gene (Table 2) and was in 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 two tagSNPs (rs233109, rs233113) located in the 3´UTR 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 one of two 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 (Supplemental Figure S1b) including *MED23* (mediator complex subunit 23), which is a co-factor required for SP-1 transcriptional activation. Four SNPs reached genome wide significance of which three 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}) (Supplemental Table S2). 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 due to 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 (Supplemental Figure S2). The mean L-arginine/ADMA ratios (±SD) for FHS, GHS I and KORA were 149.37 (±44.38), 149.86 (±47.44) and 146.91 (±55.42), respectively.

Genetic loci associated with SDMA plasma concentrations

SDMA plasma levels were associated with various SNPs located at chromosome 5p13 (Supplemental Figure S1c) including the *AGXT2* gene. *AGXT2* is one of two 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 modelling of AGXT2

To evaluate whether the polymorphism rs37369 could affect AGXT2 activity we performed computer-based 3D 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 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 one 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 Supplemental Figure S3. 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 human embryonic kidney (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 to the AGXT2 rs37369 T-allele (Supplemental Figure S4). Overexpression of the AGXT2 rs37369 C-allele resulted in a significantly enhanced d6-SDMA-metabolizing activity which was significantly reduced when the AGXT2 rs37369 T-allele was over-expressed (Figure 2).

Phenotypic associations of *AGXT2* variants with stroke and post-stroke 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. Due to the low minor allele frequency (8%) of rs37369 we chose two additional SNPs with a minor allele frequency greater than 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 (Supplemental Table S3). Univariate

Cox regression analysis revealed a trend towards 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 [11.13, 8.22], *P*=0.022). After adjustment for age, atrial fibrillation, previous stroke, stroke subtype and renal function this association became non-significant (Supplemental Table S4). We also observed significant and borderline significant associations between *AGXT2* variants and subtypes of ischemic stroke (Supplemental Table S5). Analysis of the relationships between *AGXT2* variants and cardiometabolic risk factors in the Leeds Stroke Study (see Supplemental Table S6), was carried out 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, eGFR), markers of inflammation (CRP), 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, nor 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 three 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 post-stroke 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 co-workers 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 supports 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 concentration[27] to indicate differential regulation of these two dimethylarginines. The strong association of SDMA with biomarkers of renal function and calculated glomerular filtration rate[31] 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 Cardiovascular Health Study (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 al 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[33]. However, our identification of *AGXT2* as the SDMA metabolizing enzyme may have therapeutic implications, as 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 co-factor 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 one of two arginase subtypes that regulate L-arginine bioavailability[35]. The *ARG1* SNPs (rs2248551) identified in this analysis is in LD with one *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 utilization of L-arginine by the inducible isoform of NOS (iNOS) 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 due to 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, whilst in many cases metabolite ratios have been applied to better characterize one common metabolic pathway.

Associations between SDMA, AGXT2 and stroke

Since two 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 towards poorer survival in unadjusted analyses. This trend was lost, however, after adjusting for previously identified determinants of post-stroke 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 Cardiovascular Health 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 following acute ischemic stroke, potentially mediated through the indirect link between *AGXT2*, SDMA, and renal function. However, it must be remembered that only 18% of inter-individual 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 further investigate this hypothesis.

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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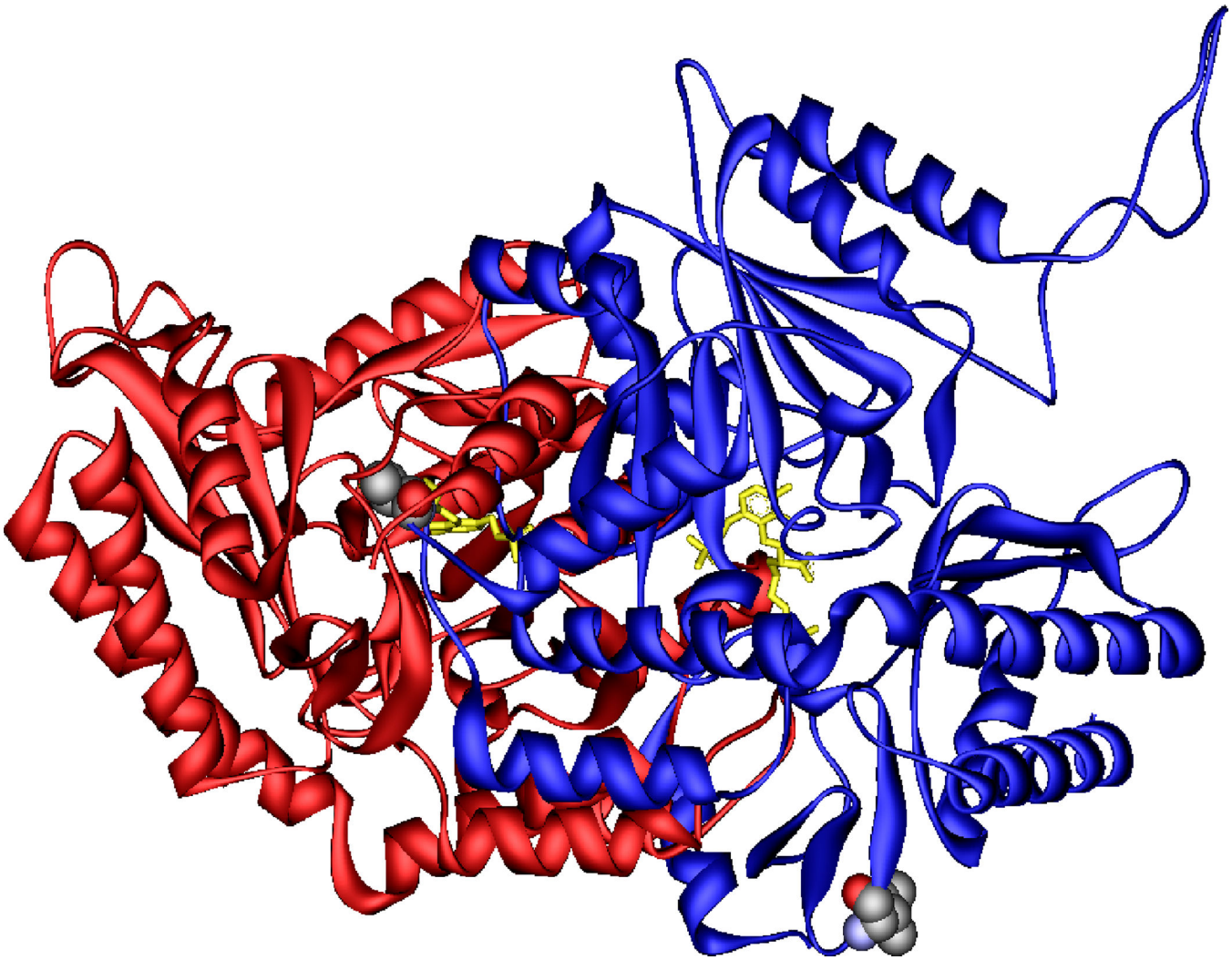

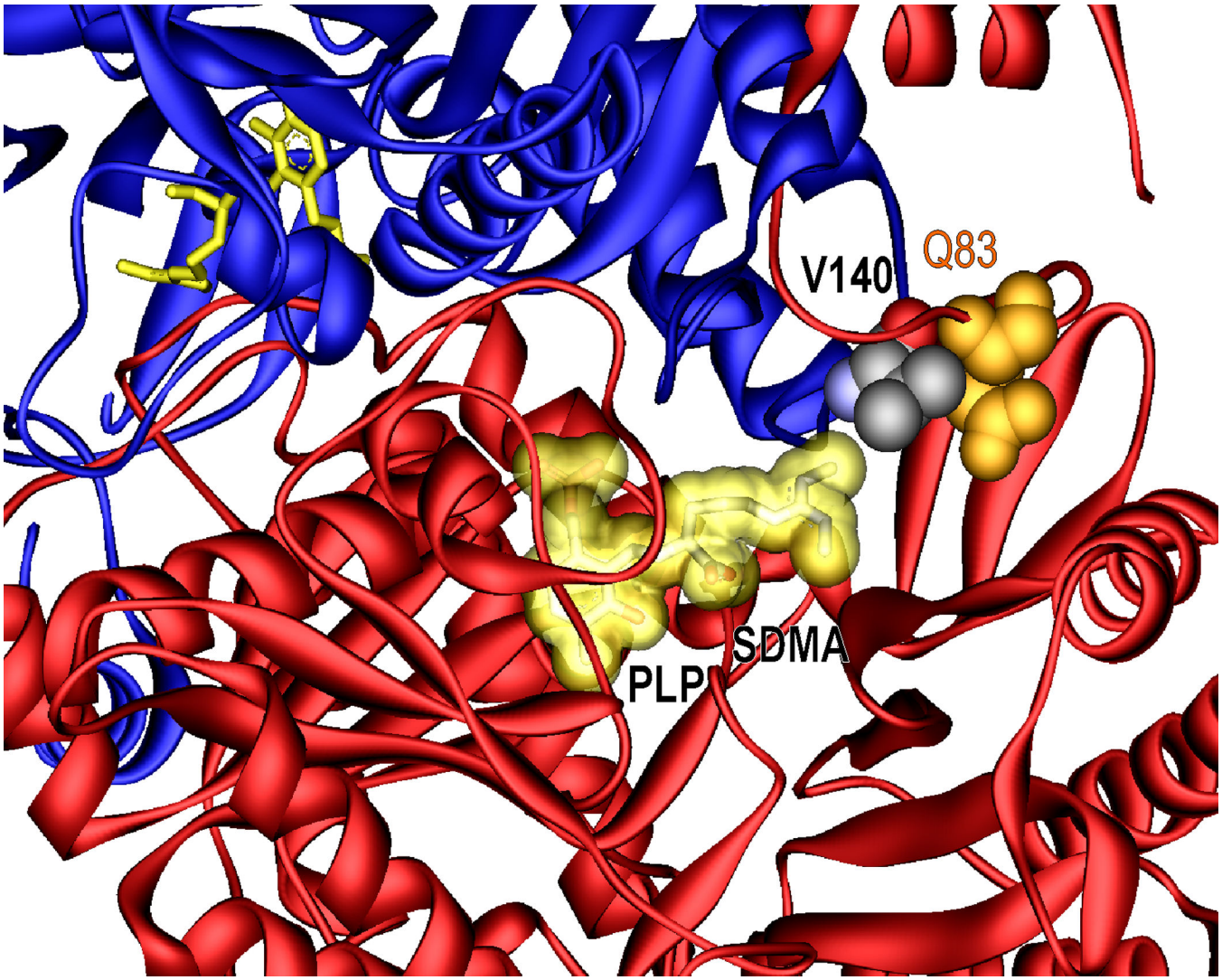

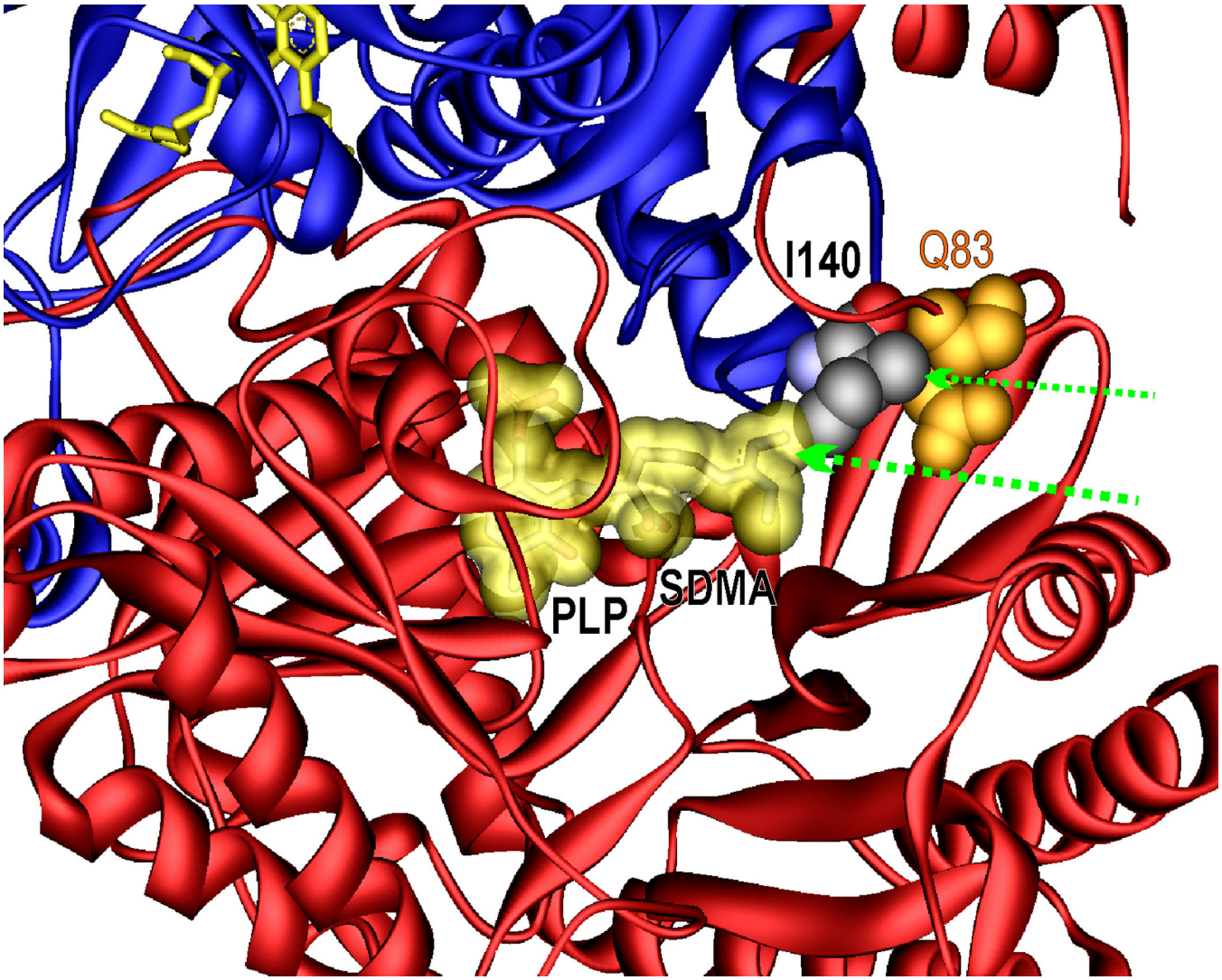

Figure 1. Results of the computerized 3D structure analysis of AGXT2

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 (PDB 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 SDMA and the cofactor pyridoxal phosphate (PLP) are highlighted as yellow sticks. The sites of mutation detected in the present 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 wildtype 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 wildtype. The thickness of the arrows reflects the relative magnitude of the clashes.

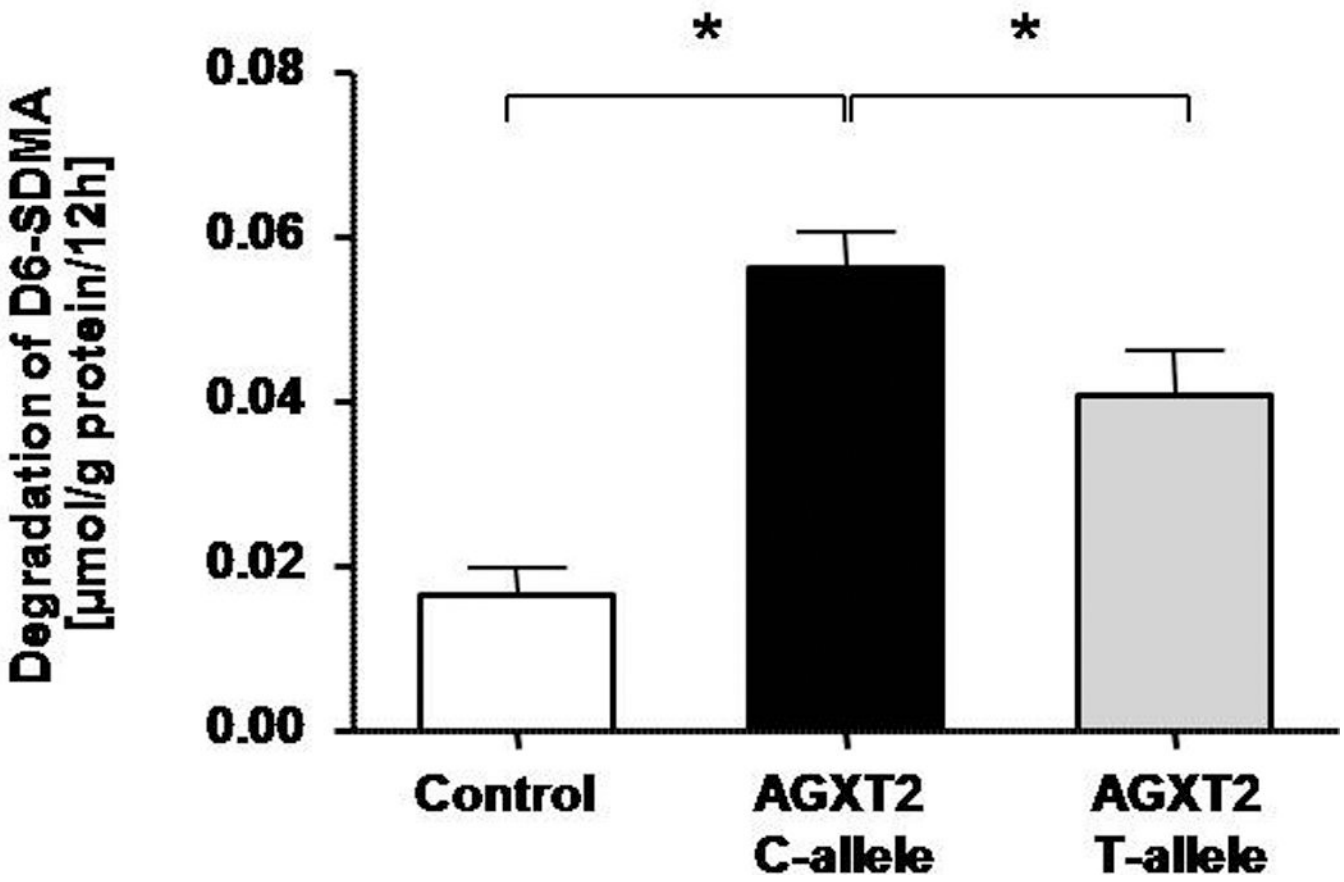

Figure 2. AGXT2 overexpression in human embryonic kidney cells

Degradation of stable isotope labeled d6-SDMA by HEK cells overexpressing wildtype*AGXT2* in comparison to HEK cells overexpressing the *AGXT2* rs37369 variant and control cells transfected with empty pcDNA3.1 vector. n=10 in each group; (two-tailed t-test; *P=0.006)

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 [Years]	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 [%]	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 [µmol/l]	0.55 (0.13)	0.52 (0.11)	0.64 (0.20)	0.52 (0.08)
Plasma L-arginine [µmol/l]	78.9 (27.9)	75.9 (20.9)	89.0 (31.6)	74.3 (17.8)
Plasma SDMA [µmol/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).

Table 2

Association data for the meta-analysis studies for ADMA, L-arginine and SDMA plasma levels. Detailed information about all genome wide significant SNPs including P values and corrected P values are shown in the Supplemental Table S1.

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)	6.28×10^{-26}	−0.015	−0.026	−0.026	4.95×10^{-7}	−0.017	7.63×10^{-31}	0.042
L-Arginine	rs2248551	6q23	Intronic	*MED23*	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	*AGXT2*	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

*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 [µmol/L] at genotype 0 minor alleles			Mean, median, IQR [µmol/L] at genotype 1 minor allele			Mean, median, IQR [µmol/L] 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