Homoarginine Levels Are Regulated by L-Arginine:Glycine Amidinotransferase and Affect Stroke Outcome

Results From Human and Murine Studies

Chi-un Choe, MD; Dorothee Atzler, PhD; Philipp S. Wild, MD, MSc; Angela M. Carter, PhD; Rainer H. Böger, MD; Francisco Ojeda, PhD; Olga Simova, MD; Malte Stockbrand, PhD; Karl Lackner, MD; Christine Nabuurs, PhD; Bart Marescau, PhD, DrSr; Thomas Streichert, MD; Christian Müller, MSc; Nicole Lüneburg, PhD; Peter P. De Deyn, MD, PhD; Ralf A. Benndorf, MD; Stephan Baldus, MD; Christian Gerloff, MD; Stefan Blankenberg, MD; Arend Heerschap, PhD; Peter J. Grant, MD, FMedSci; Tim Magnus, MD; Tanja Zeller, PhD; Dirk Isbrandt, MD; Edzard Schwedhelm, PhD

Background—Endogenous arginine homologues, including homoarginine, have been identified as novel biomarkers for cardiovascular disease and outcomes. Our studies of human cohorts and a confirmatory murine model associated the arginine homologue homoarginine and its metabolism with stroke pathology and outcome.

Methods and Results—Increasing homoarginine levels were independently associated with a reduction in all-cause mortality in patients with ischemic stroke (7.4 years of follow-up; hazard ratio for 1-SD homoarginine, 0.79 [95% confidence interval, 0.64–0.96]; P=0.019; n=389). Homoarginine was also independently associated with the National Institutes of Health Stroke Scale+age score and 30-day mortality after ischemic stroke (P<0.05; n=137). A genome-wide association study revealed that plasma homoarginine was strongly associated with single nucleotide polymorphisms in the L-arginine:glycine amidinotransferase (AGAT) gene (P<2.1×10⁻⁶; n=2806), and increased AGAT expression in a cell model was associated with increased homoarginine. Next, we used 2 genetic murine models to investigate the link between plasma homoarginine and outcome after experimental ischemic stroke: (1) an AGAT deletion (AGAT⁻/−) and (2) a guanidinoacetate N-methyltransferase deletion (GAMT⁻/−) causing AGAT upregulation. As suggested by the genome-wide association study, homoarginine was absent in AGAT⁻/− mice and increased in GAMT⁻/− mice. Cerebral damage and neurological deficits in experimental stroke were increased in AGAT⁻/− mice and attenuated by homoarginine supplementation, whereas infarct size in GAMT⁻/− mice was decreased compared with controls.

Conclusions—Low homoarginine appears to be related to poor outcome after ischemic stroke. Further validation in future trials may lead to therapeutic adjustments of homoarginine metabolism that alleviate stroke and other vascular disorders. (Circulation. 2013;128:1451-1461.)

Key Words: genome-wide association studies ▪ L-arginine:glycine amidinotransferase ▪ homoarginine ▪ single nucleotide polymorphism ▪ stroke

Homoarginine is an L-arginine homologue that differs from L-arginine by an additional methylene group. The physiological role of homoarginine is unknown, but structural similarity to L-arginine suggests that it may be an alternative substrate for nitric oxide synthase (NOS),1,2 and, in support of this, homoarginine levels are associated with endothelial function.3,4

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From the Departments of Neurology (C.C., O.S., C.G., T.M.), Experimental Neuropediatrics (C.C., M.S., D.I.), Department of Clinical Pharmacology and Toxicology (D.A., R.H.B., N.L., R.A.B., E.S.), and German Center for Cardiovascular Research (D.A., P.S.W., R.H.B., S.B., T.Z., E.S.), Clinic for General and Interventional Cardiology, University Heart Center Hamburg, Hamburg, Germany (F.O., C.M., S. Baldus, S. Blankenberg, T.Z.); Department of Clinical Chemistry, University Medical Center Hamburg–Eppendorf, Hamburg, Germany (T.S.); Department of Cardiology and Angiology (P.S.W.), Gutenberg Health Study (P.S.W.), and Department of Clinical Chemistry (K.L.), Johannes Gutenberg University, Mainz, Germany; Experimental Neurophysiology, University Hospital Cologne, Köln, Germany (D.I.); German Center for Neurodegenerative Diseases, Bonn, Germany (D.I.); Department of Clinical Pharmacy and Pharmacotherapy, Martin-Luther-University Halle Wittenberg, Halle Germany (R.A.B.); Division of Cardiovascular and Diabetes Research, Multidisciplinary Cardiovascular Research Centre, University of Leeds, Leeds, UK (A.M.C., P.J.G.); Department of Radiology, Radboud University Medical Centre Nijmegen, Nijmegen, Netherlands (C.N., A.H.); and Laboratory of Neurochemistry and Behavior, Born-Bunge Foundation, University of Antwerp, Antwerp, Belgium (B.M., P.P. De D.).

*Dr. Choe and Atzler contributed equally to this work. Drs. Grant, Magnus, Zeller, Isbrandt, and Schwedhelm are joint senior authors.

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Correspondence to Chi-un Choe, MD, Department of Neurology, Martinistraße 52, Hamburg 20246, Germany. E-mail cchoe@uke.de and Dorothee Atzler, PhD, Department of Clinical Pharmacology and Toxicology, Martinistraße 52, Hamburg 20246, Germany. E-mail d.atzler@uke.de

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NO-dependent neurotoxic and neuroprotective mechanisms influence the pathophysiology of cerebrovascular disease, and strategies that modulate NO in brain and cerebral blood vessels have been suggested as a potential treatment for cerebrovascular disease. Low plasma homoarginine concentrations have been associated with cardiovascular and all-cause mortality in patients undergoing coronary angiography and in patients with type 2 diabetes mellitus receiving maintenance hemodialysis. In a small subgroup of 61 patients, low homoarginine levels were also correlated with fatal stroke. In these and other populations, low homoarginine concentrations were inversely associated with flow-mediated vasodilation, estimated glomerular filtration rate (eGFR), ejection fraction, fibrinogen, D-dimer, and adhesion molecules. The strong association between low homoarginine and cardiovascular outcomes raises the question of whether these observations reflect a direct causal effect that could provide important insights into the pathophysiology of vascular disease. The aim of the present study was to investigate (1) the role of homoarginine in ischemic cerebrovascular disease; (2) the genetic association of homoarginine in humans by a genome-wide association study (GWAS); (3) relevant genetic murine models to confirm the genetic association of homoarginine in mice; and (4) the influence of homoarginine on stroke severity in an in vivo stroke model.

**Analytical Methods**

**Measurement of Guanidino Compounds**

After deproteinization of plasma, guanidino compounds were separated with a strong cation exchange resin with sodium citrate buffers. Subsequently, post-column derivatization of the separated compounds with ninhydrin in basic medium was followed by measurement of the fluorophores (excitation=395 and emission=500 nm). Homoarginine was determined by liquid chromatography–tandem mass spectrometry after derivatization with butanolic hydrochloric acid. Interassay accuracy (bias) and precision (coefficient of variation) were <5% and <9%, respectively. Asymmetric dimethylarginine, symmetric dimethylarginine, and N6-monomethyl-l-arginine were determined by liquid chromatography–tandem mass spectrometry as described previously. eGFR was analyzed according to the abbreviated Modification of Diet in Renal Disease equation for whites: 186×(Creatinine/88.4)−1.154×(Age)−0.203×(0.742 for females).

**Measurement of [13C6]N3-l-Homoarginine Formation**

l-Arginine:glycine amidinotransferase (AGAT)–transfected and untransfected human embryonic kidney (HEK293) cells were incubated with 100 μmol/L l-[15N2]-guanidino-arginine/100 μmol/L [13C6]-lysine for 24 hours at 37°C. Samples were analyzed by liquid chromatography–tandem mass spectrometry with the use of l-[15N2]-arginine as internal standard. [13C6]-Homoarginine and l-[15N2]-arginine were detected by selected ion monitoring of the transitions m/z 253.1→m/z 77.0 (collision energy −16 eV) and m/z 238.1→m/z 77.0 (collision energy −22 eV), respectively.

**Experimental Methods**

**Mouse Models**

Mating, housing, and generation of mice were performed as described previously (Methods in the online-only Data Supplement).

**Temporary Middle Cerebral Artery Occlusion**

Temporary middle cerebral artery occlusion (tMCAO) was achieved as described previously (Methods, Figures I through V, and Table I in the online-only Data Supplement).
Determination of Infarct Size
Stroke analysis was performed as described previously.21-24 (Methods and Figure VI in the online-only Data Supplement).

Assessment of Functional Outcome
One and 24 hours after tMCAO, mice were scored with the use of an extended Bederson score with a scale from 0 to 4, as follows: 0, no observable deficit; 1, preferential turning/forelimb weakness; 2, unidirectional circling; 3, longitudinal rolling; and 4, no movement.25 Exclusion criteria were as follows: (1) death within 24 hours of tMCAO; (2) subarachnoid hemorrhage (as macroscopically assessed during brain sampling); and (3) modified Bederson score of 0 immediately after recovery from tMCAO.

Tail-Cuff Measurements
For hemodynamic measurements, mice were anesthetized with 1.5% to 2.0% isoflurane. After 3 prepulse protocols, systolic blood pressure, pulse, and mean arterial pressure were recorded and analyzed with a Hatteras system and software (model SC-1000; Figure II in the online-only Data Supplement).

Cerebrovasculature Analysis
To visualize the cerebrovasculature, mice were anesthetized and perfused with PBS followed by India ink (20% India ink, 5% gelatin in PBS). After immersion fixation in 4% paraformaldehyde at 4°C overnight, mice were dissected under a binocular microscope and visualized with a digital camera (Figures IVA and IVB and VA and VB in the online-only Data Supplement). For posterior communicating artery scores, the posterior communicating artery anatomy was scored as follows: 0, absent; 1, poorly developed; and 2, well formed (Figure IVC and IVD in the online-only Data Supplement).26 Furthermore, cerebral angiography on the dorsal surface was evaluated by the distance from the midline to the line of anastomoses at 2, 4, and 6 mm from the frontal pole (Figure VC and VD in the online-only Data Supplement).27

Magnetic Resonance Spectroscopy Measurement
In vivo $^3$P magnetic resonance spectroscopy measurements were performed as described previously.28 At 7-T field strength with the use of a quadrature $^3$P coil, spectra were selected from voxels of 160 to 230 $\mu$L in the cerebrum of AGAT wild-type (WT; n=5), AGAT$^{-/-}$ (n=4), and AGAT$^{-/-}$Cr (n=1) mice. To obtain phosphocreatine/nucleoside triphosphate ratios, the signal intensities of phosphocreatine and nucleoside triphosphate were fitted with the use of Java-based Magnetic Resonance User Interface and corrected for TI relaxation.

Western Blot
Equal amounts of cleared lysates (20-40 $\mu$g protein) were separated by SDS-PAGE on precast gels (Invitrogen) and transferred to nitrocellulose membrane. AGAT-specific polyclonal antibodies were kindly provided by Brian Tseng (Harvard University, Cambridge, MA).18 Enhanced chemiluminescence (Luminata Crescendo, Millipore) signals were detected with a luminescent image analyzer (LAS-4000, FujiFilm). Total protein was measured with a laser scanner (FLA-9000 with LPG filter, FujiFilm) on blot membranes stained with Lava Purple (Fluorotechnics, GelCompany) according to the manufacturer’s instructions. Signal quantification was performed on nonun saturated images with the use of ImageJ software.25

Statistical Analysis
Data were expressed as median (interquartile range [IQR]) for clinical cohorts unless indicated otherwise.

For the Leeds Stroke Study, partial correlation coefficients between homoarginine and other variables were evaluated after adjustment for age and sex. Not normally distributed variables (eg, homoarginine) were log-transformed. For all other studies, correlations between homoarginine and other variables were assessed by Spearman’s rho. Correlations are reported only where the coefficient was $0.2 \pm 0.1$. Differences between 2 unrelated groups were compared with the unpaired Student’s t test, and differences between >2 groups by 1-way ANOVA with the Newman-Keuls posttest. Differences between categorical data were analyzed by $\chi^2$ test. Univariate associations between tertiles of homoarginine and mortality were assessed with Kaplan–Meier survival analysis with significance determined by the log-rank test. The independent association between homoarginine and mortality was determined by multivariable Cox regression analyses with results presented as hazard ratios (HRs) for a 1-SD increase in log homoarginine (with 95% confidence intervals). Different models were evaluated with adjustment for the demographic, clinical, biochemical, hematologic, and hemostatic determinants that we have previously shown to predict poststroke mortality in this cohort.10,20 C statistics were calculated with the use of area under the curve values from receiver operating characteristic curves, and the net reclassification index was calculated for models including and excluding homoarginine to evaluate the predictive value of homoarginine for survival.29 The relationships between genotype at selected single nucleotide polymorphisms (SNPs; 0–2 copies of the minor allele) and homoarginine were assessed by linear regression analysis, adjusted for age and sex. Statistical analyses were performed with the use of SPSS version 20 (IBM). Genome-wide significance of association in the Gutenberg Health Study was adjusted for multiple testing ($P<2.1 \times 10^{-8}$; Bonferroni correction for 2,400,000 SNPs). All genotyped SNPs had a genotype call rate >98%; imputation quality threshold was $R^2 > 0.8$. In the Gutenberg Health Study, a linear regression analysis relating the trait to genotype dosage (0–2 copies of the minor allele) for each SNP was performed, adjusted for age and sex. The association between trait and genotype was quantified by the regression slope ($\beta$), standard error (SE), and $P$ value.

Results
Homoarginine and Mortality in Patients With Acute Ischemic Stroke
To investigate the impact of homoarginine on long-term post-stroke mortality, we analyzed homoarginine plasma concentrations in 389 patients with acute ischemic stroke followed up for a median of 7.4 (range, 5.3–8.5) years.19 The median (IQR) plasma homoarginine concentration of the entire cohort was 1.07 [0.74] µmol/L. Homoarginine was higher in patients who survived (1.27 [0.72] µmol/L; n=160) compared with those who died during follow-up (0.96 [0.64] µmol/L; n=229; $P<0.001$). Homoarginine concentrations correlated with creatinine ($r=0.19$ [95% confidence interval, $-0.30$ to $-0.05$]; $P=0.001$), eGFR ($r=0.19$ [0.07 to 0.31]; $P<0.01$), plasma l-arginine ($r=0.41$ [0.33 to 0.49]), C-reactive protein ($r=-0.38$ [−0.45 to −0.30]), β-thromboglobulin ($r=-0.31$ [−0.41 to −0.20]), fibrinogen ($r=-0.25$ [−0.35 to −0.16]), and von Willebrand factor ($r=-0.31$ [−0.42 to −0.19]; $P<0.001$ for all others).

In Kaplan–Meier analysis, survival decreased from the highest to the lowest tertile of homoarginine (Figure 1A; $P<0.001$). In univariate and multivariable Cox regression analyses, decreasing plasma homoarginine concentrations were associated with increased all-cause mortality (Table 1). The HR for a 1-SD increase in log homoarginine concentration (anti-log: 0.215 µmol/L) was 0.75 after adjustment for previously identified predictors of poststroke mortality (age, atrial fibrillation, previous stroke, and stroke subtype; Table 1, model 2); there was no evidence of an interaction between homoarginine and the time from stroke to blood sampling ($P=0.48$). Inclusion of hemostasis markers β-thromboglobulin, von Willebrand factor (model 3), and
kidney function (eGFR, model 4) did not markedly alter the models (Table 1, models 3 and 4). Homoarginine did not improve risk prediction incrementally over conventional risk factors as assessed by C statistics (C statistics for model 2 without and with homoarginine, 0.831 [0.781–0.882] and 0.833 [0.784–0.883], respectively) or when other biomarkers were added (model 3; C statistics for models without and with homoarginine, 0.842 [0.794–0.889] and 0.841 [0.793–0.889], respectively). Similarly, homoarginine did not improve risk prediction incrementally over conventional risk factors as assessed by net reclassification index (eg, net reclassification index for model 2 without and with homoarginine = −0.03). In a subgroup analysis, homoarginine was inversely associated with stroke severity with the use of the Oxfordshire Community Stroke Project classification as a surrogate parameter. Patients with probable small-vessel disease (lacunar infarct) had higher median homoarginine levels (1.22 [IQR, 0.60] µmol/L; n=137) compared with patients with large-vessel disease (total or partial anterior circulation infarcts; 0.92 [0.66] µmol/L; n=213; P<0.001; Figure 1B). To investigate the role of homoarginine in the acute phase after ischemic stroke, we determined plasma homoarginine in 137 patients of the Harburg Stroke Study followed for 30 days after acute ischemic stroke.12 In this cohort, median plasma homoarginine concentrations (lacunar infarct) had higher median homoarginine levels (1.22 [IQR, 0.60] µmol/L; n=137) compared with patients with large-vessel disease (total or partial anterior circulation infarcts: 0.92 [0.66] µmol/L; n=213; P<0.001; Figure 1B) or when other biomarkers were added (model 3; C statistics for models without and with homoarginine, 0.842 [0.794–0.889] and 0.841 [0.793–0.889], respectively). Similarly, homoarginine did not improve risk prediction incrementally over conventional risk factors as assessed by net reclassification index (eg, net reclassification index for model 2 without and with homoarginine = −0.03). In a subgroup analysis, homoarginine was inversely associated with stroke severity with the use of the Oxfordshire Community Stroke Project classification as a surrogate parameter. Patients with probable small-vessel disease (lacunar infarct) had higher median homoarginine levels (1.22 [IQR, 0.60] µmol/L; n=137) compared with patients with large-vessel disease (total or partial anterior circulation infarcts: 0.92 [0.66] µmol/L; n=213; P<0.001; Figure 1B).

To investigate the role of homoarginine in the acute phase after ischemic stroke, we determined plasma homoarginine in 137 patients of the Harburg Stroke Study followed for 30 days after acute ischemic stroke. In this cohort, median plasma homoarginine was significantly lower in patients who had an event during follow-up (1.48 [1.01] µmol/L; n=130) compared with those without an event (1.93 [1.04] µmol/L; P<0.05). Cox regression analysis for continuous homoarginine revealed an association between decreased plasma homoarginine and risk of events (HR for a 1-SD increase in log homoarginine concentration [anti-log: 1.65 µmol/L]; 0.68 [95% confidence interval, 0.50–0.92; P<0.05]). After adjustment for age, sex, and NIHSS score at admission, this association remained significant (HR, 0.69 [0.50–0.94]; P<0.05). Plasma homoarginine correlated with age (ρ=−0.24; [−0.40 to −0.07]); eGFR (ρ=0.29 [0.12 to 0.44]); C-reactive protein (ρ=−0.44 [−0.58 to −0.28]; all P<0.01); NIHSS score (ρ=−0.20 [−0.36 to −0.03]; P<0.05); and NIHSS+age (ρ=−0.27 [−0.42 to −0.10]; P=0.001; Figure 2).

Genetic Determinants of Plasma Homoarginine
To identify genetic determinants of plasma homoarginine concentration in humans, we performed a GWAS analysis in the community-based Gutenberg Health Study (n=2806). Among ≈2.4 million genetic variants analyzed, we identified 31 directly genotyped SNPs and 110 imputed SNPs with genome-wide significant association with plasma homoarginine concentrations (P<2.1×10−8 for all). The top SNPs were all located within the same chromosomal locus on 15q21 and solely associated with the 1-arginine:glycine amidinotransferase (AGAT) gene (Figure 3A). One directly genotyped SNP and 6 imputed SNPs with lesser associations were located on chromosome 2 (2q34) contiguous with...
to the carbamoyl-phosphate-synthase-1 (CPS-1) gene ($P=3.6$–$5.4\times10^{-9}$). In-depth analyses of the 14 top SNPs revealed strong linkage disequilibrium across the AGAT gene (Figure 3B). The intronic SNP rs10519022 (T/C), the upstream SNP rs1145077 (T/G), and an imputed missense SNP (rs1288775; T/A) were selected for replication analysis in the Leeds Stroke Study. All 3 SNPs were significantly associated with plasma homoarginine concentrations in the replication cohort (Table 2). In both studies, a stepwise increase in the homoarginine concentration was associated with each minor allele for each AGAT SNP (Figure 4 and Figure VII in the online-only Data Supplement).

Stable Isotope Labeling of Homoarginine in AGAT-Expressing Cells
To investigate the transamidation of lysine to homoarginine by AGAT, we incubated AGAT-expressing HEK293 cells with stable isotope labeled $[^{13}C_6]$-lysine and confirmed the in vitro formation of $[^{13}C_6,^{15}N_2]$-homoarginine in AGAT-expressing HEK293 cells (Figure VIII in the online-only Data Supplement).

In Vivo Regulation of Homoarginine Levels in AGAT-Deficient and -Overexpressing Mice
The in vivo results with the use of AGAT-expressing cells prompted us to analyze homoarginine in AGAT−/− mice. Analysis of guanidino compounds revealed that both the intermediate product of creatine synthesis, guanidinoacetate, and creatine were deficient in plasma of AGAT−/− mice (Figure 5A through 5C). Plasma homoarginine concentration was 0.17±0.03 µmol/L in WT mice, whereas homoarginine was barely detectable in AGAT−/− mice (Figure 5D). Although AGAT−/− mice showed a reduction in body weight and adiposity and improved glucose tolerance, creatine levels and all metabolic parameters normalized on creatine supplementation (AGAT−/−Cr).18 We also studied

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**Table 2. Genetic Loci in Which SNPs Were Associated With Homoarginine in the Gutenberg Health Study (Stage 1) and Replication of These SNPs in the Leeds Stroke Study (Stage 2)**

<table>
<thead>
<tr>
<th>Trait</th>
<th>SNP</th>
<th>Locus</th>
<th>SNP Type</th>
<th>Nearest Gene</th>
<th>Effect Allele (Frequency)</th>
<th>Stage 1 [β (SE)*, P Value]</th>
<th>Stage 2 [β (SE)*, P Value]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homoarginine</td>
<td>rs10519022†</td>
<td>15q21</td>
<td>Intron</td>
<td>AGAT</td>
<td>C (0.26)</td>
<td>0.13 (0.01), 6.1×10⁻⁶</td>
<td>0.16 (0.04), 1.1×10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>rs2467864</td>
<td></td>
<td>Downstream</td>
<td></td>
<td>C (0.26)</td>
<td>0.13 (0.01), 8.1×10⁻⁶</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>rs1145077†</td>
<td></td>
<td>Upstream</td>
<td></td>
<td>T (0.62)</td>
<td>0.09 (0.01), 5.3×10⁻⁵</td>
<td>0.13 (0.04), 5.9×10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>rs1288775‡‡</td>
<td></td>
<td>Missense</td>
<td></td>
<td>A (0.26)</td>
<td>0.13 (0.01), 6.5×10⁻⁵</td>
<td>0.16 (0.04), 5.5×10⁻⁵</td>
</tr>
</tbody>
</table>

ND indicates not determined; and SNP, single nucleotide polymorphism.

*Effect size (β) estimates represent the change in homoarginine plasma concentrations per unit difference in minor allele dose.

†These SNPs were replicated in the Leeds Stroke Study ($P<0.05$ adjusted for age and sex).

‡Imputed SNP.
a previously generated knockout mouse model with guanidinoacetate N-methyltransferase deficiency (GAMT−/−, second enzyme of creatine synthesis).20 In GAMT−/− mice, a >2-fold upregulation of AGAT protein expression (Figure 5E and Figure IX in the online-only Data Supplement) was observed along with creatine deficiency and increased guanidinoacetate levels (Figure 5F and 5G). In agreement with homoarginine deficiency in the absence of AGAT, AGAT upregulation in GAMT−/− was associated with increased plasma homoarginine (Figure 5H).

Detailed analysis revealed a gene-dose effect, with a stepwise increase in plasma and brain homoarginine in GAMT+/− and GAMT−/− mice (Figure X in the online-only Data Supplement), suggesting that AGAT regulates homoarginine levels in a dose-dependent manner in vivo (Figure 5I and 5J).

**Acute Ischemic Stroke in AGAT−/−, GAMT−/−, and C57BL6 Mice**

To investigate the link between homoarginine and stroke outcome, we subjected WT and AGAT−/− mice to experimental acute ischemic stroke. Infarct volumes after 30 minutes of ischemia were almost 3-fold larger in AGAT−/− mice (51.8±6.1%) compared with WT littermates (17.0±4.0%; P<0.01), and neurological impairment after 24 hours was more severe in AGAT−/− mice (WT versus AGAT−/−: 1.1±0.3% versus 2.7±0.3%; P<0.05; Figure 6A through 6C). Dietary supplementation with 0.5% or 1.0% creatine in chow did not influence infarct size (0.5% creatine: 49.9±9.3%; 1.0% creatine: 36.8±7.0%; P>0.05 compared with AGAT−/−) or neurological score (0.5% creatine: 2.0±0.0; 1.0% creatine: 2.2±0.3; P>0.05 compared with AGAT−/−). Magnetic resonance spectroscopy measurements revealed that oral creatine supplementation effectively replenished brain phosphocreatine in AGAT−/− mice (Figure XI in the online-only Data Supplement). Furthermore, brain histology showed no differences between WT and AGAT−/− mice (Figure III in the online-only Data Supplement). To investigate whether stroke size and neurological outcome were AGAT dependent, we studied the influence of AGAT upregulation in GAMT−/− mice subjected to tMCAO. Experiments revealed that GAMT−/− mice had reduced infarct sizes and improved neurological scores after 24 hours compared with WT littermates (WT versus GAMT−/−: 25.4±5.2% versus 13.3±1.8%; P<0.05; and 2.0±0.3 versus 0.9±0.2; P<0.01; Figure 6D through 6F).

To determine whether differences in stroke sizes and outcome were attributable to homoarginine, we compared homoarginine- and vehicle-treated AGAT−/−Cr mice. Homoarginine supplementation via osmotic minipumps resulted in increased plasma homoarginine levels (0.23±0.05 μmol/L) compared with vehicle-treated AGAT−/−Cr mice (0.07±0.02 μmol/L; P<0.01; Figure 7A). Cerebral infarct volume was significantly decreased in homoarginine-treated compared with vehicle-treated AGAT−/−Cr mice (Figure 7). Plasma homoarginine concentrations were strongly and dose-dependently correlated with infarct sizes and neurological scores in mice subjected to 30-minute tMCAO (Figure 8).

Finally, C57BL6 mice were orally supplemented with homoarginine, resulting in increased homoarginine plasma levels compared with vehicle-treated C57BL6 mice (vehicle versus homoarginine: 0.15±0.01 versus 0.20±0.02 μmol/L; P<0.05; Figure XII in the online-only Data Supplement). Homoarginine supplementation in C57BL6 mice resulted in decreased infarct sizes and improved neurological scores after 24 hours compared with vehicle-treated C57BL6 mice (vehicle versus homoarginine: 59.0±1.7% versus 37.4±6.7% and 2.6±0.3% versus 1.5±0.2%; Figure XII in the online-only Data Supplement). Plasma homoarginine concentrations were dose-dependently correlated with infarct sizes in C57BL6 mice subjected to 60-minute tMCAO (Figure XII in the online-only Data Supplement).
Discussion

The results from the present study demonstrate that homoarginine is (1) a potential novel biomarker for short- and long-term outcome after ischemic stroke; (2) regulated by AGAT in both humans (indicated by GWAS) and mice (demonstrated with the use of genetically engineered mouse models); and (3) a...
determinant of cerebral infarct size and neurological outcome in experimental stroke in murine models of low and high homoarginine concentration. Increased stroke volume in low-homoarginine (AGAT−/−) mice was unaffected by creatine supplementation but ameliorated by prior supplementation with homoarginine, indicating a causative relationship between homoarginine and stroke severity. These results support the view that homoarginine metabolism may be a valid therapeutic target in high-risk individuals to diminish the deleterious effects of cerebral infarction.

Homoarginine is a naturally occurring amino acid derived from lysine, produced mainly in the kidney and found at low concentrations in most bodily fluids. Low plasma homoarginine has been associated with decreased renal function, energy metabolism, and NO regulation. The metabolism of homoarginine in humans is poorly understood; however, evidence indicates that homoarginine is an intermediate product of an analogous urea cycle and, in bacteria, the amidinotransferase AGAT catalyzes the generation of homoarginine. A recent study in which lymphoblasts from an AGAT-deficient patient were used demonstrated a link between AGAT and homoarginine synthesis. In support of this, we identified an association between plasma homoarginine and the AGAT gene in a GWAS of the Gutenberg Health Study. Strong linkage disequilibrium across the AGAT gene was identified, and a dose-dependent increase in plasma homoarginine associated with each minor allele was replicated in the Leeds Stroke Study. A lead SNP from the GWAS, the A→T exchange of the missense SNP rs1288775, leads to an exchange of glutamine with histidine on position 110 located at the end of an α-helix structure that might affect protein conformation and AGAT activity.

In vertebrates, AGAT is the first and rate-limiting enzyme in creatine synthesis that catalyzes the transfer of the amidino group from l-arginine to glycine, resulting in the formation of ornithine and guanidinoacetate. Subsequently, GAMT methylates guanidinoacetate to yield creatine. To clarify the link between AGAT and homoarginine synthesis, we transfected HEK cells with AGAT and identified stable isotope-labeled homoarginine, providing evidence for a functional role for AGAT in homoarginine synthesis. In support of this, homoarginine was virtually undetectable in plasma of AGAT−/− and upregulated in plasma and brain of GAMT−/− mice. Our GWAS, murine, and cell-based studies demonstrate unequivocally that AGAT is involved in homoarginine synthesis in both humans and mice, providing the opportunity to study the role of homoarginine in experimental stroke with the use of murine (AGAT−/− and GAMT−/−) genetic models.

AGAT deficiency (and consequently homoarginine and creatine deficiency) was associated with larger infarct volumes and worse neurological deficits compared with WT littermates. Although creatine has neuroprotective effects in cerebral ischemia, normalizing brain creatine by supplementation in AGAT−/− mice did not normalize infarct sizes and neurological scores. In contrast, homoarginine supplementation dramatically decreased stroke volumes and neurological deficits in AGAT−/−Cr mice (Figure 7).

**Figure 7.** Homoarginine supplementation in l-arginine:glycine amidinotransferase (AGAT)−/−Cr mice subjected to temporary middle cerebral artery occlusion for 60 minutes. A, Comparison of homoarginine plasma concentrations in AGAT−/−Cr mice supplemented with vehicle (0.9% NaCl; n=9) or homoarginine (n=5) for 4 weeks. B through D, Representative triphenyltetrazolium chloride–stained brain slices (B), infarct sizes (C), and neurological scores (D) of AGAT−/−Cr mice subjected to 60-minute middle cerebral artery occlusion and 24-hour reperfusion (NaCl, n=11; homoarginine [homoarg], n=7). Statistical significance between saline- and homoarginine-supplemented AGAT−/−Cr mice: *P<0.05, **P<0.01 by Student’s t test.

**Figure 8.** Correlation of homoarginine plasma concentrations with infarct size (A) and neurological score after 24 hours (B) in l-arginine:glycine amidinotransferase (AGAT)−/−, wild-type, and guanidinoacetate N-methyltransferase (GAMT)−/− mice after 30 minutes of temporary middle cerebral artery occlusion (n=27). *P<0.05, ***P<0.001 by Spearman correlation analysis.
and elevated homoarginine levels in GAMT−/− mice were also associated with smaller stroke size and less severe neurological deficits. Finally, oral homoarginine supplementation in C57BL6 mice attenuated acute infarct size and ameliorated neurological deficits. These results provide evidence that higher homoarginine levels attenuate stroke severity and improve outcome in mice and suggest that homoarginine is causally involved in the (patho)physiology of ischemic stroke.

Recently, data from population studies have indicated that low homoarginine independently predicts mortality from cardiovascular disease, including sudden cardiac death, heart failure, acute myocardial infarction, and fatal ischemic stroke.7,8,39 The associations between low plasma homoarginine and poor outcome after ischemic stroke in the Leeds and Harburg stroke studies support these previous clinical findings. Low plasma homoarginine was also associated with large-vessel disease (associated with increased stroke volume and increased morbidity and mortality) and neurological deficit (based on age and NIHSS score),40 supporting results from our murine studies indicating that homoarginine influences severity of stroke and stroke outcome. The potential mechanisms linking low levels of homoarginine with stroke outcome remain unclear; however, it is believed that homoarginine increases NO by acting as a substrate for NOS. Our experiments to evaluate the role of homoarginine in NO metabolism did not reveal differences in neuronal NOS, endothelial NOS, inducible NOS, arginase 1 and 2, and dimethylarginine dimethylaminohydrolase 1 and 2 mRNA expression between WT and AGAT−/− mice (Figure XIII in the online-only Data Supplement) or plasma asymmetric dimethylarginine, symmetric dimethylarginine, and other guanidino compounds (Table II in the online-only Data Supplement). However, these analyses do not exclude an influence of homoarginine on NO bioavailability. As an alternative substrate for NOS, homoarginine may increase NO bioavailability directly. In addition, in vitro studies have demonstrated that homoarginine is a weak inhibitor of arginase activity, suggesting that homoarginine might indirectly increase NO production by increasing availability of l-arginine.2,41,42 The regulation and biological effects of NO during ischemia/reperfusion are complex, involving both protective (endothelial NOS and inducible NOS) and detrimental effects (neuronal NOS and inducible NOS) on inflammatory and thrombotic processes.5,43,44 Our data suggest that homoarginine may positively influence the beneficial pathways of NO. Clinically, this view is supported by the association between homoarginine levels and endothelial function, blood pressure, and left ventricular ejection fraction5,39 as well as by evidence of a role for homoarginine in insulin secretion and inhibition of platelet aggregation.22 Previous studies have reported inverse associations between homoarginine and vascular cell adhesion molecule-1 and intercellular adhesion molecule-1,7 and in the Leeds Stroke Study we observed significant inverse associations between homoarginine and C-reactive protein, β-thromboglobulin, fibrinogen, and von Willebrand factor. These latter findings suggest that homoarginine may influence inflammatory and thrombotic components of ischemic vascular disease, including vessel wall/leukocyte, vessel wall/platelet, and platelet/platelet interactions, which warrant further investigation.

Strengths and Limitations
The strength of our investigation is the translational approach combining in vitro and in vivo experimental studies in addition to epidemiology and clinical research. In 2 patient cohorts, we identified low homoarginine as independently associated with stroke outcome; however, despite adjustment, unidentified confounding effects cannot be ruled out, and homoarginine did not improve poststroke mortality risk prediction incrementally over conventional risk factors. Additional studies in larger cohorts of stroke patients are required for validation of our results and stratification of cause of mortality. The data from our clinical studies do not provide information on the value of homoarginine as a diagnostic marker for stroke. NIHSS and magnetic resonance imaging data were not available in the Leeds Stroke Study. However, homoarginine was correlated with NIHSS score and age in the Harburg Stroke Study, and experimental data causally linked homoarginine levels with stroke size and outcome.

Conclusion
Our data provide evidence that homoarginine predicts post-stroke mortality in 2 prospective clinical studies. Our experimental results extend the importance of homoarginine from a potential biomarker for secondary events after stroke to a functional modulator of stroke severity and poststroke neurological deficit. In contrast to nonmodifiable stroke predictors, like age and NIHSS score, homoarginine could open new therapeutic avenues of stroke management.

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Disclosures
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Disclosures
None.

References


CLINICAL PERSPECTIVE

Homoarginine is an endogenous l-arginine homologue that is suggested to be an alternative substrate for nitric oxide synthase. Low homoarginine plasma concentrations have been associated with cardiovascular diseases in patients with diabetes mellitus and in patients referred to coronary angiography. We identified homoarginine as a novel biomarker for short- and long-term outcome after acute ischemic stroke. In multivariable models, adjusted for previously identified risk factors, a 21% to 31% reduction in risk for secondary events was observed by increasing plasma homoarginine. Furthermore, homoarginine was significantly correlated with neurological impairment quantified by National Institutes of Health Stroke Scale score and age. Using genome-wide association studies and genetically modified mice, we showed that homoarginine is regulated by the enzyme l-arginine:glycine amidinotransferase (AGAT) in humans and mice and demonstrated that this pathway is the major source for homoarginine synthesis in mice. Applying an experimental model of ischemic stroke in AGAT-deficient mice, we confirmed a causal link between homoarginine and stroke. These findings suggest that homoarginine is a novel biomarker and potential therapeutic target in the management of stroke in humans. Clinical trials evaluating the efficacy of homoarginine supplementation in relation to stroke prevention and poststroke outcomes are required to clarify the potential clinical benefits of homoarginine.
Homoarginine Levels Are Regulated by l-Arginine:Glycine Amidinotransferase and Affect Stroke Outcome: Results From Human and Murine Studies

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SUPPLEMENTAL MATERIAL

1) Supplemental Methods

Experimental methods

i) Mouse models Mating, housing and generation of mice were performed as previously described.\textsuperscript{1-3} For all experiments only littermates from heterozygous matings were used. C57Bl/6 wild-type mice were obtained from Charles River (Sulzfeld, Germany). Animals were kept under standard housing conditions with free access to food and water. All procedures were in accordance with the German animal welfare laws and approved by the local animal research committee (TV-Nr. 08/08 and 10/110). As previously described, creatine deficiency in AGAT\textsuperscript{−/−} and guanidinoacetate N-methyltransferase (GAMT)-deficient mice resulted in reduced body weight, less relative body fat mass, decreased muscle strength and volume\textsuperscript{1-3}. In addition, cholesterol levels were reduced and glucose tolerance was increased in AGAT\textsuperscript{−/−} mice\textsuperscript{1}. On a special diet containing 1% Cr \textit{ad libitum} after weaning (Ssniff, Germany), the metabolic phenotype of AGAT\textsuperscript{+/+} and AGAT\textsuperscript{−/−}Cr did not differ.\textsuperscript{1} The 4-week-long supplementation with homoarginine or vehicle in AGAT\textsuperscript{−/−} mice was achieved \textit{via} osmotic mini pumps (Alzet, Cupertino, CA, USA) loaded with homoarginine dissolved in saline (30 \(\mu\)g/kg/h) or saline alone, whereas C57Bl/6 mice were supplemented with homoarginine via drinking water (14mg/l).

ii) Temporary middle cerebral artery occlusion Temporary middle cerebral artery occlusion (tMCAO) was achieved as previously described.\textsuperscript{4-5} Mice were randomly assigned to the surgeons who were blinded to the experimental groups. Focal cerebral ischemia was induced by transient occlusion of the MCA using the intraluminal filament technique in 12 to 15-week-old male mice (weights: AGAT\textsuperscript{+/+} 28.9g, AGAT\textsuperscript{−/−} 18.6g, AGAT\textsuperscript{+/−}Cr 27.4g, GAMT\textsuperscript{+/+} 30.2g, GAMT\textsuperscript{−/−} 26.0g, C57Bl/6 with vehicle 29.8g, C57Bl/6 with homoarginine 28.8g). Mice were anesthetized with 1.5-2% isoflurane in 100% O\textsubscript{2} and underwent analgesia with buprenorphine (0.03 mg/kg body weight intraperitoneally every 12 hours for 24 hours). The
left common, internal and external carotid arteries were isolated and ligated. A 6-0 silicon-rubber-coated nylon filament (Doccol Cooperation, USA) was introduced through a small incision into the external carotid artery and advanced 10 mm distal to the carotid bifurcation for occlusion of the MCA. In most animals, laser Doppler flowmetry (Moor Instruments) was used to monitor regional cerebral blood flow (rCBF) in the territory of the left middle cerebral artery (3 mm lateral and 6 mm posterior to bregma) to verify occlusion after insertion (ischemia) and after removal of filament (reperfusion). The cerebral blood flow showed a reduction of >80%, which did not differ between groups (Suppl. Fig. 1). After indicated periods (30 min or 1 h), the monofilament was removed to allow reperfusion of the MCA. The internal carotid artery was then ligated and the skin incision was closed. In separate cohorts of animals, additional physiological parameters that affect stroke outcome were analyzed (brain structure, cerebrovasculature, blood gas, blood pressure, heart rate) (Suppl. Fig. 2-5, Suppl. Tab. 1).

iii) Determination of infarct size Stroke analysis was performed as previously described.4-7 After 24 h, mice were sacrificed using isoflurane, cardiac blood samples were obtained and brains were cut in 1-mm-thick coronal sections using a mouse brain slice matrix (Braintree Scientific, USA). The slices were stained with 2% (w/v) 2,3,5-triphenyl-2-hydroxytetrazolium chloride; (TTC, Sigma) to visualize the infarcts. Planimetric measurements were calculated to determine lesion volumes (ImageJ software, NIH).6 Absolute infarct volume was corrected for the brain swelling according to the following formula: $V_{\text{corrected}} = V_{\text{infarct}} \times (1 - (V_i - V_c)/V_c)$, where the term $(V_i - V_c)$ represents the volume difference between the ischemic hemisphere and the contralateral control hemisphere and $(V_i - V_c)/V_c$ represents this difference as a percentage of the contralateral hemisphere.7 The resulting edema-corrected infarct volume was expressed as a percentage of the control hemisphere. Later time points were not possible due to high mortality of AGAT−/− mice (Suppl. Fig. 6).
2) Supplemental Tables

**Supplemental Table 1.** Blood gas analysis in wildtype (WT), L-arginine:glycine-amidinotransferase (AGAT)-KO, and creatine supplemented AGAT-KO mice under anesthesia with 1.5-2% isoflurane.

<table>
<thead>
<tr>
<th>Retrobulbar</th>
<th>AGATwt (n = 6)</th>
<th>AGATd/d (n = 5)</th>
<th>AGATd/d+Cr (n = 6)</th>
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<tr>
<td>pO2 (mmHg)</td>
<td>92.7 ± 6.1</td>
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<tr>
<td>pCO2 (mmHg)</td>
<td>52.6 ± 0.8</td>
<td>60.8 ± 1.8*</td>
<td>48.7 ± 4.2</td>
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<tr>
<td>pH</td>
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<td>7.21 ± 0.01</td>
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</table>

<table>
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<th>Retrobulbar</th>
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<th>GAMTd/d (n = 3)</th>
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<tbody>
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<td>pO2 (mmHg)</td>
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<td>70.4 ± 7.3</td>
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</tr>
<tr>
<td>pCO2 (mmHg)</td>
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<td>ns</td>
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<tr>
<td>pH</td>
<td>7.25 ± 0.02</td>
<td>7.22 ± 0.01</td>
<td>ns</td>
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</tbody>
</table>

*P<0.05 compared with AGATwt and AGATd/d+Cr*
Supplemental Table 2. Plasma guanidino compounds in wildtype (WT), L-arginine:glycine-amidinotransferase (AGAT)-KO, and creatine supplemented AGAT-KO mice.

<table>
<thead>
<tr>
<th>Guanidino Compound</th>
<th>mean±SD</th>
<th>P-value</th>
</tr>
</thead>
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<tr>
<td></td>
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<td>AGAT-KO</td>
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<tr>
<td>CTN</td>
<td>7.76 ± 1.77</td>
<td>0.40 ± 0.00</td>
</tr>
<tr>
<td>Arg</td>
<td>113.5 ± 29.3</td>
<td>97.4 ± 26.3</td>
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<tr>
<td>ADMA*</td>
<td>0.71 ± 0.16</td>
<td>0.70 ± 0.19</td>
</tr>
<tr>
<td>SDMA*</td>
<td>0.24 ± 0.04</td>
<td>0.26 ± 0.07</td>
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<tr>
<td>L-NMMA*</td>
<td>0.19 ± 0.06</td>
<td>0.16 ± 0.04</td>
</tr>
<tr>
<td>α-K-δ-GVA</td>
<td>0.23 ± 0.08</td>
<td>0.27 ± 0.08</td>
</tr>
<tr>
<td>GSA</td>
<td>0.32 ± 0.15</td>
<td>0.33 ± 0.13</td>
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<tr>
<td>α-N-AA</td>
<td>0.80 ± 0.21</td>
<td>0.97 ± 0.22</td>
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<tr>
<td>ArgA</td>
<td>0.05 ± 0.05</td>
<td>0.05 ± 0.02</td>
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<tr>
<td>γ-GBA</td>
<td>0.26 ± 0.11</td>
<td>0.47 ± 0.15</td>
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<td>G</td>
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<td>0.40 ± 0.24</td>
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<td>MG</td>
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<td>β-GPA</td>
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<td>0.02 ± 0.04</td>
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</table>

Supplemental Figure 1. Cerebral laser doppler analysis.
Regional cerebral blood flow before, during and after occlusion of middle cerebral artery. Statistical analysis was not significant (n.s., P>0.05) by 1-way ANOVA with Newman-Keuls post-test (n=4-6 per group).
Supplemental Figure 2. Systolic blood pressure, pulse and mean arterial pressure. (A-B) Systolic blood pressure in AGATwt, AGAT-/- and AGAT-/- Cr mice (A), as well as GAMTwt and GAMT-/- mice (B). (C-D) Pulse in AGATwt, AGAT-/- and AGAT-/- Cr mice (C), as well as GAMTwt and GAMT-/- mice (D). (E-F) Mean arterial pressure (MAP) in AGATwt, AGAT-/- and AGAT-/- Cr mice (E), as well as GAMTwt and GAMT-/- mice (F). Statistical analysis was not significant (n.s., P>0.05) by 1-way ANOVA with Newman-Keuls post-test for AGAT and Student's t-test for GAMT (n=5-6 per group).
Supplemental Figure 3. Brain histology of wt and AGAT-/- mice. 
(A-B) Histology of hippocampus (top left), cortex (bottom left), and cerebellum (right) in (A) wt and (B) AGAT-/- mice, images representative of n = 3 per group.
Supplemental Figure 4. Cerebrovasculature of wt, AGAT-/- and GAMT-/- mice. (A-B) Visualization of circle of Willisi of AGATwt (A left), AGAT-/- (A right), GAMTwt (B left) and GAMT-/- (B right). (C-D) Pcom-scores of AGATwt (C left), AGAT-/- (C right), GAMTwt (D left) and GAMT-/- (D right). Statistical analysis was not significant (n.s., P>0.05) by Student’s t-test (n=5-7 per group).
Supplemental Figure 5. Cerebral angioarchitecture of AGATwt, AGAT-/-, GAMTwt and GAMT-/- mice.

(A-B) Anastomoses between peripheral branches of anterior and middle cerebral arteries are marked by circles and interconnected by a line of AGATwt (A left), AGAT-/- (A right), GAMTwt (B left) and GAMT-/- (B right). Bar graph indicates 1 mm. (C-D) Distance from midline in AGATwt and AGAT-/- (C), as well as GAMTwt and GAMT-/- (D). Statistical analysis was not significant (P>0.05) by Student's t-test (n=3-6 per group).
Supplemental Figure 6. Kaplan-Meier survival analysis. AGATwt and AGAT-/- mice were subjected to 30min MCAO (n=6/7). Statistical significance was calculated between AGATwt and AGAT-/- mice: ***P<0.001 by Log-rank (Mantel-Cox) test.
Supplemental Figure 7. Allele-specific homoarginine plasma levels across the AGAT genotypes in the Leeds Stroke Cohort. For the SNPs rs1288775 (missense) (A), rs10519022 (intron) (B), and rs1145077 (upstream) (C), homozygous allele carriers of the minor allele had significantly higher plasma homoarginine as compared with heterozygous or homozygous allele carriers of the major allele. Linear regression analysis relating the trait to genotype dosage (0-2 copies of the minor allele) for each SNP was performed ($P<0.01$ for all). The association between trait and genotype was quantified by the regression slope ($\beta$), its standard error [SE($\beta$)], and $P$ value.
Supplemental Figure 8. In vitro generation of homoarginine. LC-MS/MS chromatograms of L-[\textsuperscript{2H}_7]-arginine (m/z 238 → m/z 77), and [\textsuperscript{13}C_6\textsuperscript{15}N_2]-homoarginine (m/z 253 → m/z 89). In contrast to untransfected HEK cells (A), the incubation of AGAT-expressing HEK cells (B) with [\textsuperscript{13}C_6]-lysine and L-[\textsuperscript{15}N_2]-guanidino-arginine resulted in the formation of [\textsuperscript{13}C_6\textsuperscript{15}N_2]-homoarginine.
Supplemental Figure 9. AGAT protein expression, neurological deficit and relative weight loss in AGAT-deficient and -overexpressing mice. (A) AGAT protein expression (normalized to total protein) is absent in AGAT−/- and increased in GAMT+/− mice. (B-C) Neurological score and relative weight loss after 24h correlated with AGAT genotype in mice subjected to 30 min tMCAO. *P<0.05; ***P<0.001 by 1-way ANOVA with Newman-Keuls post-test (n = 5-11).
Supplemental Figure 10. Correlation of homoarginine concentration and GAMT-genotype. Homoarginine is increased in a genotype-dependent manner in (A) serum and (B) brain tissue of wt, GAMT+/-, and GAMT-/- mice, *P<0.05, **P<0.01, ***P<0.001 by 1-way ANOVA with Newman-Keuls post-test (n=6-16).
Supplemental Figure 11. Magnetic resonance spectroscopy (MRS) measurements of AGATwt, AGAT-/- and AGAT-/-Cr mice. In vivo cerebral 31P MR spectra and PCr/NTP ratios of AGATwt, AGAT-/- and AGAT-/-Cr. Note the complete absence of PCr in the brain of AGAT-/- mice, which is normalized after Cr supplementation (AGAT-/-Cr). PME: phospho-monoesters, Pi: inorganic phosphate, PDE phosphodiesters, PCr: phosphocreatine, NTP: nucleosine triphosphate (including ATP).
Supplemental Figure 12. Homoarginine supplementation in C57BL6 reduces cerebral brain damage after 60 min tMCAO. (A) Comparison of homoarginine plasma concentrations in C57BL6 mice supplemented orally with vehicle (0 mg/L) or homoarginine (14 mg/L) for four weeks (n = 7/6). (B-D) Representative TTC-stained brain slices (B), infarct sizes (C), and neurological scores (D) of C57BL6 mice subjected to 60 min tMCAO and 24 h reperfusion (n = 7/6). (E) Correlation of homoarginine plasma concentrations with infarct size (n=13). Statistical significance was calculated between saline and homoarginine supplemented mice: *P<0.05; **P<0.01 by Student’s t-test (A,C,D) and Spearman’s correlation analysis (E).
Supplemental Figure 13. Quantitative PCR analysis of NO metabolism in brains of WT and AGAT-/- mice. Expression analysis of (A and B) neuronal, endothelial, and inducible nitric oxide synthase (NOS) isoforms, (D and E) arginase 1 and 2 isoforms, (F and G) dimethylarginine dimethylaminohydrolase (DDAH) 1 and 2 isoforms by qPCR. Statistical analysis was not significant by Student's t-test with P>0.05 for all comparisons (n=6-7 per group).
4) Supplemental References


