Homoarginine Levels are Regulated by L-Arginine:Glycine Amidinotransferase and Affect Stroke Outcome: Results from Human and Murine Studies

Running title: Choe et al.; Homoarginine and stroke outcome

Chi-un Choe, MD1,2*; Dorothee Atzler, PhD3,4*; Philipp S Wild, MD, MSc4,5,6; Angela M. Carter, PhD7; Rainer H. Böger, MD3,4; Francisco Ojeda, PhD8; Olga Simova, MD1; Malte Stockebrand, PhD2; Karl Lackner, MD9; Christine Nabuurs, PhD10; Bart Marescau, PhD, DrSr11; Thomas Streichert, MD12; Christian Müller, MSc8; Nicole Lüneburg, PhD7; Peter P. De Deyn, MD, PhD11; Ralf A. Benndorf, MD3,13; Stephan Baldus, MD8; Christian Gerloff, MD1; Stefan Blankenberg, MD4,8; Arend Heerschap, PhD10; Peter J. Grant, MD, FMedSci7*; Tim Magnus, MD8; Tanja Zeller, PhD4,8*; Dirk Isbrandt, MD2*; Edzard Schwedhelm, PhD3,4*

1Dept of Neurology; 2Experimental Neuropediatrics; 3Dept of Clinical Pharmacology and Toxicology; 4German Center for Cardiovascular Research (DZHK e.V.), 8Clinic for General and Interventional Cardiology, University Heart Center Hamburg; 12Dept of Clinical Chemistry, University Medical Center Hamburg-Eppendorf, Hamburg; 3Dept of Cardiology and Angiology; 9Gutenberg Health Study; 5Dept of Clinical Chemistry, Johannes Gutenberg University, Mainz; 13Institute of Anatomy and Cell Biology, University of Würzburg, Würzburg, Germany; 7Division of Cardiovascular and Diabetes Research, the Multidisciplinary Cardiovascular Research Centre, University of Leeds, Leeds, UK; 10Dept of Radiology, Radboud University Medical Centre Nijmegen, The Netherlands; 11Laboratory of Neurochemistry and Behaviour, Born-Bunge Foundation, University of Antwerp, Antwerp, Belgium

*contributed equally to the manuscript

Address for Correspondence:
Chi-un Choe, MD
Department of Neurology
German Center for Cardiovascular Research
Martinistraße 52
Hamburg, 20246, Germany
Tel: +4940741051959
Fax: +4940741056263
E-mail: cchoe@uke.de

Abstract

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 in stroke pathology and outcome.

Methods and Results—Increasing homoarginine levels independently associated with a reduction in all-cause mortality in patients with ischemic stroke (7.4 years follow-up, HR for 1 SD homoarginine: 0.79 [95% CI: 0.64, 0.96], P=0.019, n=389). Homoarginine was also independently associated with the NIHSS+age score and 30-day mortality after ischemic stroke (P<0.05, n=137). Genome-wide association study (GWAS) revealed that plasma homoarginine strongly associated with SNPs in the L-arginine:glycine amidinotransferase (AGAT) gene (P<2.1x10^-8, n=2,806) and increased AGAT expression in a cell model was associated with increased homoarginine. Next, we employed two genetic murine models to investigate the link between plasma homoarginine and outcome after experimental ischemic stroke: i) an AGAT deletion (AGAT^-/-) and ii) a guanidinoacetate N-methyltransferase deletion, (GAMT^-/-) causing AGAT upregulation. As suggested by the GWAS, homoarginine was absent in AGAT^-/-, 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 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.

Key words: Genome Wide Association Study, ischemic stroke, single nucleotide polymorphism, homoarginine, L-arginine:glycine amidinotransferase
Introduction

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 suggested it may be an alternative substrate for nitric oxide synthase (NOS) and in support of this, homoarginine levels are associated with endothelial function. NO-dependent neurotoxic and neuroprotective mechanisms influence the pathophysiology of cerebrovascular disease and strategies which 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 type 2 diabetes patients receiving maintenance hemodialysis. In a small subgroup of 61 patients, low homoarginine levels also correlated with fatal stroke. In these and other populations, low homoarginine concentrations were inversely associated with flow mediated vasodilatation, 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 as to whether these observations reflect a direct causal effect which could provide important insights into the pathophysiology of vascular disease. The aim of the present study was to investigate, i) the role of homoarginine in ischemic cerebrovascular disease ii) the genetic association of homoarginine in man by GWAS iii) relevant genetic murine models to confirm the genetic association of homoarginine in mice and iv) the influence of homoarginine on stroke severity in an in vivo stroke model.
Clinical cohorts, analytical and experimental methods

Clinical cohorts

i) Leeds Stroke Study

The recruitment and characteristics of patients in the Leeds Stroke Study have been described elsewhere.9–10 In brief, consecutive white European patients (n=609) with a clinical diagnosis of acute ischemic stroke were recruited from hospitals in Leeds between August 1993 and April 1996. Surviving patients were censored on Jan 19th 2000. According to the Oxfordshire Community Stroke Project (OCSP) classification, ischemic stroke, confirmed by noncontrast cranial CT scan, was subclassified as lacunar, partial anterior, total anterior, or posterior circulation infarction (LACI, TACI, PACI, and POCI). LACI represents stroke of probable small vessel origin, PACI and TACI represent stroke of probable large vessel origin and POCI represents stroke of mixed vascular pathology.11 Venous blood samples were taken within 10 days of the acute event, and plasma was stored at 40 °C until use. Subjects were classified as current, former, or non-smokers, and a medical history of previous stroke or transient ischemic attack, ischemic heart disease, and peripheral vascular disease was documented. Atrial fibrillation (AF) was confirmed by 12-lead electrocardiogram. Diabetes and hypertension were determined from case notes, as were current use of hypoglycemic and antihypertensive agents. Patients were flagged with the Office for National Statistics for notification of death, as previously described.10 All subjects provided informed consent according to a protocol approved by the Leeds Teaching Hospitals Research Ethics Committee. To assess determinants of long-term outcome following stroke, only patients surviving more than 30 days after the acute event, and with sufficient plasma for the analysis of homoarginine, were included in the present study (n=389). There were no differences in baseline characteristics between the entire cohort and the subsample.
ii) Harburg Stroke Study

The Harburg Stroke Study was conducted to investigate the acute phase after ischemic stroke. Briefly, 137 consecutive patients with an acute ischemic stroke, confirmed by cranial CT scan, were recruited from the stroke unit of the Department of Neurology at the Asklepios Clinic Hamburg-Harburg between May 2007 and May 2008. Blood samples were taken directly after admission, and plasma stored at -80 °C. The NIHSS score was determined on admission to assess stroke severity. Patients were followed daily during admission and, after discharge until day 30 post-stroke for the combined endpoint of death, non-fatal recurrent stroke, non-fatal myocardial infarction, and rehospitalisation. All patients provided written informed consent according to a protocol approved by the Ethics Committee at the Hamburg Board of Physicians.

iii) Gutenberg Health Study and GWAS

The Gutenberg Health Study is a community-based, prospective cohort study including 15,000 subjects aged 35 to 74 years from the city of Mainz and the district Mainz-Bingen. Participants of the Gutenberg Health Study were randomly recruited to the study from government local registry offices, as described. Written, informed consent was obtained from all participants and the study protocol and sampling design were approved by the local ethics committee and by the local and federal data safety commissioners. For the present investigation, a cross-sectional analysis was performed for 2,806 participants of the study recruited from 2007 until 2008. There were no differences in baseline characteristics between the first 5,000 subjects of the cohort and the subsample. The sample was stratified according to gender (50% women, decades of age, and places of residence (urban or rural)). Genotyping was performed on the Affymetrix Human SNP Array 6.0. Extensive quality control analyses were performed before data were analyzed.
Analytical methods

i) Measurement of guanidino compounds

After deproteinization of plasma, guanidino compounds were separated using a strong cation exchange resin using sodium citrate buffers. Subsequently, post column derivatization of the separated compounds with ninhydrin in basic medium was followed by measurement of the fluorophores (ex=395 and em=500 nm). Homoarginine was determined by LC-MS/MS after derivatization with butanolic hydrochloric acid. Interassay accuracy (bias) and precision (CV) were below 5% and 9%, respectively. ADMA, SDMA and L-NMMA were determined by LC-MS/MS as described previously. Venous blood samples from stroke patients were previously analyzed for biochemical, hematological, and hemostatic factors. eGFR was analyzed according to the abbreviated MDRD equation for Caucasians: \(186 \times \frac{\text{Creat}}{88.4} - 1.154 \times \text{Age} - 0.203 \times (0.742 \text{ for females})\).

ii) Measurement of \([^{13}\text{C}_6^{15}\text{N}_2]-\text{homoarginine}\) formation in vitro

L-arginine-glycine amidinotransferase (AGAT)-transfected and untransfected Human Embryonic Kidney (HEK293) cells were incubated with 100 µM L-[\(^{15}\text{N}_2\)-guanidino]-arginine/100 µM \([^{13}\text{C}_6]\)-lysine for 24h at 37°C. Samples were analysed by LC-MS/MS using L-[\(^2\text{H}_7\)]-arginine as internal standard. \([^{13}\text{C}_6^{15}\text{N}_2]\)-Homoarginine and L-[\(^2\text{H}_7\)]-arginine were detected by selected ion monitoring of the transitions \(m/z\) 253.1→\(m/z\) 89.0 (CE -16eV) and \(m/z\) 238.1→\(m/z\) 77.0 (CE -22 eV), respectively.

Experimental methods

i) Mouse models

Mating, housing and generation of mice were performed as previously described (Supplemental Methods in the online-only Data Supplement).
ii) Temporary middle cerebral artery occlusion

Temporary middle cerebral artery occlusion (tMCAO) was achieved as previously described21-22 (Supplemental Methods, **Suppl. Fig 1-5**, **Suppl. Tab. 1** in the online-only Data Supplement).

iii) Determination of infarct size

Stroke analysis was performed as previously described21-24 (Supplemental Methods and **Suppl. Fig. 6** in online-only Data Supplement).

iv) Assessment of functional outcome

One and 24h after MCAO, mice were scored using an extended Bederson score with a scale from 0 to 4: (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 24h of tMCAO, (2) subarachnoid hemorrhage (as macroscopically assessed during the brain sampling), and (3) modified Bederson score 0 immediately after recovery from tMCAO.

v) Tail-cuff measurements

For hemodynamic measurements, mice were anesthetized with 1.5-2.0% isoflurane. After 3 pre-pulse protocols, systolic blood pressure, pulse and mean arterial pressure were recorded and analyzed with a Hatteras system and software (Modell SC-1000, USA) (**Suppl. Fig. 2**).

vi) 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 using a digital camera (**Suppl. Fig. 4 A, B** and **5 A, B**). For posterior communicating artery (Pcom)-scores, the Pcom anatomy was scored as follows: absent (0), poorly developed (1), and well
formed (2) (Suppl. Fig. 4 C-D). Furthermore, cerebral angioarchitecture on the dorsal surface was evaluated by the distance from the midline to the line of anastomoses at 2 mm, 4 mm, and 6 mm from the frontal pole (Suppl. Fig. 5 C-D).

vii) Magnetic resonance spectroscopy measurement

In vivo 31P magnetic resonance spectroscopy (MRS) measurements were performed as previously described. At 7T field strength using a quadrature 31P coil, spectra were selected from voxels of 160-230 µl in the cerebrum of AGATwt (n=5), AGAT<sup>−/−</sup> (n=4) and AGAT<sup>−/−</sup>Cr (n=1) mice. To obtain PCr/NTP ratios the signal intensities of PCr and NTP were fitted using jMRUI and corrected for T1 relaxation.

viii) Western blot

Equal amounts of cleared lysates (20-40 µg 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, USA). 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 non-saturated images using ImageJ software.

Statistical analysis

Data were expressed as median (interquartile range [IQR]) for clinical cohorts unless indicated. 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, e.g. 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 and/or P<0.01. Differences between two unrelated groups were compared using unpaired Student’s t-test and differences between >2 groups by one-way ANOVA with Newman-Keuls post-test. Differences between categorical data were analyzed by X² test. Univariate associations between tertiles of homoarginine and mortality were assessed using Kaplan-Meier survival analysis with significance determined using the log-rank test. The independent association between homoarginine and mortality was determined using multivariable Cox regression analyses with results presented as hazard ratios (HR) for a 1 SD increase in log homoarginine (with 95% confidence intervals). Different models were evaluated adjusting for the demographic, clinical, biochemical, hematological, and hemostatic determinants that we have previously shown to predict post-stroke mortality in this cohort.¹⁰,²⁹ C-statistics were calculated using AUCs from receiver operating curve characteristics (ROC) curves and the net reclassification index (NRI) was calculated for models including and excluding homoarginine to evaluate the predictive value of homoarginine for survival.³⁰ The relationships between genotype at selected 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 using SPSS v20 (IBM). Genome-wide significance of association in the Gutenberg Health Study was adjusted for multiple testing (P<2.1x10⁻⁸, Bonferroni correction for 2,400,000 SNPs). All genotyped SNPs had a genotype call rate > 98%; imputation quality threshold was R² > 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 (β), 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 to 8.5] years. The median [IQR] plasma homoarginine concentration of the entire cohort was 1.07 [0.74] μM. Homoarginine was higher in patients who survived (1.27 [0.72] μM, n=160) compared with those who died during follow-up (0.96 [0.64] μM, n=229; P<0.001). Homoarginine concentrations correlated with creatinine (r=-0.19 [95% CI: -0.30,-0.05]; P=0.001), eGFR (r=0.19 [0.07,0.31]; P<0.01), plasma L-arginine (r=0.41 [0.33, 0.49]), C-reactive protein (CRP) (r=-0.38 [-0.45,-0.30]), -thromboglobulin (β-TG, r=-0.31 [-0.41,-0.20]), fibrinogen (r=-0.25 [-0.35,-0.16]), and von Willebrand factor (vWF, r=-0.31 [-0.42, -0.19], P<0.001 for all other).

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 hazard ratio for a 1 SD increase in log homoarginine concentration (anti-log: 0.215 μM) was 0.75 after adjustment for previously identified predictors of post-stroke mortality (age, AF, 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-TG, vWF (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 (e.g. NRI for model 2 without and with homoarginine = -0.03). In a subgroup analysis, homoarginine was inversely associated with stroke severity using the OCSP classification as a surrogate parameter. Patients with probable small vessel disease (lacunar infarct) had higher median homoarginine levels (1.22 [IQR: 0.60] μM, n=137) compared with patients with large vessel disease (total or partial anterior circulation infarcts: 0.92 [0.66] μM, 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 was significantly lower in patients who had an event during follow-up (1.48 [1.01] μmol/L, n=25) 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 μM]: 0.68 [95% CI: 0.50,0.92; P<0.05]). After adjustment for age, sex, and NIHSS 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, -0.07]), eGFR (ρ=0.29 [0.12,0.44]), CRP (ρ=0.44 [-0.58, -0.28]; all P<0.01), NIHSS (ρ=0.20 [-0.36,-0.03]; P<0.05), and NIHSS + age (ρ=-0.27 [-0.42, -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 genome-wide association (GWA) analysis in the community-based Gutenberg Health Study (n=2,806). Among approximately 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 ($P<2.1 \times 10^{-8}$ for all). The top SNPs were all located within the same chromosomal locus on 15q21 and solely associated with the L-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 to the carbamoyl-phosphate-synthase-1 (CPS-1) gene ($P=3.6-5.4 \times 10^{-9}$). 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 three 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, Suppl. Fig. 7).

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 L-$[^{15}\text{N}_2]$-guanidino-arginine and $[^{13}\text{C}_6]$-lysine and confirmed the in vitro formation of $[^{13}\text{C}_6][^{15}\text{N}_2]$-guanidino-homoarginine in AGAT-expressing HEK293 cells (Suppl. Fig. 8).

In vivo regulation of homoarginine levels in AGAT-deficient and –overexpressing mice

The in vitro results using 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 (GAA) and creatine were deficient in plasma of AGAT$^{-/}$
mice (Figure 5A-C). Plasma homoarginine concentration was 0.17±0.03 μM in wild-type (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 upon creatine supplementation (AGAT^{-/-} Cr). We also studied a previously generated knockout mouse model with GAMT deficiency (GAMT^{-/-}, second enzyme of creatine synthesis). In GAMT^{-/-} mice, a more than two-fold upregulation of AGAT protein expression (Figure 5E, Suppl. Fig. 9) was observed along with creatine deficiency and increased GAA levels (Figure 5F-G). In line 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 (Suppl. Fig. 10), suggesting that AGAT regulates homoarginine levels in a dose-dependent manner in vivo (Figure 5I and J).

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 min of ischemia were almost two-fold larger in AGAT^{-/-} mice (51.8±6.1%) compared with wt littermates (17.0±4.0%, P<0.01), and neurological impairment after 24h was more severe in AGAT^{-/-} mice (wt vs. AGAT^{-/-}: 1.1±0.3 vs. 2.7±0.3; P<0.05; Figure 6A-C). Dietary supplementation with 0.5% or 1.0% creatine in chow did not influence infarct size (0.5% Cr: 49.9%±9.3%; 1.0% Cr: 36.8%±7.0%; P>0.05 compared with AGAT^{-/-}) or neurological score (0.5% Cr: 2.0±0.0; 1.0% Cr: 2.2±0.3; P>0.05 compared with AGAT^{-/-}). MRS measurements revealed that oral Cr supplementation effectively replenished brain PCr in AGAT^{-/-} mice (Suppl. Fig. 11).
Furthermore, brain histology showed no differences between wt and AGAT°/° mice (Suppl. Fig. 3). 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 h compared with wt littermates (wt vs. GAMT°/°: 25.4±5.2% vs. 13.3±1.8%; *P*<0.05 and 2.0±0.3 vs. 0.9±0.2; *P*<0.01; **Figure 6D-F**).

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 μM) compared with vehicle-treated AGAT°/°Cr mice (0.07±0.02 μM; *P*<0.01; **Figure 7A**). Cerebral infarct volume was significantly decreased in homoarginine-treated compared with vehicle-treated AGAT°/°Cr mice (**Figure 7B-D**). Plasma homoarginine concentrations were strongly and dose-dependently correlated with infarct sizes and neurological scores in mice subjected to 30 min tMCAO (**Figure 8**).

Finally, C57BL6 mice were orally supplemented with homoarginine resulting in increased homoarginine plasma levels compared with vehicle-treated C57BL6 mice (vehicle vs. homoarginine: 0.15±0.01 μM vs. 0.20±0.02 μM; *P*<0.05; Suppl. Fig. 12). Homoarginine supplementation in C57BL6 mice resulted in decreased infarct sizes and improved neurological scores after 24h compared with vehicle-treated C57BL6 mice (vehicle vs. homoarginine: 59.0±1.7% vs. 37.4±6.7% and 2.6±0.3% vs. 1.5±0.2%; Suppl. Fig. 12). Plasma homoarginine concentrations were dose-dependently correlated with infarct sizes in C57BL6 mice subjected to 60 min tMCAO (Suppl. Fig. 12).
Discussion

The results from the present study demonstrate that homoarginine is i) a potential novel biomarker for short- and long-term outcome after ischemic stroke, (ii) regulated by AGAT in both humans (indicated by GWAS) and mice (demonstrated using genetically engineered mouse models), and (iii) a determinant of cerebral infarct size and neurological outcome in experimental stroke in murine models of low and high homoarginine expression. 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 kidney31 and found at low concentrations in most bodily fluids.32-33 Low plasma homoarginine has been associated with decreased renal function, energy metabolism, and NO regulation.7-8, 32 The metabolism of homoarginine in humans is poorly understood, however, evidence indicates that homoarginine is an intermediate product of an analogous urea cycle7 and, in bacteria, the amidinotransferase AGAT catalyzes the generation of homoarginine.34 A recent study using lymphoblasts from an AGAT-deficient patient demonstrated a link between AGAT and homoarginine35 and 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 which might affect protein conformation and AGAT activity.36

In vertebrates, AGAT is the first and rate-limiting enzyme in creatine synthesis which catalyzes the transfer of the amidino group from L-arginine to glycine, resulting in the formation of ornithine and GAA. Subsequently, GAMT methylates GAA to yield creatine.37 To clarify the link between AGAT and homoarginine synthesis, we transfected HEK cells with AGAT and identified stable isotope-labelled 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 using 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 ischemia38, 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), 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 following 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 nNOS, eNOS, iNOS, arginase 1&2, and DDAH 1&2 mRNA expression between wt and AGAT\(^{-/-}\) mice (Suppl. Fig. 13) or plasma ADMA, SDMA and other guanidino compounds (Suppl. Tab. 2). 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 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 (eNOS, and iNOS) and detrimental effects (nNOS and iNOS) 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 fraction\(^4\),\(^7\),\(^39\) as well
as by evidence of a role for homoarginine in insulin secretion and inhibition of platelet aggregation.42 Previous studies have reported inverse associations between homoarginine and VCAM-1 and ICAM-17 and in the Leeds Stroke study we observed significant inverse associations between homoarginine and CRP, β-TG, fibrinogen and vWF. These latter findings suggest 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 two 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 post-stroke 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 MRI data were not available in the Leeds Stroke Study. However, homoarginine was correlated with NIHSS and age in the Harburg Stroke Study and experimental data causally linked homoarginine levels with stroke size and outcome.

**Conclusions**

Our data provide evidence that homoarginine predicts post-stroke mortality in two 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 post-stroke neurological deficit. In contrast to non-modifiable stroke predictors, like age and NIHSS, homoarginine could open new therapeutic avenues of stroke management.

**Acknowledgments:** The excellent technical assistance of Mariola Kastner, Ellen Orthey, Kathrin Sauter, Stefan Schillemeit, Anna Steenpaß, and Hartwig Wiebolt is gratefully acknowledged.

**Funding Sources:** The study was supported by the Deutsche Forschungsgemeinschaft (CH872/1-1,SFB545/A3,IS63/3-1,IS63/3-2), by an Else Kröner Memorial Stipendium from the Else Kröner-Fresenius-Stiftung, the UK Stroke Association, Stiftung Rheinland-Pfalz “Wissenschaft-Zukunft” (AZ961-386261/733), by “Schwerpunkt Vaskuläre Prämvention” (Mainz University, Boehringer Ingelheim), by the „Integrierte Verbünde der Medizinischen Genomforschung – NGFN-Plus” (A3 01GS0833 and 01GS0831), by the Stiftung Pathobiochemie of the Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin e.V. and by the Werner-Otto-Stiftung.

**Conflict of Interest Disclosures:** None.

**References:**


17. Schwedhelm E, Maas R, Tan-Andresen J, Schulze F, Riederer U, Böger RH. High-


**Table 1.** Cox regression analyses for homoarginine in the Leeds Stroke Study.

<table>
<thead>
<tr>
<th>Cox Regression Models</th>
<th>Hazard Ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model 1</strong>: unadjusted</td>
<td>0.67 [0.59,0.76]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Model 2</strong>: adjusted for age, AF, previous stroke, and stroke subtype</td>
<td>0.75 [0.66,0.86]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Model 3</strong>: adjusted for Model 2 + β-TG and vWF</td>
<td>0.84 [0.72,0.98]</td>
<td>0.024</td>
</tr>
<tr>
<td><strong>Model 4</strong>: adjusted for Model 2 + eGFR</td>
<td>0.79 [0.64,0.96]</td>
<td>0.019</td>
</tr>
</tbody>
</table>

Data presented as hazard ratio [95% CI] for 1 SD increase in log homoarginine.
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</th>
<th>Stage 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homoarginine</td>
<td>rs10519022</td>
<td>15q21</td>
<td>Intronic</td>
<td>C (0.26)</td>
<td>0.13 (0.01) 6.1x10⁻³⁰</td>
<td>0.16 (0.04) 1.1x10⁻⁴</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs2467864</td>
<td></td>
<td>Downstream</td>
<td>C (0.26)</td>
<td>0.13 (0.01) 8.1x10⁻³⁰</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>rs1145077</td>
<td></td>
<td>Upstream</td>
<td>T (0.62)</td>
<td>0.09 (0.01) 5.3x10⁻¹⁸</td>
<td>0.13 (0.04) 5.9x10⁻⁴</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs1288775²</td>
<td></td>
<td>Missense</td>
<td>A (0.26)</td>
<td>0.13 (0.01) 6.5x10⁻³⁰</td>
<td>0.16 (0.04) 5.5x10⁻⁵</td>
<td></td>
</tr>
</tbody>
</table>

*Effect size (β) estimates represent the change in homoarginine plasma concentrations per unit difference in minor allele dose. Bold SNPs indicate SNPs that were replicated in the Leeds Stroke Study (P<0.05 adjusted for age and sex). Imputed SNP. ND indicates ‘not determined’.
Figure Legends:

**Figure 1.** Homoarginine plasma levels predict survival and stroke severity. (A) Kaplan-Meier survival curves indicating the relationship of tertiles of homoarginine with all-cause mortality after ischemic stroke during a median follow-up of 7.4 years (n=389). Statistical significance by log-rank test was $P<0.001$. (B) Homoarginine plasma levels in patients with lacunar infarcts (LACI, n=137) and partial/total anterior circulation infarcts (PACI/TACI, n=213, ***$P<0.001$ by Student’s t-test on log-transformed homoarginine).

**Figure 2.** Homoarginine plasma levels in patients of the Harburg Stroke Study. Plasma homoarginine correlated with NIHSS+age ($\rho=-0.27$; 95% CI:-0.42,-0.10.; n=137; **$P<0.01$ by Spearman’s correlation analysis).

**Figure 3.** Association of homoarginine with genetic variations. (A) Genome-wide association (GWA) analysis between homoarginine plasma levels and 2.4 million SNPs. The SNPs reaching genome-wide significance were all within the same locus on 15q21 which includes the $AGAT$ gene. Genome-wide significance of association in the Gutenberg Health Study was adjusted for multiple testing ($P<2.1x10^{-8}$, Bonferroni). (B) Heat map of the 14 top SNPs illustrating the linkage disequilibrium across the $AGAT$ gene (n=2,806).

**Figure 4.** Allele-specific homoarginine plasma levels across the AGAT genotypes in the Gutenberg Health Study (n=2,806). For the SNPs (A) rs10519022 (AGAT intron), (B) rs1145077 (upstream of AGAT), (C) rs1288775 (AGAT missense), and (D) rs2467864
(downstream of AGAT), 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, adjusted for age and sex ($P<2.1\times10^{-8}$ for all). The association between trait and genotype was quantified by the regression slope (β [SE]).

**Figure 5.** Metabolic characterization of AGAT$^{-/-}$, GAMT$^{-/-}$, and wt littermates. (A-H) Western blot expression analysis of kidneys from AGAT$^{-/-}$ (A), GAMT$^{-/-}$ (E), and wt mice (n=3). Plasma concentrations of Cr (B and F), guanidinoacetate (GAA) (C and G), and homoarginine (D and H) in wt, AGAT$^{-/-}$, Cr-supplemented AGAT$^{-/-}$ mice (B-D) (n=6-12), wt, and GAMT$^{-/-}$ mice (F-H) (n=6-10). (I and J) Metabolic scheme of Cr, GAA, and homoarginine in AGAT (I) and GAMT deficiency (J). **$P<0.01$; ***$P<0.001$ by one-way ANOVA with Newman-Keuls post-test.

**Figure 6.** Temporary middle cerebral artery occlusion (tMCAO) in AGAT$^{-/-}$, GAMT$^{-/-}$, and wt mice. (A and D) Representative triphenyltetrazolium chloride (TTC)-stained brain slices of AGAT$^{-/-}$ (A), GAMT$^{-/-}$ (D) and wt mice subjected to 30 min tMCAO and 24h reperfusion. (B-C and E-F) Infarct sizes and neurological scores were analyzed in wt (n=11), AGAT$^{-/-}$ (n=10), Cr-supplemented AGAT$^{-/-}$ mice (n=10) (B and C), wt (n=5), and GAMT$^{-/-}$ (n=11) mice (E and F). *$P<0.05$; **$P<0.01$ one-way ANOVA with Newman-Keuls post-test.

**Figure 7.** Homoarginine supplementation in AGAT$^{-/-}$Cr mice subjected to tMCAO for 60min (A) Comparison of homarginine plasma concentrations in AGAT$^{-/-}$Cr mice supplemented with
vehicle (0.9% NaCl) (n=9) or homoarginine (n=5) for four weeks. (B-D) Representative TTC-stained brain slices (B), infarct sizes (C), and neurological scores (D) of AGAT⁻/⁻Cr mice subjected to 60min MCAO and 24h reperfusion (NaCl n=11, homoarginine 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 24h (B) in AGAT⁻/⁻, wt, and GAMT⁻/⁻ mice after 30 min tMCAO (n=27). *P<0.05; ***P<0.001 by Spearman’s correlation analysis.
Figure 3
Figure 4

A. rs10519022 (AGAT intron)  
B. rs1145077 (12.8 kb upstream of AGAT)  
C. rs1288775 (imputed, exon of AGAT)  
D. rs2467864 (12.2 kb downstream of AGAT)
Figure 5
Figure 6
Figure 7
Figure 8

(A) Spearman ρ = -0.67 ***

(B) Spearman ρ = -0.47 *
Homoarginine Levels are Regulated by L-Arginine:Glycine Amidinotransferase and Affect Stroke Outcome: Results from Human and Murine Studies


_Circulation_. published online September 4, 2013;
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2013/09/04/CIRCULATIONAHA.112.000580

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2013/09/04/CIRCULATIONAHA.112.000580.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL

1) Supplemental Methods

Experimental methods

i) Mouse models Mating, housing and generation of mice were performed as previously described. 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\(^{-/-}\) and guanidinoacetate N-methyltransferase (GAMT)-deficient mice resulted in reduced body weight, less relative body fat mass, decreased muscle strength and volume\(^{1-3}\). In addition, cholesterol levels were reduced and glucose tolerance was increased in AGAT\(^{-/-}\) mice\(^{1}\). On a special diet containing 1% Cr ad libitum after weaning (Ssniff, Germany), the metabolic phenotype of AGAT\(^{+/+}\) and AGAT\(^{-/-}\)Cr did not differ.\(^{1}\) The 4-week-long supplementation with homoarginine or vehicle in AGAT\(^{-/-}\) mice was achieved via osmotic mini pumps (Alzet, Cupertino, CA, USA) loaded with homoarginine dissolved in saline (30 µg/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.\(^{4-5}\) 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\(^{+/+}\) 28.9g, AGAT\(^{-/-}\) 18.6g, AGAT\(^{+/+}\)Cr 27.4g, GAMT\(^{+/+}\) 30.2g, GAMT\(^{-/-}\) 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\(_2\) 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: \( V_{\text{corrected}} = V_{\text{infarct}} \times \left(1 - \frac{V_i - V_c}{V_c}\right) \), 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>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>pO2 (mmHg)</td>
<td>92.7 ± 6.1</td>
<td>81.7 ± 4.2</td>
<td>82.4 ± 10.7</td>
<td>ns</td>
</tr>
<tr>
<td>pCO2 (mmHg)</td>
<td>52.6 ± 0.8</td>
<td>60.8± 1.8*</td>
<td>48.7 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.23 ± 0.01</td>
<td>7.21 ± 0.01</td>
<td>7.28 ± 0.03</td>
<td>ns</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Retrobulbar</th>
<th>GAMTwt (n = 3)</th>
<th>GAMTd/d (n = 3)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>pO2 (mmHg)</td>
<td>84.7 ± 2.4</td>
<td>70.4 ± 7.3</td>
<td>ns</td>
</tr>
<tr>
<td>pCO2 (mmHg)</td>
<td>45.5 ± 4.5</td>
<td>49.3 ± 1.9</td>
<td>ns</td>
</tr>
<tr>
<td>pH</td>
<td>7.25 ± 0.02</td>
<td>7.22 ± 0.01</td>
<td>ns</td>
</tr>
</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>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>AGAT-KO</td>
</tr>
<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>
</tr>
<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>
</tr>
<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>
</tr>
<tr>
<td>α-N-AA</td>
<td>0.80 ± 0.21</td>
<td>0.97 ± 0.22</td>
</tr>
<tr>
<td>ArgA</td>
<td>0.05 ± 0.05</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>γ-GBA</td>
<td>0.26 ± 0.11</td>
<td>0.47 ± 0.15</td>
</tr>
<tr>
<td>G</td>
<td>0.45 ± 0.20</td>
<td>0.40 ± 0.24</td>
</tr>
<tr>
<td>MG</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>β-GPA</td>
<td>0.01 ± 0.01</td>
<td>0.02 ± 0.04</td>
</tr>
</tbody>
</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-[2H7]-arginine (m/z 238 → m/z 77), and [13C6,15N2]-homoarginine (m/z 253 → m/z 89). In contrast to untransfected HEK cells (A), the incubation of AGAT-expressing HEK cells (B) with [13C6]-lysine and L-[15N2-guanidino]-arginine resulted in the formation of [13C6,15N2]-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-monooesters, 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


