High plasma guanidinoacetate-to-homoarginine ratio is associated with high all-cause and cardiovascular mortality rate in adult renal transplant recipients

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ORIGINAL ARTICLE

High plasma guanidinoacetate-to-homoarginine ratio is associated with high all-cause and cardiovascular mortality rate in adult renal transplant recipients

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Abstract

L-Arginine:glycine amidinotransferase (AGAT) is the main producer of the creatine precursor, guanidinoacetate (GAA), and L-homoarginine (hArg). We and others previously reported lower levels of circulating and urinary hArg in renal transplant recipients (RTR) compared to healthy subjects. In adults, hArg emerged as a novel risk factor for renal and cardiovascular adverse outcome. Urinary GAA was found to be lower in children and adolescents with kidney transplants compared to healthy controls. Whether GAA is also a risk factor in the renal and cardiovascular systems of adults, is not yet known. In the present study, we aimed to investigate the significance of circulating GAA and the GAA-to-hArg molar ratio (GAA/hArg) in adult RTR. We hypothesized that GAA/hArg represents a measure of the balanced state of the AGAT activity in the kidneys, and would prospectively allow assessing a potential association between GAA/hArg and long-term outcome in RTR. The median follow-up period was 5.4 years. Confounders and potential mediators of GAA/hArg associations were evaluated with multivariate linear regression analyses, and the association with all-cause and cardiovascular mortality or death-censored graft loss was studied with Cox regression analyses. The study cohort consisted of 686 stable RTR and 140 healthy kidney donors. Median plasma GAA concentration was significantly lower in the RTR compared to the kidney donors before kidney donation: 2.19 [1.77–2.70] µM vs. 2.78 [2.89–3.35] µM ($P < 0.001$). In cross-sectional multivariable analyses in RTR, HDL cholesterol showed the strongest association with GAA/hArg. In prospective analyses in RTR, GAA/hArg was associated with a higher risk for all-cause mortality (hazard ratio (HR): 1.35 [95% CI 1.19–1.53]) and cardiovascular mortality (HR: 1.46 [95% CI 1.24–1.73]), independent of potential confounders. GAA but not GAA/hArg was associated with death-censored graft loss in crude survival and Cox regression analyses. The association of GAA and death-censored graft loss was lost after adjustment for eGFR. Our study suggests that in the kidneys of RTR, the AGAT-catalyzed biosynthesis of GAA is decreased. That high GAA/hArg is associated with a higher risk for all-cause and cardiovascular mortality may suggest that low plasma hArg is a stronger contributor to these adverse outcomes in RTR than GAA.

Keywords Arginine · Cardiovascular risk · Graft survival · Kidney · Transplantation

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Abbreviations

ADMA	Asymmetric dimethylarginine
AGAT	Arginine:glycine amidinotransferase
BSA	Body surface area

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BMI	Body mass index
CI	Confidence interval
CKD	Chronic kidney disease
eGFR	Estimated glomerular filtration rate
GAA	Guanidinoacetate
hArg	Homoarginine
HR	Hazard ratio
IQR	Interquartile range
MMF	Mycophenolate mofetil
NO	Nitric oxide
NOS	Nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
NT-pro-BNP	N-Terminal pro-hormone of brain natriuretic peptide
PTH	Parathyroid hormone
QC	Quality control
RTR	Renal transplant recipients

Introduction

Guanidine-groups-containing substances play important roles in living organisms. Guanidinoacetate (GAA) and L-homoarginine (hArg) are products formed from L-arginine (Arg), i.e., 2-amino-5-guanidinopentanoic acid, by the activity of renal arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1). GAA is subsequently converted to creatine by guanidinoacetate *N*-methyltransferase (GAMT, EC 2.1.1.2) (Fig. 1). The AGAT-catalyzed formation of GAA is considered the rate-limiting step of creatine biosynthesis. In the past decade, low concentrations of hArg were found to be associated with a higher risk for adverse outcomes in the renal and cardiovascular systems (Choe et al. 2013; Frenay et al. 2015a; Pilz et al. 2011a, b). Furthermore, supplementary hArg has been shown to be cardioprotective in a model of mice after myocardial infarction (Atzler et al. 2017). Being the direct precursor of creatine, GAA has been thoroughly investigated over several decades mainly from an energetic perspective. To our knowledge, and in contrast to hArg, GAA has not been investigated as a potential risk factor in the renal and cardiovascular systems of adults so far. There is solely a report proposing the urinary molar ratio of GAA to guanidinosuccinate as an indicator of kidney function (Sasaki et al. 1973). More recently, urinary GAA excretion was found to be lower in children and adolescents with kidney transplants than in healthy controls (Andrade et al. 2008, 2011), suggesting a role of GAA in chronic kidney disease (CKD). Previous investigations mainly focused on circulating GAA in patients with AGAT and/or GAMT deficiency and the serious implications in energy homeostasis (Battini et al. 2002; Das et al. 2000; Item et al. 2001; Stockler et al. 1996; Wyss and Wallimann 1994).

Asymmetric dimethylarginine (ADMA), another physiological Arg derivative and risk factor for endothelial dysfunction (Böger et al. 1998), cardiovascular disease, and CKD progression (Ueda et al. 2007), was found to be inversely associated with long-term outcome in adult renal transplant recipients (RTR) (Kayacelebi et al. 2017). The mechanisms that underlie the detrimental effects of ADMA in the renal and cardiovascular systems are still unexplored. It is generally assumed that the main mechanism involves inhibition by ADMA of the activity of endothelial nitric oxide synthase (eNOS), which converts Arg to nitric oxide (NO), one of the most potent endogenous vasodilators (Tsikas et al. 2018). hArg may also serve as a substrate for NOS (Hecker et al. 1991; Moali et al. 1998). Yet, hArg rather seems to compete with Arg for eNOS and other nitric oxide synthase (NOS) isoforms and to decrease NO synthesis (Alesutan et al. 2016). Alpha-guanidinoglutaric acid, another guanidine compound, was found to potently inhibit neuronal NOS (nNOS) activity in rat brain (Yokoi et al. 1994). We recently reported that supplementation of therapeutical doses of the biguanidine compound metformin (1,1-dimethylbiguanide), an oral antidiabetic drug that contains an asymmetrically dimethylated and a non-methylated guanidine group, influenced the circulating concentration of both, GAA and hArg (Hanff et al. 2018). These observations suggest that methylated and non-methylated guanidine compounds of endogenous and exogenous origin may exert biological activities in the renal and cardiovascular systems by interacting with NOS, AGAT, and other biological structures.

In the circulation of healthy humans, GAA and hArg are found in similar quantities being in the very low µM-range, whereas the urinary excretion rate of GAA is much higher than that of hArg (Wyss and Wallimann 1994), indicating a higher biosynthesis rate of GAA compared to hArg (Fig. 1). AGAT is mainly expressed in the kidney, which is a major contributor to circulating hArg (Kayacelebi et al. 2017). The relative contribution of the kidney to the circulating GAA in humans is not yet known. Also, the importance of circulating GAA and its association with circulating hArg in adult RTR, i.e., the balanced state of AGAT activity, have not been investigated before. We assumed that measurement of circulating GAA and hArg in patients suffering from CKD and in healthy subjects would provide a promising approach to evaluate the kidney's contribution to the homeostasis of GAA and hArg and could be expressed as the GAA-to-hArg molar ratio, i.e., GAA/hArg.

The aim of the present study was to gain a deeper insight into the AGAT-dependent homeostasis of GAA and hArg in healthy humans and in a state of impaired renal function, as it is found in stable RTR, and to evaluate the kidney's contribution to these guanidine substances. To reach this goal, we measured the concentration of

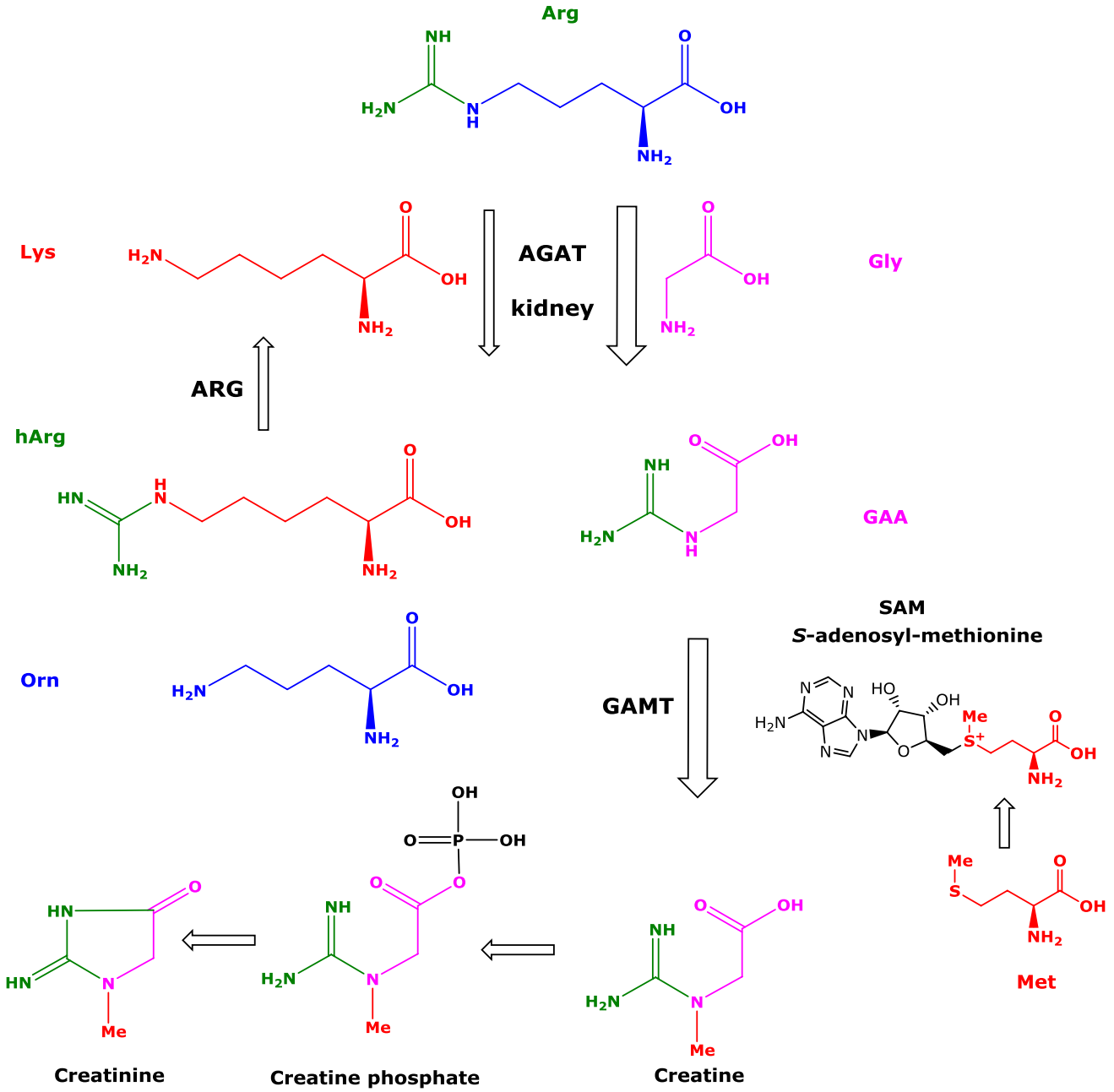

Fig. 1 Biosynthesis of GAA and hArg from Arg by AGAT and downstream pathways. In the kidneys, mitochondrial AGAT catalyzes the formation of GAA from Arg and Gly, and the formation of hArg from Arg and Lys; Orn is the second common product of these reactions. GAMT catalyzes the formation of creatine from GAA and the methyl (Me) donor *S*-adenosylmethionine (SAM), which itself derives from Met. Creatine is then converted to creatine phosphate, which cyclizes to creatinine. hArg is hydrolyzed by arginase (ARG) to urea and Lys. The size of the arrows is a rough quantitative estimate

circulating GAA in adult RTR and in healthy kidney donors before and after kidney transplantation, and we determined the prospective association of both, GAA and GAA/hArg, with all-cause and cardiovascular mortality and death-censored graft loss in RTR.

Methods

Design and study population

The single center RTR cohort of this prospective study has been previously described in detail (van den Berg et al.

2012a, b, 2013). A total of 706 RTR and 297 living kidney donors signed informed consent and participated in the present study. All transplantations were conducted in the University Medical Center Groningen (UMCG). Data of 20 RTR and 17 kidney donors with missing biomaterials were excluded from statistical analyses. Plasma samples of 687 RTR were collected for measurement of circulating biomarkers including Arg and its metabolite GAA. We also collected plasma samples of 140 healthy kidney donors before and after donation of a kidney. Median time between kidney donation and the second plasma collection was 1.6 [1.6–1.9] months. The Institutional Review Board of the UMCG approved the study protocol (METc 2008/186) in adherence to the Declaration of Helsinki. The primary outcome measure of the study was all-cause mortality. Secondary endpoints were cardiovascular mortality and death-censored graft loss; the latter was defined as return to dialysis or re-transplantation. Participants were followed up till the end of August 2015.

Measurements

All study participants were asked to collect a 24-h urine sample on the day prior to their visit to the outpatient clinic. RTR and healthy kidney donors visited the outpatient clinic in the morning, after an overnight fasting period. A strict protocol was followed to measure both blood pressure (mmHg) and heart rate with a semi-automatic device (Dinamap® 1846, Critikon, Tampa, FL, USA) every minute for the duration of 15 min. The final value was defined as the average of the last three values. Stature and body weight were measured and were used for calculating the body mass index (BMI; weight divided by height square). The body surface area (BSA) was calculated using the universally adopted formula of Du Bois and Du Bois (1989). Venous gas analysis was determined spectrophotometrically in freshly drawn blood. Cholesterol, HbA1c, uric acid, hs-CRP, electrolytes, phosphate, glucose, albumin, urea, and creatinine were measured in plasma and urine samples by validated routine clinical chemistry laboratory methods (Frenay et al. 2015a, b). The estimated glomerular filtration rate (eGFR) was calculated using the CKD Epidemiology Collaboration (CKD-EPI creatinine-cystatin C) equation (Levey et al. 2009). Participants registered their dietary intake using a validated semi-quantitative FFQ as described elsewhere (van den Berg et al. 2013). All information on participants' medical history, medication use, and health status was obtained from patient records. Relevant transplant information was extracted from the UMCG renal transplant database. Information on smoking behavior was obtained by using a questionnaire, with classification as current, past or never smokers. Plasma hArg was quantified with a fully validated and previously in detail reported gas chromatography–mass spectrometry (GC–MS) method, which is similar to the method used to measure plasma GAA (Kayacelebi et al. 2014).

Measurement of plasma GAA

The concentration of GAA in plasma samples was quantified with a fully validated and previously published method (Hanff et al. 2016b). In brief, ultrafiltration cartridges with a cutoff of 10 kDa were used for plasma protein removal. For esterification of GAA, 10-µL aliquots of plasma ultrafiltrates were transferred into 1.5-mL glass vials and evaporated under a stream of nitrogen. Residues of entirely evaporated plasma ultrafiltrate samples were reconstituted in 100-µL aliquots of a 2 M HCl/methanol solution. The glass vials were tightly sealed and esterification was performed by heating the samples for 60 min at 80 °C. After cooling to room temperature, the samples were spiked with 10-µL aliquots of the newly synthesized trideuteromethyl ester of GAA (d_3Me-GAA) to reach a concentration of 5 µM with respect to plasma. Then solvents were evaporated to dryness under a stream of nitrogen and residues were reconstituted in 100-µL aliquots of pentafluoropropionic anhydride in ethyl acetate (1:4, v/v). The glass vials were tightly sealed and heated for 30 min at 65 °C to prepare *N*-pentafluoropropionic amides of the unlabelled methyl (Me) esters of endogenous GAA (d_0Me-GAA) and of the internal standard d_3Me-GAA. The samples were then cooled to room temperature, and solvents and reagents excess were evaporated under a stream of nitrogen. The dried residues were treated first with 200-µL aliquots of 400 mM borate buffer (pH 8.5) and immediately thereafter with 200-µL aliquots of toluene for solvent extraction. Subsequently the samples were vortex mixed for 60 s and centrifuged (4000×*g*, 5 min, 18 °C). Aliquots (150 µL) of the upper organic phase were transferred into autosampler glass vials equipped with micro inserts and the samples were sealed and subjected to GC–MS analysis on a gas chromatograph-single quadrupole mass spectrometer model ISQ from ThermoFisher (Dreieich, Germany).

Plasma donated by a healthy volunteer served as a quality control (QC) sample. QC samples were analyzed in duplicate alongside the study plasma samples within eight runs to determine the precision of the GC–MS method for GAA. The GAA concentration in the QC samples was determined to be 1.64 ± 0.064 µM, i.e., with a mean imprecision (relative standard deviation; RSD, %) of 3.9% (range 0–5.4%), supporting the validity of the GC–MS method for measuring GAA in the plasma samples of the RTR and kidney donors of the study.

Statistical analyses

Statistical data analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± standard deviation for normally distributed data, as median [interquartile range (IQR)] for non-normally distributed data, and as proportions (percentages) for nominal data. GAA/hArg data of RTR were divided in sex-stratified tertiles. Differences in GAA/hArg between kidney donors and RTR, and differences between sex-stratified tertiles were analyzed by independent *t* test and ANOVA for normally distributed continuous data, Mann–Whitney *U* test and Kruskal–Wallis test for non-normally distributed data and Chi-squared test or Fisher's exact test for nominal data as appropriate. A two-sided *P* value of less than 0.05 was considered statistically significant.

Univariable and multivariable regression models were employed to test for cross-sectional associations of $\log_2$-transformed GAA/hArg and $\log_2$-transformed GAA data with baseline variables. Backward selection was used for inclusion of parameters in the multivariable analyses that were significant in the univariable analyses ($P_{out} > 0.05$). Percentage change in beta was calculated by the formula (Oterdoom et al. 2009): [(beta after adjustment − beta before adjustment)/beta before adjustment] × 100.

Cox regression analyses were used to explore the prospective associations. Potential confounders (model 1–5) and potential mediators (model 6, 7, 8) were included in the Cox regression model construction. A stepwise process was used to avoid overfitting and to keep the number of predictors in relevant proportion to the number of events (Harrell et al. 1996). First, a crude Cox regression analysis (model 1) was performed followed by an analysis adjusting for age, gender, and BMI (model 2). Further associations were adjusted in a parallel manner for potential confounders, including eGFR (model 3), dialysis vintage, transplantation vintage, donor type, pre-emptive transplantation, use of mycophenolate mofetil (MMF) (model 4), and smoking, diabetes and NT-proBNP (model 5). Variables that may act as intermediaries in the causal path between GAA/hArg or GAA and outcome were added in a parallel manner to model 3 to evaluate their role in the association. Causal path models included HDL cholesterol, LDL cholesterol, triglycerides (model 6), venous bicarbonate and uric acid excretion (model 7), and urea excretion (model 8). Schoenfeld residuals were inspected to control for the proportionality of hazards for covariates.

Results

Cohort characteristics, plasma GAA, and GAA/hArg

RTR were included in the study after a median post-transplantation time of 5.4 [1.8-12.1] years. Baseline characteristics of the total RTR cohort and across sex-specific tertiles of GAA/hArg are summarized in Table 1. As reported previously (Frenay et al. 2015a), there were no differences between RTR and controls with regard to dietary habits.

Median plasma GAA concentration was significantly lower (by about 21%) in RTR (2.19 [1.77–2.70] µM) compared to healthy donors pre-donation (2.78 [2.89–3.35] µM) ($P < 0.001$). Healthy donors had a lower median plasma GAA concentration post-donation (2.21 [1.87–2.55] µM; $P < 0.001$ vs. pre-donation; $P = 0.23$ vs. RTR). Thus, donation of a kidney decreased the plasma GAA concentration on average by about 21%, closely approaching the baseline median plasma GAA concentration in RTR.

Spearman correlation between the plasma concentrations of GAA and hArg revealed significant correlation in RTR ($r = 0.23$, $P < 0.0001$), but not in donors before ($r = 0.02$, $P = 0.85$) and after ($r = 0.13$, $P = 0.13$) kidney donation.

In the RTR cohort, men had higher (on average by 5.4%) plasma GAA concentrations than women ($P = 0.008$), yet GAA/hArg was higher (on average by 2.3%) in women ($P = 0.006$). RTR in the highest GAA/hArg tertile were older, had a higher dialysis vintage, received kidneys more often from deceased donors, and had a higher acute rejection rate. Additionally, RTR in the highest GAA/hArg tertile had the highest levels of NT-proBNP and received antidiabetic medication more often compared to RTR in the tertile with the lowest GAA/hArg. RTR in the lowest GAA/hArg tertile had the highest BMI, triglyceride, BSA, and serum albumin levels. Uric acid, urea excretion, and eGFR were higher in the tertile with the lowest GAA/hArg. An overview of the tested associations between the $\log_2$-transformed GAA/hArg values and different parameters in univariable and multivariable regression analyses is provided in Table 2. Associations between the $\log_2$-transformed plasma GAA concentrations and various parameters in univariable and multivariable regression analyses are shown in Table S1 (see Supplement). In RTR, GAA/hArg was positively associated with age, dialysis vintage, transplant vintage, transplant rejection, antidiabetic medication, HDL cholesterol, urea, NT-pro-BNP, and vitamin K antagonist use. Inverse associations were found between the GAA/hArg ratio and BMI, BSA, donor mortality, pre-emptive renal transplantation, serum albumin, LDL cholesterol, hemoglobin, uric acid excretion, urea

Table 1 Baseline characteristics of RTR presented as sex-stratified tertiles of plasma GAA/hArg ratio

	Sex-specific tertiles of plasma GAA/hArg				
	Total cohort ($N=686$)	I 0.22–1.49 ($N=228$)	II 1.42–2.26 ($N=229$)	III 1.99–9.27 ($N=229$)	$P_{difference}$
Plasma GAA/hArg ratio	1.73 [1.31–2.30]	1.16 [0.92–1.31]	1.73 [1.59–1.88]	2.57 [2.30–3.13]	**< 0.001**
Men	1.71 [1.29–2.17]	1.15 [0.95–1.30]	1.71 [1.56–1.84]	2.41 [2.17–2.88]	**< 0.001**
Women	1.75 [1.33–2.50]	1.18 [0.90–1.33]	1.76 [1.63–2.00]	2.79 [2.50–3.63]	**< 0.001**
Plasma GAA (μM)	2.19 [1.77–2.70]	1.85 [1.52–2.26]	2.16 [1.77–2.56]	2.62 [2.17–3.18]	**< 0.001**
Men	2.23 [1.81–2.78]	1.88 [1.58–2.35]	2.20 [1.80–2.66]	2.73 [2.18–3.29]	**< 0.001**
Women	2.11 [1.70–2.55]	1.77 [1.45–2.13]	2.10 [1.75–2.46]	2.51 [2.14–3.06]	**< 0.001**
Demographics					
Age (years)	53.0 ± 12.8	50.6 ± 13.5	53.5 ± 11.8	54.8 ± 12.6	**0.002**
Male gender [*n* (%)]	391 (57)	130 (57)	131 (57)	130 (57)	1.00
BMI (kg/m^2)	26.0 [23.2–29.3]	26.7 [24.6–29.7]	26.0 [23.2–29.4]	24.9 [22.4–27.9]	**< 0.001**
BSA (m^2)	1.94 ± 0.22	1.97 ± 0.20	1.95 ± 0.22	1.91 ± 0.23	**0.028**
Smokers [*n* (%)][a]					0.79
Never	269 (39.2)	90 (41.9)	97 (43.9)	82 (39.2)	
Past	293 (42.7)	100 (46.5)	97 (43.9)	96 (45.9)	
Current	83 (12.1)	25 (11.6)	27 (12.2)	31 (14.8)	
Heart rate (bpm)	68.7 ± 12.0	67.9 ± 11.1	69.6 ± 12.3	68.7 ± 12.5	0.35
SBP (mmHg)	136 ± 17	135 ± 17	136 ± 18	137 ± 18	0.40
DBP (mmHg)	83 ± 11	83 ± 11	82 ± 10	82 ± 11	0.78
Antihypertensives [*n* (%)]	605 (88.2)	208 (91.2)	195 (84.8)	202 (88.6)	0.10
Dialysis vintage (months[a])	27 [9–51]	24 [6–51]	25 [7–49]	32 [14–57]	**0.02**
Transplant vintage (years[b])	5.4 [1.8–12.1]	5.2 [2.2–10.0]	5.2 [1.3–11.4]	5.8 [2.2–14.0]	0.14
Deceased donor [*n* (%)]	452 (65.9)	136 (59.6)	149 (64.8)	167 (73.2)	**0.01**
Pre-emptive Rtx [*n* (%)]	107 (15.6)	43 (18.9)	40 (17.4)	24 (10.5)	**0.03**
HLA mismatches (*n*)	2.0 [1.0–3.0]	2.0 [1.0–3.0]	2.0 [1.0–3.0]	2.0 [1.0–3.0]	0.83
Rejection [*n* (%)]	182 (686)	49 (21.5)	60 (26.1)	73 (32.0)	**0.04**
Glucose homeostasis					
Diabetes [*n* (%)]	163 (23.8)	44 (19.3)	64 (27.8)	55 (24.1)	0.10
Glucose (mM)	5.2 [4.8–6.0]	5.2 [4.8–5.7]	5.3 [4.8–6.2]	5.3 [4.7–6.1]	0.15
HbA_{1c} (%)	5.8 [5.5–6.2]	5.8 [5.5–6.1]	5.8 [5.5–6.3]	5.7 [5.5–6.2]	0.14
Antidiabetic medication [*n* (%)]	107 (15.6)	24 (10.5)	42 (18.3)	41 (18.0)	**0.04**
Inflammation					
hsCRP (mg/L)	1.6 [0.7–4.6]	1.5 [0.7–3.5]	1.6 [0.8–5.2]	1.7 [0.8–4.7]	0.27
Leucocytes (10E9/L)	8.1 ± 2.6	8.2 ± 2.5	8.2 ± 2.7	8.0 ± 2.7	0.57
Serum albumin (g/L)	43.0 ± 3.0	43.5 ± 2.8	42.8 ± 3.0	42.7 ± 3.0	**0.01**
Cardiovascular and endocrinology					
Total cholesterol (mM)	5.12 ± 1.11	5.08 ± 1.12	5.19 ± 1.05	5.10 ± 1.18	0.53
HDL-cholesterol (mM)	1.3 [1.1–1.6]	1.3 [1.0–1.6]	1.3 [1.1–1.6]	1.3 [1.1–1.7]	**0.01**
LDL-cholesterol (mM)	2.98 ± 0.92	3.01 ± 0.93	3.02 ± 0.89	2.90 ± 0.95	0.31
Triglycerides (mM)	1.68 [1.25–2.30]	1.73 [1.26–2.28]	1.72 [1.30–2.41]	1.52 [1.13–2.18]	**0.02**
Statins [*n* (%)]	364 (53.1)	126 (55.3)	120 (52.2)	118 (51.8)	0.71
Hemoglobin (mM)	8.22 ± 1.08	8.31 ± 1.14	8.26 ± 1.03	8.09 ± 1.04	0.07
Corrected calcium (mM)[d]	2.34 ± 0.15	2.34 ± 0.16	2.35 ± 0.14	2.34 ± 0.15	0.86
Phosphate (mM)	0.97 ± 0.21	0.95 ± 0.21	0.96 ± 0.22	0.99 ± 0.21	0.14
Magnesium (mM)	0.95 ± 0.12	0.95 ± 0.12	0.95 ± 0.13	0.96 ± 0.11	0.81
PTH (pM)	9.01 [5.94–14.76]	9.65 [6.05–14.37]	8.68 [5.43–15.27]	8.89 [6.22–15.00]	0.67
Venous pH	7.37 ± 0.04	7.37 ± 0.04	7.37 ± 0.04	7.37 ± 0.04	0.48
Venous HCO_3^- (mM)	24.6 ± 3.1	24.5 ± 3.1	24.5 ± 3.1	24.8 ± 3.0	0.37

Table 1 (continued)

	Sex-specific tertiles of plasma GAA/hArg				
	Total cohort ($N=686$)	I 0.22–1.49 ($N=228$)	II 1.42–2.26 ($N=229$)	III 1.99–9.27 ($N=229$)	$P_{difference}$
Uric acid (mM)	0.43 ± 0.11	0.43 ± 0.11	0.43 ± 0.12	0.44 ± 0.11	0.82
Urea (mM)	9.5 [7.2–13.3]	9.7 [7.2–12.6]	9.4 [7.3–12.9]	9.7 [7.2–14.2]	0.82
NT-pro-BNP (ng/L)	249 [107–632]	164 [78–388]	289 [121–655]	361 [121–779]	**< 0.001**
Uric acid excretion (mmol/24 h	2.61 ± 0.99	2.84 ± 0.90	2.66 ± 1.04	2.31 ± 0.97	**< 0.001**
Urea excretion (mmol/24 h)	388 ± 115	417 ± 111	387 ± 117	360 ± 109	**< 0.001**
Vitamin K antagonists [*n* (%)]	77 (11.2)	21 (9.2)	23 (10.0)	33 (14.5)	0.16
Renal function					
Serum creatinine (µM)	125 [99–162]	122 [98–154]	122 [101–164]	131 [99–168]	0.43
eGFR (mL/min/1.73 m^2)	45.1 ± 18.8	47.7 ± 18.8	44.5 ± 18.5	42.9 ± 18.9	**0.02**
Albuminuria (mg/24 h)	39.0 [10.6–176.6]	36.6 [9.9–193.9]	36.5 [9.9–182.9]	47.8 [11.0–168.7]	0.78
Liver parameters					
LDH (U/L)	207 ± 55	209 ± 52	207 ± 57	206 ± 55	0.90
Alkaline phosphatase (U/L)	67 [54–83]	68 [53–82]	68 [55–84]	66 [53–84]	0.85
Dietary supplementation					
Calcium [*n* (%)]	147 (21.4)	40 (17.5)	54 (23.5)	53 (23.2)	0.22
Vitamin D [*n* (%)]	168 (24.5)	48 (21.2)	58 (25.2)	62 (27.2)	0.30
Immunosuppression					
CNI [*n* (%)]	391 (57.0)	128 (56.1)	138 (60.0)	125 (54.8)	0.51
Proliferation inhibitor [*n* (%)]					
MMF	451 (65.7)	164 (71.9)	155 (67.4)	132 (57.9)	**0.01**
Everolimus	7 (1.0)	3 (1.3)	1 (0.4)	3 (1.3)	0.58
Sirolimus	13 (1.9)	5 (2.2)	3 (1.3)	5 (2.2)	0.74
Prednisolone (mg/day)	10.0 [7.5 – 10.0]	10.0 [7.5–10.0]	10.0 [7.5–10.0]	10.0 [7.5–10.0]	0.06

Data are presented as mean ± SD for normally distributed variables, as median [IQR] for non-normally distributed variables or as number (percentage) for categorical variables

Bold values indicate statistical significance ($P<0.05$)

BMI body mass index, *BSA* body surface area, *CNI* calcineurin inhibitor, *DBP* diastolic blood pressure, *eGFR* estimated glomerular filtration rate, *HbA1c* glycated hemoglobin, *HDL* high-density lipoprotein, *HLA* human leukocyte antigen, *hsCRP* high-sensitivity C-reactive protein, *LDH* lactate dehydrogenase, *LDL* low-density lipoprotein, *MMF* mycophenolate mofetil, *NT-pro-BNP* N-terminal prohormone of brain natriuretic peptide, *PTH* parathyroid hormone, *RTx* renal transplantation, *SBP* systolic blood pressure

[a]Percentages do not add up to 100% because of missing cases

[b]Total dialysis time (also of previous dialysis episodes of previous transplantations)

[c]Time between transplantation and baseline visit

[d][serum calcium (mM)] + (0.02 × (40 – [blood albumin (g/L)]))

excretion, eGFR, and MMF medication use. Adjustments for eGFR increased the beta values of the association of GAA/hArg with transplant vintage, HDL cholesterol, and LDL cholesterol by 11%, 23%, and 16%, respectively. Adjustments for eGFR also lowered the standardized beta values of the association of GAA/hArg with previous rejection, pre-emptive renal transplantation, uric acid excretion, and MMF medication by 20%, 11%, 5%, and 18%, respectively. The same adjustment revealed a positive association with venous bicarbonate ($\beta=0.02$ mM, $P=0.02$) and an inverse association with triglycerides ($\beta=-0.06$ mM, $P=0.03$). The associations between GAA/hArg and hemoglobin, urea, vitamin K antagonist use, serum creatinine or serum albumin were lost by eGFR adjustment. However, additional multivariable adjustment using backward selection, which included all parameters that were significantly associated with GAA/hArg in the eGFR-corrected model, lowered the beta value of uric acid excretion, antidiabetic medication use, HDL cholesterol use, and MMF medication by 56%, 12%, 33%, and 57%, respectively. Plasma GAA was strongly and positively associated with gender, transplant rejection, hemoglobin, phosphate, magnesium, venous pH, venous bicarbonate, and eGFR. Inverse associations were found between

Table 2 Associations of log_2-transformed plasma GAA/hArg ratio with clinical parameters in RTR

	Log_2-transformed plasma GAA/hArg					
	Univariable		eGFR-corrected		Multivariable	
	β	*P* value	β	*P* value	β	*P* value
Demographics						
Age (years)	0.01	**< 0.001**	0.01	**< 0.001**	0.004	0.06
Male gender [*n* (%)]	– 0.10	0.06	– 0.10	0.06		
BMI (kg/m^2)	– 0.02	**< 0.001**	– 0.02	**< 0.001**	– 0.02	**0.01**
BSA (m^2)	– 0.46	**< 0.001**	– 0.50	**< 0.001**	0.10	0.57
Smokers [*n* (%)]						
Never	Ref.		Ref.			
Past	0.03	0.63	0.02	0.69		
Current	0.05	0.57	0.04	0.67		
Heart rate (bpm)	0.003	0.19	0.003	0.17		
SBP (mmHg)	0.002	0.13	0.001	0.31		
DBP (mmHg)	– 0.001	0.54	– 0.002	0.40		
Antihypertensives [*n* (%)]	– 0.06	0.44	– 0.12	0.13		
Dialysis vintage (months)	0.002	**0.01**	0.002	**0.02**	0.001	0.38
Transplant vintage (years)	0.009	**0.01**	0.01	**0.004**	– 0.001	0.81
Deceased donor [*n* (%)]	– 0.016	**0.002**	– 0.14	**0.01**	– 0.004	0.95
Pre-emptive Rtx [*n* (%)]	– 0.19	**0.01**	– 0.17	**0.02**	– 0.10	0.21
HLA mismatches, *n*	– 0.01	0.50	– 0.01	0.41		
Rejection [*n* (%)]	0.15	**0.01**	0.12	**0.03**	0.12	0.05
Glucose homeostasis						
Diabetes [*n* (%)]	0.11	0.06	0.10	0.08		
Glucose (mM	0.02	0.17	0.02	0.12		
HbA_{1c} (%)	0.02	0.54	0.02	0.50		
Antidiabetic medication [*n* (%)]	0.18	**0.01**	0.17	**0.02**	0.15	**0.04**
Inflammation						
hsCRP (mg/L)	– 0.0004	0.88	– 0.002	0.52		
Leucocytes (10E9/L)	– 0.01	0.23	– 0.01	0.22		
Serum albumin (g/L)	– 0.02	**0.02**	– 0.01	0.17		
hsCRP (mg/L)	– 0.0004	0.88	– 0.002	0.52		
Cardiovascular and endocrinology						
Total cholesterol (mM)	0.003	0.89	– 0.01	0.66		
HDL-cholesterol (mM)	0.22	**< 0.001**	0.27	**< 0.001**	0.18	**0.004**
LDL-cholesterol (mM)	– 0.06	**0.04**	– 0.07	**0.02**	– 0.05	0.06
Triglycerides (mM)	– 0.03	0.25	– 0.06	**0.03**	– 0.02	0.46
Statins [*n* (%)]	– 0.01	0.90	– 0.01	0.79		
Hemoglobin (mM)	– 0.05	**0.02**	– 0.02	0.45		
Corrected calcium (mM)	0.13	0.45	0.10	0.56		
Phosphate (mM)	0.34	0.01	0.21	0.10		
Magnesium (mM)	0.30	0.14	0.28	0.18		
PTH (pM)	– 0.001	0.40	– 0.001	0.23		
Venous pH	– 0.17	0.80	0.48	0.48		
Venous HCO_3^- (mM)	0.01	0.24	0.02	**0.02**	0.01	0.15
Uric acid (mM)	0.18	0.41	– 0.43	0.11		
Urea (mM)	0.01	**0.03**	– 0.01	0.20		
Nt-pro-BNP (ng/L)	< 0.001	**0.001**	< 0.001	**0.01**	< 0.001	0.10
Log-NT-pro-BNP						
Uric acid excretion (mmol/24 h)	– 0.19	**< 0.001**	– 0.18	**< 0.001**	– 0.08	**0.04**
Urea excretion (mmol/24 h)	– 0.001	**< 0.001**	– 0.001	**< 0.001**	– 0.001	**0.01**

Table 2 (continued)

	Log_2-transformed plasma GAA/hArg					
	Univariable		eGFR-corrected		Multivariable	
	β	*P* value	β	*P* value	β	*P* value
Vitamin K antagonists [*n* (%)]	0.17	**0.04**	0.14	0.09		
Renal function						
Serum creatinine (µM)	0.001	**0.03**	− 0.001	0.23		
eGFR (mL/min/1.73 m^2)	− 0.01	**< 0.001**	N/A		− 0.004	**0.01**
Albuminuria (mg/24 h)	< 0.001	0.87	− 0.001	0.47		
Liver parameters						
LDH (U/L)	< 0.001	0.32	< 0.001	0.91		
Alkaline phosphatase (U/L)	< 0.001	0.61	< 0.001	0.88		
Dietary supplementation						
Calcium [*n* (%)]	0.10	0.11	0.08	0.19		
Vitamin D [*n* (%)]	0.08	0.18	0.05	0.44		
Immunosuppression						
CNI [*n* (%)]	0.001	0.99	− 0.05	0.35		
Proliferation inhibitor [*n* (%)]	− 0.07	0.26	− 0.06	0.40		
MM [*n* (%)]	− 0.17	**0.001**	− 0.14	**0.01**	− 0.06	0.39
Prednisolone (mg/day)	0.01	0.57	0.01	0.58		
Sirolimus [*n* (%)]	− 0.01	0.97	0.01	0.97		

Multivariable model is created with significant variables from the eGFR-corrected model and adjustment for eGFR. Regression coefficients are given as betas for the log_2-transformed GAA/hArg ratio, showing the effect per doubling of the GAA/hArg ratio. Proliferation inhibitors include azathioprine and mycophenolate medication. CNIs include cyclosporine and tacrolimus medication

Bold values indicate statistical significance ($P < 0.05$)

BMI body mass index, *BSA* body surface area, *CNI* calcineurin inhibitor, *DBP* diastolic blood pressure, *eGFR* estimated glomerular filtration rate, *HbA1c* glycated hemoglobin, *HDL* high-density lipoprotein, *HLA* human leukocyte antigen, *hsCRP* high-sensitivity C-reactive protein, *LDH* lactate dehydrogenase, *LDL* low-density lipoprotein, *MMF* mycophenolate mofetil, *NT-pro-BNP* N-terminal prohormone of brain natriuretic peptide, *PTH* parathyroid hormone, *RTx* renal transplantation, *SBP* systolic blood pressure

plasma GAA and hs-CRP, uric acid excretion, alkaline phosphatase, and MMF medication use (Table S1).

Association of plasma GAA concentration and GAA/hArg with mortality and death-censored graft loss

In the RTR cohort, 146 out of 686 (21%) RTR died within a median follow-up period of 5.4 [4.9–6.1] years, of whom 58 (8%) died due to cardiovascular disease. Death-censored graft loss was experienced by 82 (12%) RTR. Figures 2 and 3 show crude sex-stratified Kaplan–Meier survival curves of GAA/hArg tertiles and the plasma GAA concentration, respectively, for all-cause mortality, cardiovascular mortality, and death-censored graft loss. Statistically significant differences were found for GAA/hArg tertiles in RTR with all-cause mortality (Fig. 2a) (log-rank test, $P < 0.001$) and cardiovascular mortality (Fig. 2b) (log-rank test $P < 0.02$), but not for tertiles of RTR with death-censored graft loss (Fig. 2c). In contrast, sex-stratified Kaplan–Meier analyses for tertiles of plasma GAA concentration with all-cause mortality (Fig. 3a) and cardiovascular mortality (Fig. 3b) did not result in statistically significant differences and revealed non-proportional hazards. A statistically significant difference was observed for GAA and death-censored graft loss in crude analysis (log-rank test, $P < 0.001$) (Fig. 3c). Cox regression analysis was also performed to assess the associations between plasma GAA and mortality as well as death-censored graft loss. Cox model regression analysis for the association of plasma GAA with all-cause (Table S2A) and cardiovascular mortality (Table S2B) did not reach statistical significance, but plasma GAA was significantly associated with death-censored graft loss (HR: 0.55 [95% CI 0.39–0.77];$P = 0.001$) (Table S2C) in the crude Cox regression model (model 1). The fact that plasma GAA is not associated with all-cause and cardiovascular mortality supports the notion that the association of the GAA/hArg ratio is mainly driven by hArg. Adjustment for age, gender, and BMI showed no change for the association of plasma GAA with death-censored graft loss (model 2). After eGFR adjustment, the association of plasma GAA with death-censored graft loss became borderline significant (HR: 0.78 [95% CI 0.58–1.04]; $P = 0.09$). Significance was lost for the

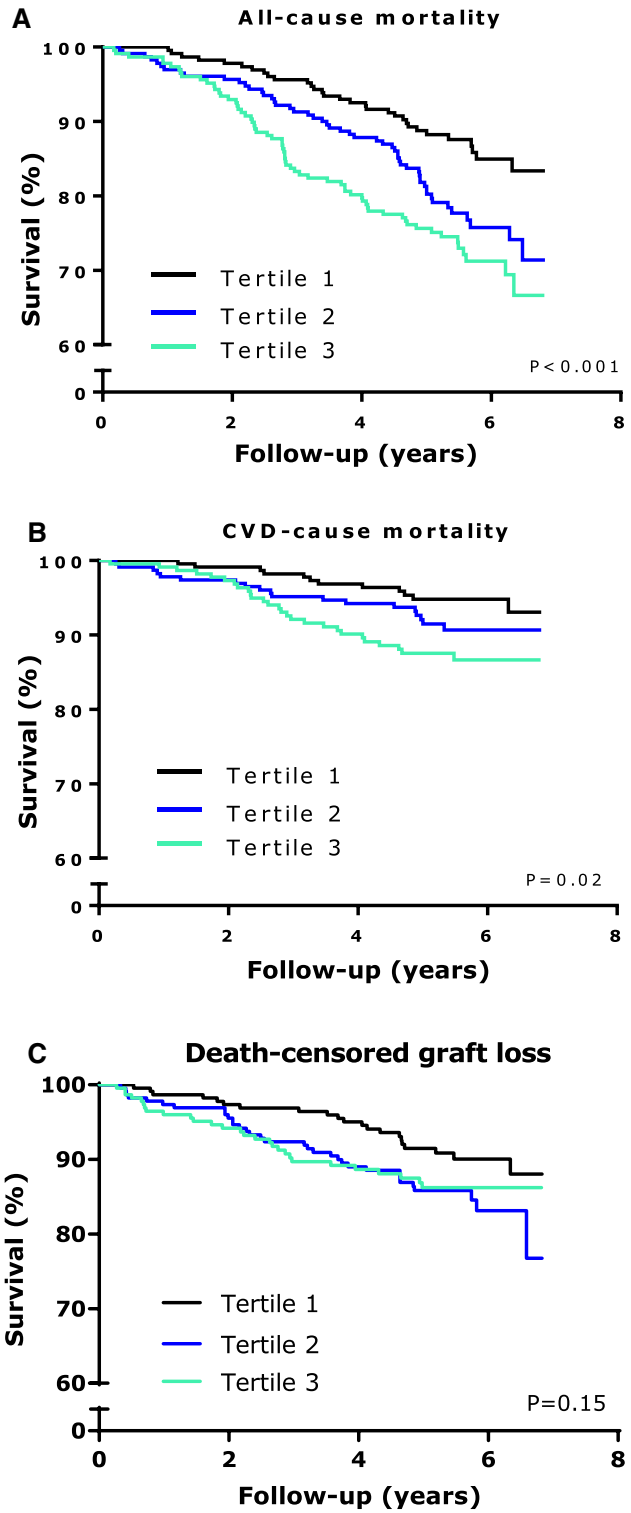

Fig. 2 Kaplan–Meier analyses of the association of sex-stratified plasma GAA/hArg ratio tertiles with **a** all-cause mortality (log-rank test $P<0.001$), **b** cardiovascular mortality (log-rank test $P=0.02$), and **c** death-censored graft loss (log-rank test $P=0.15$). Median follow-up period was 5.4 [4.9–6.1] years

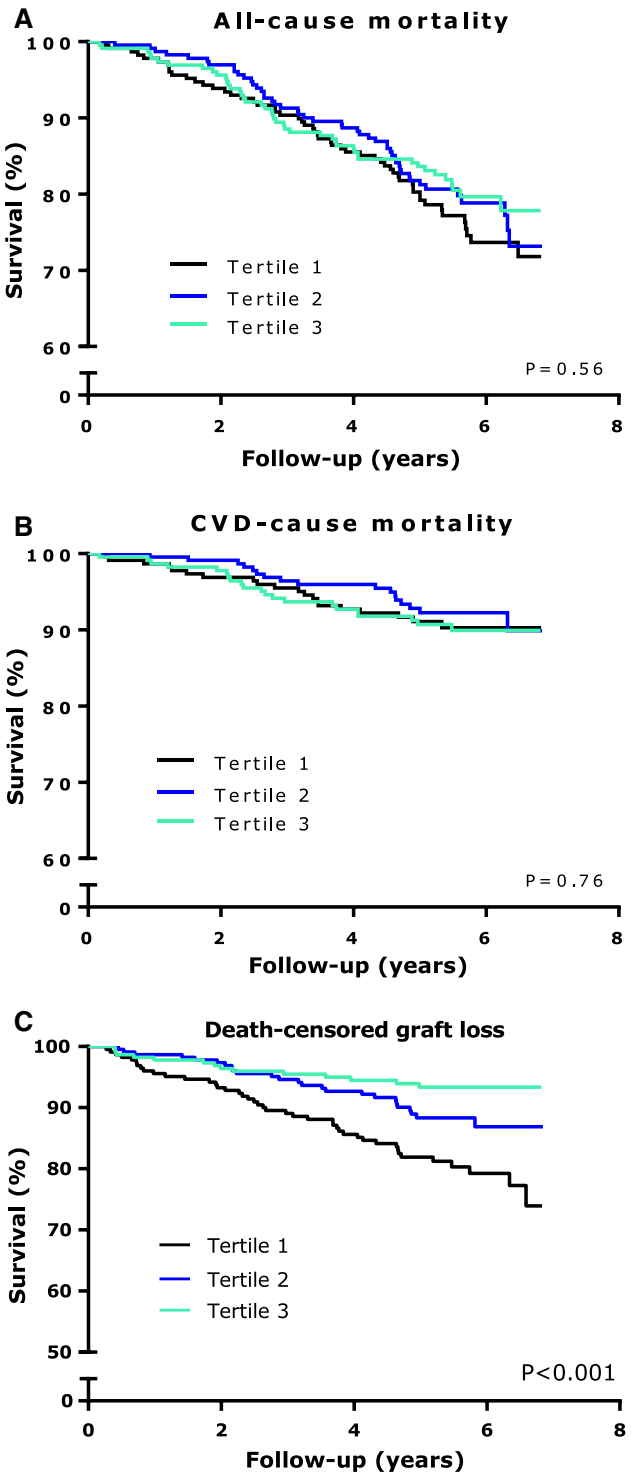

Fig. 3 Kaplan–Meier analyses of the association of sex-stratified plasma GAA tertiles with **a** all-cause mortality (log-rank test $P=0.056$), **b** cardiovascular mortality (log-rank test $P=0.76$), and **c** death-censored graft loss (log-rank test $P<0.001$). Median follow-up period was 5.4 [4.9–6.1] years

Table 3 Associations of plasma GAA/hArg ratio with risk for (a) all-cause mortality, (b) cardiovascular mortality, and (c) death-censored graft loss, adjusted for confounders (models 1–5) and causal path analyses (models 6–8) in the RTR cohort

	Tertiles of plasma GAA/hArg					Continuous plasma GAA/hArg	
	I ($N=228$)		II ($N=231$)		III ($N=228$)	Continuous ($N=687$)	
	HR [95% CI]	*P* value	HR [95% CI]	*P* value	HR (ref.)	HR [95% CI]	*P* value
(a)							
Model							
1	0.48 [0.31–0.74]	0.001	0.88 [0.61–1.27]	0.48	1.00	1.35 [1.19-1.53]	< 0.001
2	0.54 [0.35–0.84]	0.01	0.89 [0.61–1.30]	0.55	1.00	1.36 [1.18–1.56]	< 0.001
3	0.60 [0.39–0.94]	0.02	0.94 [0.64–1.37]	0.74	1.00	1.24 [1.08–1.43]	0.002
4	0.61 [0.39–0.96]	0.03	1.03 [0.70–1.52]	0.87	1.00	1.25 [1.08–1.45]	0.003
5	0.56 [0.34–0.92]	0.02	0.88 [0.58–1.32]	0.53	1.00	1.32 [1.12–1.56]	< 0.001
6	0.51 [0.32–0.79]	0.003	0.88 [0.60–1.28]	0.49	1.00	1.29 [1.12–1.48]	< 0.001
7	0.66 [0.41–1.06]	0.08	1.07 [0.72–1.60]	0.73	1.00	1.18 [1.02–1.36]	0.02
8	0.70 [0.45–1.10]	0.12	1.09 [0.74–1.60]	0.66	1.00	1.17 [1.01–1.36]	0.04
(b)							
Model							
1	0.43 [0.22–0.85]	0.02	0.75 [0.42–1.35]	0.34	1.00	1.46 [1.24–1.73]	< 0.001
2	0.42 [0.21–0.85]	0.02	0.70 [0.39–1.27]	0.24	1.00	1.58 [1.31–1.91]	< 0.001
3	0.46 [0.23–0.94]	0.03	0.74 [0.41–1.34]	0.32	1.00	1.44 [1.19–1.75]	< 0.001
4	0.47 [0.23–0.95]	0.04	0.81 [0.44–1.48]	0.49	1.00	1.49 [1.21–1.82]	< 0.001
5	0.49 [0.23–1.08]	0.08	0.65 [0.34–1.24]	0.19	1.00	1.50 [1.18–1.90]	0.001
6	0.39 [0.19–0.78]	0.01	0.69 [0.38–1.24]	0.21	1.00	1.50 [1.24–1.82]	< 0.001
7	0.46 [0.22–0.95]	0.04	0.73 [0.39–1.36]	0.32	1.00	1.38 [1.14–1.66]	0.001
8	0.51 [0.25–1.04]	0.07	0.82 [0.45–1.50]	0.52	1.00	1.39 [1.14–1.69]	0.001
(c)							
Model							
1	0.71 [0.41–1.23]	0.22	0.99 [0.60–1.66]	0.98	1.00	1.16 [0.95–1.42]	0.14
2	0.65 [0.37–1.14]	0.13	0.98 [0.58–1.64]	0.94	1.00	1.20 [0.99–1.47]	0.07
3	0.86 [0.49–1.52]	0.61	1.20 [0.71–2.03]	0.49	1.00	0.98 [0.81–1.18]	0.80
4	0.94 [0.53–1.68]	0.84	1.15 [0.67–1.97]	0.61	1.00	0.95 [0.78–1.16]	0.63
5	0.74 [0.41–1.36]	0.33	0.92 [0.53–1.61]	0.78	1.00	1.13 [0.90–1.40]	0.29
6	0.80 [0.45–1.44]	0.46	1.17 [0.69–1.98]	0.57	1.00	0.99 [0.82–1.20]	0.94
7	0.65 [0.35–1.19]	0.16	1.06 [0.62–1.84]	0.82	1.00	1.05 [0.87–1.26]	0.62
8	0.93 [0.52–1.66]	0.80	1.23 [0.73–2.08]	0.44	1.00	0.96 [0.79–1.16]	0.65

Model 1: crude

Model 2: adjusted for age, gender, and BMI

Model 3: as model 2, additionally adjusted for eGFR

Model 4: as model 3, additionally adjusted for dialysis vintage, transplantation vintage, donor type, pre-emptive transplantation, and MMF use

Model 5: as model 3, additionally adjusted for smoking, diabetes, and NT-proBNP

Model 6: as model 3, additionally adjusted for HDL cholesterol, LDL cholesterol, triglycerides

Model 7: as model 3, additionally adjusted for venous HCO_3^-, uric acid excretion

Model 8: as model 3, additionally adjusted for urea excretion

association of plasma GAA with death-censored graft loss (HR: 0.88 [95% CI 0.63–1.21]; $P=0.121$) when adjusted for smoking, diabetes, and NT-proBNP (model 5).

Potential confounders/mediators of GAA/hArg identified in the multivariable regression analyses were used to perform Cox regression analyses (Table 3a–c). The crude Cox regression model showed that GAA/hArg is associated with higher risk for all-cause mortality (HR 1.35: [95% CI 1.19–1.53]) and cardiovascular mortality (HR: 1.46 [95% CI 1.24–1.73]). Additional adjustments showed that the association of GAA/hArg with all-cause and cardiovascular mortality was independent of age, gender, and BMI (model 2), eGFR (model 3), dialysis vintage, transplantation vintage, donor type, pre-emptive transplantation, use of MMF (model

4), and smoking, diabetes, NT-proBNP (model 5), respectively. Causal path analysis showed no material change for the association of GAA/hArg with all-cause mortality and cardiovascular mortality when adjusted for HDL cholesterol, LDL cholesterol, and triglycerides (model 6). The association of GAA/hArg with all-cause and cardiovascular mortality remained significant when adjusted for venous bicarbonate and uric acid excretion (model 7) and urea excretion (model 8), but resulted in lower HR. After urea adjustment (model 8), significance was lost for the association with all-cause (HR: 0.70 [95% CI 0.45–1.10]; $P = 0.12$) and cardiovascular mortality (HR: 0.51 [95% CI 0.25–1.04]; $P = 0.07$) in the first sex-stratified tertile.

Cox model regression analysis for the association of GAA/hArg with death-censored graft loss did not reach statistical significance (Table 3c).

Discussion

In the present study, we found lower (on average by 21%) plasma GAA concentrations in RTR compared to healthy kidney donors before donation. In the same cohort, we previously found that RTR had also lower (on average by 22%) hArg plasma concentrations compared with the same kidney donors prior to donation (Kayacelebi et al. 2017). In that work, we observed significant associations between high plasma hArg concentration and reduced all-cause mortality. Furthermore, we found that high plasma hArg concentration is associated with reduced death-censored graft loss. The results of the present study show that low GAA/hArg is associated with reduced risk of all-cause and cardiovascular mortality, but it is not associated with death-censored graft loss in stable RTR. The absence of an association of low GAA plasma concentrations with all-cause and cardiovascular mortality suggests that the association of the GAA/hArg ratio is mainly driven by hArg.

hArg, the methylene (CH_2) homologue of Arg, is biosynthesized by the transamidination of Lys by Arg. This reaction is catalyzed by the mitochondrial AGAT mainly in the kidney (Fig. 1). AGAT also catalyzes the transamidination of glycine (Gly) by Arg to form guanidinoacetate (GAA), thereby releasing L-ornithine (Orn) (Fig. 1). GAA is further converted to creatine by GAMT, which is mainly expressed in the liver. The AGAT-catalyzed synthesis of GAA is considered the rate-limiting step of creatine biosynthesis. Both ADMA and hArg emerged as cardiovascular risk factors (Hanff et al. 2018; Faller et al. 2018; Valtonen et al. 2008). Yet, the underlying mechanisms are still unexplored (Tsikas et al. 2018). The importance of circulating GAA in adult RTR has not been reported thus far. As hArg and GAA are Arg metabolites produced by the catalytic action of the same enzyme, i.e., AGAT (Fig. 1), we assumed that the concentration of GAA in blood and its relation to circulating hArg, i.e., the GAA/hArg molar ratio, may be suitable parameters to assess the relative contribution of the renal AGAT activity to GAA and hArg in humans, and may also be risk factors in the renal and cardiovascular systems. Interestingly lower plasma concentrations of GAA and hArg are associated with death-censored graft loss before adjustment for eGFR, but the molar GAA/hArg ratio is not associated with death-censored graft loss. This indicates a link between functional kidney mass and AGAT activity (Post et al. 2019). A possible mechanism could be the decline of proximal tubules cells, which are the main site of AGAT expression, by progression of tubular atrophy throughout the stages of CKD, especially in patients with renal transplants (Schelling 2016). Tubular atrophy is also a strong predictor of GFR decline in CKD patients (Issa et al. 2019) and could explain the strong association of eGFR with GAA and hArg. GAA is also the precursor of endogenously synthesized creatinine, which could also contribute to the strong association between eGFR and GAA.

Another important physiological distinction between GAA and hArg refers to their excretion in the urine. In humans, hArg is mainly excreted without prior metabolization (Frenay et al. 2015b). Only a small part of circulating hArg is metabolized by AGXT2 to form 6-guanidino-2-oxocaproic acid (Martens-Lobenhoffer et al. 2018; Rodionov et al. 2016). The urinary excretion of GAA is much higher than that of hArg. As a result, the GAA/hArg molar ratio is about 1.3 in the serum and 250 in the urine of healthy adults (Hanff et al. 2018). These data suggest that the kidneys reabsorb hArg from the primary urine and facilitate excretion of GAA into the urine. The direct association of higher circulating GAA/hArg with higher all-cause and cardiovascular mortality in RTR suggests that effects secondary to renal AGAT activity may contribute to these associations, such as changes in the metabolism/catabolism of GAA and hArg (Hanff et al. 2016a; Atzler et al. 2016, 2017). This is supported by studies on AGAT-deficient mice showing that a fully functional creatine biosynthesis is not absolutely required for normal cardiac function (Faller et al. 2018). However, myocardial AGAT expression is still upregulated in heart failure patients (Cullen et al. 2006). Consequently, a shift of the GAA-hArg homeostasis in favor of hArg, for instance by hArg supplementation, may be considered as beneficial.

The lower circulating GAA and hArg concentrations measured in the RTR of our study in comparison to those in the kidney donors before kidney donation are likely to result from a decreased activity of AGAT in the kidneys of the RTR. As GAA and hArg undergo distinctly different subsequent metabolism (Fig. 1), downstream processes primarily in extra-renal organs such as the liver and the heart may also influence the concentration of GAA and hArg in the blood.

The metabolic fate of GAA is quite well understood and includes rapid conversion of GAA to creatine which is further converted to creatine phosphate and finally to creatinine (Fig. 1). On the other hand, the metabolic pathways of hArg are poorly investigated thus far. hArg can be hydrolyzed by arginases to Lys (Ryan et al. 1968; Bollenbach et al. 2019), which can be reused in AGAT-catalyzed synthesis of hArg (Fig. 1). GAA/hArg is directly associated with plasma urea concentration ($\beta = 0.01$ mM, $P = 0.03$) which could in part result from the hydrolysis of hArg to urea and Lys by arginase. The extent of hArg contribution to plasma urea and Lys is unknown, but is considered rather low (Bollenbach et al. 2019). This is supported by the beta value of 0.01 mM (10 µM) which is of the same order of magnitude of circulating hArg (Table 2). It is important to note that uric acid excretion is mainly used as a marker for protein intake. Additionally, arginase 1 overexpression induced by an arginase 1 gene polymorphism is associated with increased intima-media thickness, which is a marker for myocardial infarction risk (Dumont et al. 2007). Low circulating hArg and therefore a higher molar GAA/hArg ratio could indicate increased extra-renal arginase activity.

It is interesting to note that the plasma concentrations of GAA and hArg correlated in the RTR ($r = 0.226$, $P < 0.0001$), but not in the donors before kidney donation ($r = 0.02$, $P = 0.85$), while donation of a kidney increased the correlation between the plasma concentrations of GAA and hArg in the donors, albeit just failing statistical significance ($r = 0.13$, $P = 0.13$). In healthy young men, we also did not observe a correlation between plasma GAA and hArg (Hanff et al. 2016b). These observations may suggest that the circulating GAA and hArg concentrations in humans with normal kidney function do not exclusively reflect the AGAT activity in the kidneys, but also the activity of AGAT in other organs. In the kidney donors, donation of a kidney did not halve the plasma concentration of GAA (present study) and hArg (Frenay et al. 2015b; Kayacelebi et al. 2017). This observation suggests that the remaining second kidney of the donors tries to complete the functional gap left by the kidney donation, yet to a degree of about 70% with respect to its filtration function 12 months after kidney donation (Altmann et al. 2017). It may also be expected that other organs in which AGAT is less abundantly expressed compared to the kidney, such as the liver, may also contribute to the circulating GAA/hArg. Studies in humans reporting on the contribution of individual organs to GAA and hArg are very scarce and are limited to protein and gene expression (Cullen et al. 2006). Our study suggests that RTR and kidney donors have mildly reduced kidney function after kidney transplantation and donation, respectively. Kidney donation is life saving for the RTR and life compromising to the donors. Deterioration of the kidney's function due to disease or due to loss of a kidney, for instance by donation such as in the present study, emphasize the contribution of renal AGAT to the circulating GAA and hArg.

Investigations on the effect of immunosuppressive regiments on the GAA-hArg homeostasis are scarce. An inverse association was shown between MMF and circulating hArg in a large cohort of CKD patients (Maas et al. 2018), and circulating concentrations of GAA and hArg were lower in pediatric kidney transplant patients who received MMF compared to everolimus (Hanff et al. 2019). In the present study, an inverse association between the use of MMF and circulating GAA/hArg was found, supporting findings of MMF improving cardiovascular risk profiles (Morales and Dominguez-Gil 2006) and possibly modulating atherosclerosis (van Leuven et al. 2006). Yet, further studies are needed to elucidate the underlying mechanism(s) of GAA-hArg homeostasis modulation by MMF.

Being an observational study, we acknowledge the limitation that causality cannot be proven and the possibility of residual confounding remains. Also, we did not measure and determine all known metabolites of hArg and GAA, which may have contributed to the observed associations by potentially interfering with the activity of AGAT, arginase, and other enzymes, and by transporters of relative amino acids and creatine. Eventually, our study included predominantly Caucasians, who might not be representative for other ethnicities.

In conclusion, we found that higher plasma GAA/hArg molar ratios are associated with higher risks for all-cause and cardiovascular mortality, suggesting that the GAA-hArg homeostasis is crucially important both for the kidneys and the heart. The fact that plasma GAA is not associated with all-cause and cardiovascular mortality supports the notion that the association of the GAA/hArg ratio is mainly driven by hArg. Our study suggests that in the kidneys of the RTR, the AGAT-catalyzed biosynthesis of GAA and hArg is equally decreased, apparently to the same extent seen in the kidney donors. The decrease in the AGAT-catalyzed synthesis of hArg in the kidneys of the RTR seems to be much more important than that of GAA. It could, therefore, be assumed that supplementation of hArg to RTR or kidney donors would provide stronger protection to the renal and cardiovascular systems than supplementation of GAA.

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Compliance with ethical standards

Conflict of interest All authors report no conflict of interest.

Ethical approval The Institutional Review Board approved the study protocol (METc 2008/186) which was in adherence to the Declaration of Helsinki.

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