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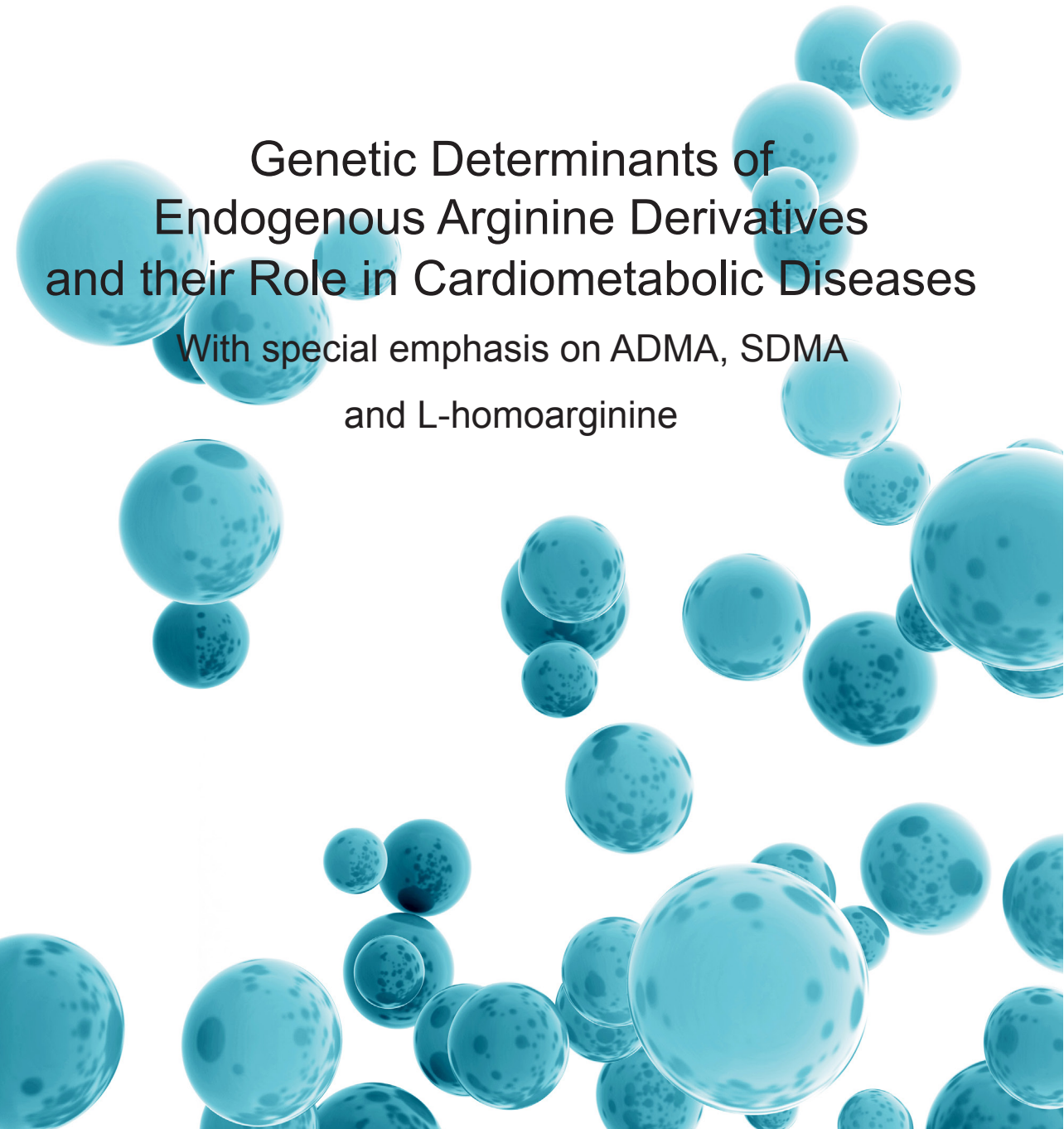
Genetic Determinants of Endogenous Arginine Derivatives and their Role in Cardiometabolic Diseases

With special emphasis on ADMA, SDMA and L-homoarginine



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ACADEMIC DISSERTATION
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of the University of Tampere,
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UNIVERSITY OF TAMPERE

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University of Tampere, Faculty of Medicine and Life Sciences
Pirkanmaa Hospital District, Department of Clinical Chemistry
Fimlab Laboratories
Finnish Cardiovascular Research Center
Finland

Supervised by
Professor Terho Lehtimäki
University of Tampere
Finland

Reviewed by
Docent Timo Hiltunen
University of Helsinki
Finland
Docent Kari Kervinen
University of Oulu
Finland

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ABSTRACT

Background: Cardiometabolic diseases are a significant burden to healthcare and one of the leading causes of morbidity and death across the world. A prominent feature of cardiometabolic diseases is endothelial dysfunction and subsequent diminished bioavailability of vasoactive molecule nitric oxide (NO). Asymmetric and symmetric dimethylarginines (ADMA and SDMA) reduce NO bioavailability and thus contribute to the loss of the protective effects of NO (e.g. antithrombotic and anti-inflammatory) in the vasculature, leading to cardiometabolic complications. High ADMA and SDMA and low L-homoarginine (hArg), a naturally occurring non-proteinogenic amino acid, predict increased risk of cardiovascular and overall mortality although their genetic determinants and the underlying molecular mechanisms are not completely understood.

Aims of the study: 1) identify novel genetic determinants of serum ADMA, SDMA and hArg by genome-wide association study (GWAS); **2)** investigate associations of these newly identified genetic variants with cardiometabolic diseases and end points; and **3)** characterise the biomarker and causal roles of serum hArg in the development of cardiometabolic diseases.

Materials and methods: In studies I and II, GWASs were conducted by using the Cardiovascular Risk in Young Finns Study (YFS, N=2,083) and the Ludwigshafen Risk and Cardiovascular Health (LURIC, N=3,027) study materials. In study III, genetic associations with atrial fibrillation were studied in LURIC, in the Finnish Cardiovascular Study (FINCAVAS, N=3,862) and in the Corogene study. Associations with ischemic stroke were studied in the Wellcome Trust Case Control Concortium 2 (WTCCC2) GWAS cohorts (3,548 cases and 5,972 controls) and in FINCAVAS. In study IV, observational association analyses between hArg and cardiometabolic risk factors and diseases were investigated in YFS. The causal effect estimates between hArg and cardiometabolic risk factors and diseases were calculated using association summary statistics for SNP-outcome associations available in the public domain. This data involved tens to hundreds of thousands of individuals from GWA meta-analyses.

In all studies, genotyping and imputation passed strict quality control measures. Serum levels of ADMA, SDMA and hArg were measured using the same reversed-phase high-performance liquid chromatography method from the

YFS and LURIC studies. The GWASs were performed using the PLINK, ProbABEL, QUICKTEST and SNPTEST software programs while Mendelian randomisation and other statistical analyses were performed under the R statistical environment.

Results: In study I, several intron variants at the *DDAH1* locus (rs28489187 as the lead variant) were associated with circulating ADMA levels and two missense variants in *AGXT2* (rs37369, Val140Ile; rs16899974, Val498Leu) were independently associated with SDMA levels. An additional association signal for SDMA was observed in *SLC25A45* (rs34400381, Arg285Cys/Arg243Cys/etc. depending on the splice variant) encoding a mitochondrial solute carrier and thus sharing the same intracellular localisation as the mitochondrial aminotransferase enzyme AGXT2. Serum hArg levels were associated with variants at the *GATM* (rs1153858), *CPS1* rs1047891 (Thr1406Asn, formerly rs7422339) and *AGXT2* (rs37369, Val140Ile) loci (study II).

The two functional *AGXT2* variants (rs37369 and rs1873737) were associated in a consistent manner with the low-frequency to high-frequency (LF/HF) ratio of spectral heart rate variability (HRV) in YFS (study I). Moreover, the same *AGXT2* variants showed strong associations with atrial fibrillation in LURIC and age at a first ischemic stroke diagnosis in FINCAVAS. However, these associations were not replicated in all the cohorts studied (study III).

In longitudinal study (IV), serum hArg predicted incident hyperglycemia and abdominal obesity in young men in YFS. In young women of reproductive age, hArg levels were elevated in those who used oestrogen-containing oral contraceptives compared to non-users. In Mendelian randomisation studies, we found no evidence that life-long exposure to higher levels of serum hArg would causally increase the risk of developing cardiometabolic risk factors, type 2 diabetes mellitus or coronary artery disease.

Conclusions: These results reveal novel genetic variants associated with circulating ADMA, SDMA and hArg levels and demonstrate that *AGXT2* is involved in cardiometabolic diseases and their complications. Thus, AGXT2 might be a promising target for novel therapeutic interventions. Although not likely to be a causal mediator, serum hArg could potentially be used as a biomarker to detect individuals that are prone to developing abdominal obesity and associated comorbidities.

TIIVISTELMÄ

Tausta: Kardiometaboliset sairaudet ovat merkittävä taakka terveydenhuollolle ja johtava sairastavuuden ja kuolleisuuden aiheuttaja mailman laajuisesti. Endoteelin dysfunktio on kardiometabolisiin sairauksiin usein liittyvä ilmiö, joka johtuu vasoaktiivisen typpioksidin (NO) puutteesta. Asymmetrinen ja symmetrinen dimetyyliarginiini (ADMA ja SDMA) vähentävät NO:n määrää ja tätä kautta NO:n verisuonistoa suojaavia vaikutuksia (mm. antitromboottiset ja anti-imflammatoriset), mikä voi johtaa kardiometabolisiin komplikaatioihin. Seerumin korkeat ADMA- ja SDMA-pitoisuudet ja matalat L-homoarginiini (hArg) -pitoisuudet ennustavat suurentunutta kardiovaskulaari- ja kokonaiskuolleisuutta. Näiden yhteyksien taustalla olevia molekyylitason mekanismeja ei tunneta tarkasti eikä näiden arginiinijohdannaisten pitoisuuksiin vaikuttavia geneettisiä tekijöitä.

Tavoitteet: 1) tunnistaa uusia seerumin ADMA-, SDMA- ja hArg-tasojen taustalla olevia geneettisiä tekijöitä genominlaajuisen assosiaatiotutkimuksen (GWAS) keinoin, **2)** tutkia tässä työssä löydettyjen geenivarianttien yhteyksiä kardiometabolisiin sairauksiin ja päätetapahtumiin ja **3)** karakterisoida hArg:n merkitystä kardiometabolisten sairauksien biomarkkerina ja kausaalisena tekijänä.

Aineisto ja menetelmät: Tutkimuksissa I ja II genominlaajuiset assosiaatioanalyysit tehtiin Lasten Sepelvaltimotaudin Riskitekijät (LASERI, N=2083) - ja Ludwigshafen Risk and Cardiovascular Health (LURIC, N=3027) -aineistoilla. Tutkimuksessa III geneettisiä yhteyksiä tutkittiin LURIC-, Finnish Cardiovascular Study (FINCAVAS, N=3862) - ja Corogene -aineistoissa. Yhteyksiä iskeemisen aivoinfarktin kanssa tutkittiin Wellcome Trust Case Control Concortium 2 (WTCCC2, 3548 tapausta ja 5972 verrokkia) GWAS -kohorteissa ja FINCAVAS-aineistossa. Tutkimuksessa IV yhteyksiä hArg:n ja kardiometabolisten riskitekijöiden ja sairauksien välillä tutkittiin LASERI-aineistossa. Kausaaliset efektiestimaatit seerumin hArg:n ja kardiometabolisten riskitekijöiden ja sairauksien välillä estimoitiin hyödyntämällä julkisesti saatavilla olevia yhden emäksen polymorfioiden (SNP) ja fenotyyppien välisiä assosiaatiotuloksia, jotka perustuvat kymmeniä ja satojatuhansia ihmisiä sisältäviin genominlaajuisiin assosiaatiotutkimuksiin.

Kaikissa tutkimuksissa näytteiden genotyyppaus ja datan imputaatio läpäisivät tiukat laatukontrollikriteerit ennen analyyseja. Seerumin ADMA-, SDMA- ja

hArg-tasot mitattiin käyttäen samaa HPLC-menetelmää keskitetysti sekä LASERI- että LURIC-aineistoissa. GWAS-laskuissa käytimme PLINK-, ProbABEL-, QUICKTEST- ja SNPTEST-ohjelmia. Mendeliaaninen randomisaatio- ja muut tilastoanalyysit tehtiin R-tilasto-ohjelmalla.

Tulokset: Tutkimuksessa I useat *DDAH1*-geenin intronеissa sijaitsevat SNP:t (rs28489187:llä pienin p-arvo) olivat yhteydessä ADMA-tasoihin ja kaksi aminohappoa vaihtavaa SNP:tä (rs37369, Val140Ile; rs16899974, Val498Leu) *AGXT2*-geenissä olivat itsenäisesti yhteydessä SDMA-tasoihin. Lisäksi havaitsimme assosiaatiosignaalin *SLC25A45*-geenissä (rs34400381, Arg285Cys/Arg243Cys), joka koodaa mitokondriaalista kuljettajaproteiinia ja lokalisoituu siksi samaan paikkaan solun sisällä kuin mitokondriaalinen aminotransferaasi AGXT2. Seerumin hArg-tasot olivat yhteydessä variantteihin *GATM*- (rs1153858), *CPS1*- (rs1047891, Thr1406Asn, aikaisemmin rs7422339) ja *AGXT2*- (rs37369, Val140Ile) lokuksissa.

Kaksi funktionaalista *AGXT2*-varianttia (rs37369 ja rs16899974) olivat systemaattisesti yhteydessä spektraalisen sykevaihtelun matala- ja korkeataajuisten komponenttien suhteeseen (HRV-LF/HF) LASERI-tutkimuksessa. Lisäksi samat *AGXT2*-variantit olivat yhteydessä eteisvärinään LURIC-tutkimuksessa ja ikään iskeemisen aivoinfarktin diagnoosihetkellä FINCAVAS-tutkimuksessa. Nämä yhteydet eivät kuitenkaan toistuneet kaikissa tutkimuksissa (tutkimus III).

Tutkimuksen IV pitkittäisanalyyseissä hArg ennusti tulevaa hyperglykemiaa ja keskivartalolihavuutta nuorilla miehillä. Naisilla estrogeenia sisältävien yhdistelmäehkäisyvalmisteiden käyttö oli yhteydessä korkeampiin seerumin hArg-tasoihin verrattuna naisiin, jotka eivät käyttäneet mitään hormonaalista ehkäisyä. Mendeliaaliset randomisaatioanalyysit eivät tukeneet hypoteesia siitä, että seerumin hArg-tasoilla olisi mitään kausaalista vaikutusta kardiometabolisten riskitekijöiden, tyypin 2 diabeteksen tai koronaaritaudin kehittymiseen.

Johtopäätökset: Löysimme uusia geenivarientteja, jotka ovat yhteydessä seerumista mitattuihin ADMA-, SDMA- ja hArg-tasoihin. Lisäksi osoitimme, että *AGXT2*-geeni liittyy useisiin eri kardiometabolisiin sairauksiin ja niiden ennusteeseen. Serumin hArg tuskin on kardiometabolisten sairauksien kausaalinen tekijä. Sitä voitaneen kuitenkin käyttää biomarkkerina tunnistamaan henkilöitä, joilla on suurentunut vaara kehittää keskivartalolihavuus ja siihen liittyviä liitännäissairauksia.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text with their Roman numerals (I-IV):

I Seppälä I*, Kleber ME*, Lyytikäinen L, Hernesniemi JA, Mäkelä K, Oksala N, Laaksonen R, Pilz S, Tomaschitz A, Silbernagel G, Boehm BO, Grammer TB, Koskinen T, Juonala M, Hutri-Kähönen N, Alfthan G, Viikari JSA, Kähonen M, Raitakari OT, März W, Meinitzer A & Lehtimäki T. (2014) Genome-wide association study on dimethylarginines reveals novel AGXT2 variants associated with heart rate variability but not with overall mortality. Eur Heart J 35(8): 524-531. *Authors contributed equally.

II Kleber ME*, Seppälä I*, Pilz S, Hoffmann MM, Tomaschitz A, Oksala N, Raitoharju E, Lyytikäinen L, Mäkelä K, Laaksonen R, Kähönen M, Raitakari OT, Huang J, Kienreich K, Fahrleitner-Pammer A, Drechsler C, Krane V, Boehm BO, Koenig W, Wanner C, Lehtimäki T, März W & Meinitzer A. (2013) Genome-wide association study identifies 3 genomic loci significantly associated with serum levels of homoarginine: the AtheroRemo Consortium. Circ Cardiovasc Genet 6(5): 505-513. *Authors contributed equally.

III Seppälä I, Kleber ME, Bevan S, Lyytikäinen L, Oksala N, Hernesniemi JA, Mäkelä K, Rothwell PM, Sudlow C, Dichgans M, Mononen N, Vlachopoulou E, Sinisalo J, Delgado GE, Laaksonen R, Koskinen T, Scharnagl H, Kähönen M, Markus HS, März W & Lehtimäki T. (2016) Associations of functional alanine-glyoxylate aminotransferase 2 gene variants with atrial fibrillation and ischemic stroke. Sci Rep 6: 23207.

IV Seppälä I, Oksala N, Jula A, Kangas AJ, Soininen P, Hutri-Kähönen N, März W, Meinitzer A, Juonala M, Kähönen M, Raitakari OT & Lehtimäki T. (2017) The biomarker and causal roles of homoarginine in the development of cardiometabolic diseases: an observational and Mendelian randomization analysis. Sci Rep 7(1): 1130.

ABBREVIATIONS

ACS	acute coronary syndrome
ADMA	N^G,N^G-dimethyl-L-arginine, asymmetric dimethylarginine
AF	atrial fibrillation
AGAT	L-arginine:glycine amidinotransferase
AGXT2	alanine-glyoxylate aminotransferase 2
BAIB	β-aminoisobuturate
BH_4	tetrahydrobiobeterin
BMI	body mass index
CAC	coronary artery calcification
CAD	coronary artery disease
cGMP	cyclic guanosine monophosphate
CPS1	carbamoyl-phosphate synthase 1
DDAH	dimethylarginine dimethylaminohydrolase
DM'GV	α-keto-δ-(N^G,N'^G-dimethylguanidino) valeric acid
DMGV	α-keto-δ-(N^G,N^G-dimethylguanidino) valeric acid
DNA	deoxyribonucleic acid
ECG	electrocardiogram
FINCAVAS	Finnish Cardiovascular Study
GAA	guanidino acetic acid
GATM	glycine amidinotransferase, encodes AGAT
GWAS	genome-wide association study
hArg	L-homoarginine
HDL	high-density lipoprotein
HF	heart failure
HPLC	high-performance liquid chromatography
HRV	heart rate variability
HWE	Hardy-Weinberg equilibrium
IMT	intima-media thickness
indel	insertion/deletion polymorphism

LD	linkage disequilibrium
LDL	low-density lipoprotein
L-NMMA	N^G-monomethyl-L-arginine, monomethylarginine
LURIC	Ludwigshafen Risk and Cardiovascular Health
LVEF	left ventricular ejection fraction
MAF	minor allele frequency
MDS	multidimensional scaling
MI	myocardial infarction
MR	Mendelian randomisation
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
NO	nitric oxide
NOS	nitric oxide synthase
NOx	nitrate/nitrite
NT-proBNP	N-terminal pro-B-type natriuretic peptide
NYHA	New York Heart Association
oxLDL	oxidised low-density lipoprotein
PRMT	protein arginine methyltransferase
QC	quality control
ROS	reactive oxygen species
SCD	sudden cardiac death
SD	standard deviation
SDMA	N^G,N'^G-dimethyl-L-arginine, symmetric dimethylarginine
SHBG	sex hormone-binding globulin
SNP	single-nucleotide polymorphism
T2DM	type 2 diabetes mellitus
TNF-α	tumor necrosis factor-α
TVS	Tampere Vascular Study
UK	the United Kingdom
VSMC	vascular smooth muscle cell
WTCCC2	Wellcome Trust Case Control Consortium 2
YFS	Cardiovascular Risk in Young Finns Study

1 INTRODUCTION

Cardiometabolic diseases – cardiovascular diseases affecting the artery tree and heart and metabolic abnormalities including type 2 diabetes mellitus (T2DM) – are the leading cause of morbidity and mortality worldwide (Grundy. 2008). Many risk factors for cardiometabolic diseases, i.e. risk factors for cardiovascular diseases, dyslipidemia, insulin resistance, high blood glucose, elevation of blood pressure and obesity, tend to cluster together, increasing the risk for major cardiometabolic complications, including T2DM as well as myocardial and cerebral infarction, more than any of the risk factors in isolation. A hallmark of cardiometabolic diseases is endothelial dysfunction, a state that is associated with a decline in the production of the vasoactive signalling molecule nitric oxide (NO) (Tziomalos *et al.* 2010). Endothelial dysfunction and reduced NO bioavailability contributes to the development and progression of cardiovascular diseases and their complications.

Asymmetric and symmetric dimethylarginines (ADMA and SDMA) are naturally occurring products of protein turnover. ADMA, though not SDMA, is capable of directly inhibiting the enzymatic NO production and is considered to be a marker of endothelial dysfunction. Elevated levels of both methylarginines have been observed in many cardiometabolic diseases. Moreover, both ADMA and SDMA are able to potentiate the processes, leading to oxidative stress and inflammation in the vasculature (Bode-Böger *et al.* 2006, Wilcox. 2012), both of which are also prominent features of cardiometabolic diseases. Moreover, high ADMA and SDMA levels have been shown to predict future cardiovascular events and mortality, both in the general population and among different patient groups.

L-homoarginine (hArg) is an endogenous amino acid and low hArg has been shown to predict poor outcome in the general population (Atzler *et al.* 2014, Pilz *et al.* 2014b) and in different patient groups (Atzler *et al.* 2013, Drechsler *et al.* 2011, März *et al.* 2010), while high hArg is directly associated with several risk factors for cardiometabolic diseases (Atzler *et al.* 2014, van der Zwan, Leonard P *et al.* 2013). Therefore, it is poorly understood whether hArg is a causal mediator or a biomarker in the context of cardiometabolic diseases.

This work aims to: **a)** increase our understanding of genetic determinants of the three arginine derivatives by genome-wide association study (GWAS); **b)** investigate associations between these newly identified genetic variants and selected cardiometabolic diseases and their manifestations; and **c)** study the predictive value of serum hArg and its causal role in the development of cardiometabolic diseases by utilising longitudinal observational data as well as summary statistics from large-scale GWASs available in the public domain to maximise the statistical power to identify potential causal associations.

2 LITERATURE REVIEW

2.1 ADMA and SDMA

2.1.1 NO pathway and arginine derivatives

NO plays an important role in many physiological processes including, among others, endothelium-dependent vasodilatation (Palmer *et al.* 1987), the immune system's response to pathogens and inflammation (Liew *et al.* 1990, Moncada *et al.* 1989), neuronal signalling (Knowles *et al.* 1989) and platelet aggregation (Palmer *et al.* 1988). NO is synthesised *in situ* by the three isoforms of the enzyme nitric oxide synthase (NOS): neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2) and endothelial NOS (eNOS, NOS3) (Förstermann *et al.* 1994). In its gaseous form, NO degrades rapidly to its inactive metabolites, i.e. to nitrates and nitrites (NOx), and thus has a relatively limited diffusion distance and duration of action in tissues. For this reason, NO is an important signalling transduction molecule in physiological processes which needs to be rapidly initiated and discontinued as a response to various stimuli. Probably due to the fact that NO is a gas and challenging to measure directly *in vivo*, it was not until the 1980s that the endothelium derived NO was discovered to act as a local signalling molecule that induces the relaxation of the arterial and venous blood vessels (Furchgott & Zawadzki. 1980, Palmer *et al.* 1987).The effects of NO are mediated via a cyclic guanosine monophosphate (cGMP)–dependent mechanism in target cells, e.g. in vascular smooth muscle cells (VSMC) in vessel walls and platelets in the circulation (Radomski *et al.* 1990). NOS utilises the semi-essential amino acid L-arginine as a substrate to form NO and L-citrulline (Palmer *et al.* 1988). The effects of blood flow induced shear stress and vasoactive ligands on endothelial cells and VSMCs via the L-arginine/NO/cGMP pathway in vessel walls under normal physiological conditions are illustrated in **Figure 1**.

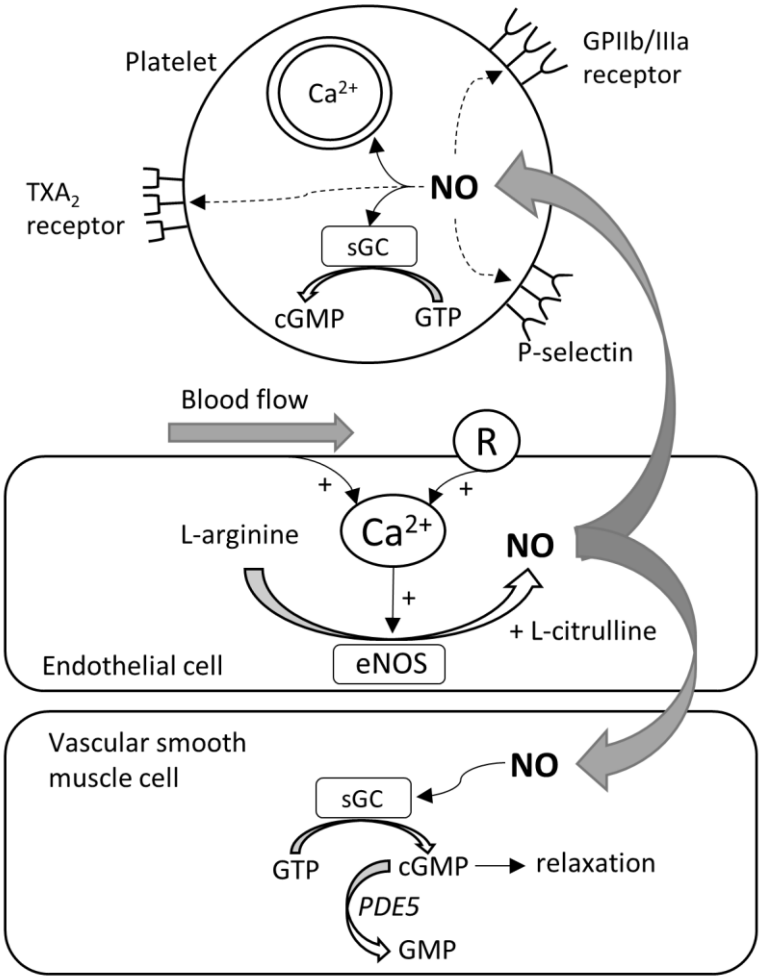

Figure 1. Depicted are the two key physiological endothelium and L-arginine/NO/cGMP pathway dependent processes in the blood and vasculature: NO dependent vasorelaxation and NO induced inhibition of platelet activation and aggregation. The activity of eNOS is calcium dependent and can be increased either by blood flow induced shear stress (flow dependent NO formation) or by receptor stimulus of vasoactive ligands (receptor-stimulated NO formation). After diffusion into the VSMCs adjacent to the endothelium, NO binds and activates the enzyme soluble guanylyl cyclase (sGC) that catalyses the dephosphorylation of GTP to cGMP. cGMP signals VSMC relaxation and is catabolised to GMP by the enzyme PDE5. PDE5 inhibitors such as sildenafil are used to treat erectile dysfunction. The anti-aggregatory effects of the endothelium derived NO in platelets are mediated through cGMP and Ca^{2+}/calmodulin dependent mechanisms as well as expressions of glycoprotein (GP) IIB/IIIa, thromboxane A2 and the platelet surface adhesion molecule P-selectin. R, receptor; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; PDE5, cGMP-dependent phosphodiesterase (type 5). Modified from (Jin & Loscalzo. 2010, Klabunde. 2012).

Endogenously formed asymmetric dimethylarginine (ADMA) and monomethylarginine (L-NMMA), although not symmetric dimethylarginine (SDMA), directly and competitively inhibit all three NOS isoforms reducing the synthesis of NO by NOS (Vallance & Leone. 1992). Therefore, SDMA was first considered to be an inert and biologically inactive arginine derivative. However, increasing *in vitro* evidence indicates that SDMA can reduce the bioavailability of NO by inhibiting cellular uptake of L-arginine and by uncoupling NOS to produce superoxide radical ($O_2^{\cdot-}$) rather than NO (Bode-Böger *et al.* 2006).

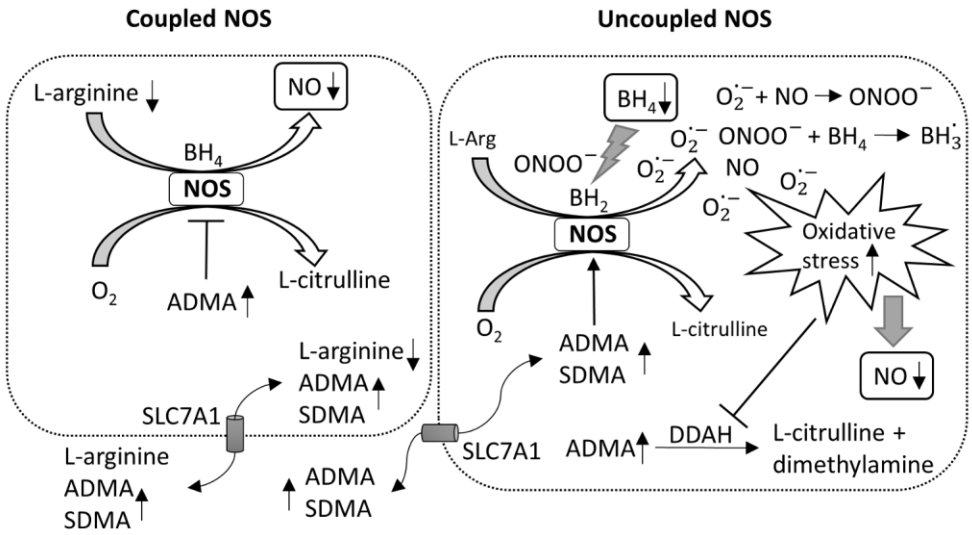

Figure 2. Schematic presentation of the mechanisms underlying reduced NO bioavailability. Under normal physiological conditions, NOS is, in its coupled form, utilising L-arginine and molecular oxygen as substrates to form NO and L-citrulline while tetrahydrobiopterin (BH_4) acts as a cofactor stabilising the enzyme. In this scenario, ADMA competes with L-arginine at the active site of NOS reducing NO formation. Both ADMA and SDMA can indirectly reduce intracellular concentrations of L-arginine and thus NO production by competitive inhibition of cellular uptake of L-arginine as they all utilise the same cationic amino acid transporter (SLC7A1, CAT-1). Under pathological conditions, an initiation of superoxide production predominantly induces NOS uncoupling due to reduced BH_4 bioavailability further potentiating superoxide production by NOS. In addition, increased concentrations of both ADMA and SDMA contribute to NOS uncoupling and superoxide production. Increasing oxidative stress further enhances this vicious cycle by inhibiting the ADMA degrading enzyme DDAH as well as transport by SLC7A1 leading to intracellular accumulation of ADMA and SDMA. SLC7A1, solute carrier family 7 member 1. (Rochette *et al.* 2013, Wilcox. 2012)

The balance between the formation of NO and reactive oxygen species (ROS) by NOS *in vitro* and *in vivo* is a complex and dynamic process which is influenced by numerous factors depicted in **Figure 2**. Under normal conditions, NOS is in its NO producing coupled state while NO formation by NOS is influenced by

intracellular concentrations of L-arginine and ADMA. The so called "L-arginine paradox" refers to the situation where L-arginine concentrations are well above the half-saturating L-arginine concentrations of NOSs (K_M ~2.9 µM for eNOS) in living organisms (~100 µM in human plasma) but still endothelium-dependent vasodilation can be induced by arginine supplementation (Förstermann *et al.* 1994, Tsikas *et al.* 2000). It has been estimated that intracellular ADMA concentrations are in the range of 10 to 20 times higher than plasma concentrations of ~0.5–1.0 µM (Teerlink *et al.* 2009), while the circulating concentrations of endogenous L-NMMA are approximately 10 times smaller compared to the concentrations of ADMA and SDMA and therefore rarely measured in epidemiological studies. A study using isolated enzymes, cellular systems and a balloon model of vascular injury demonstrated that ADMA and L-NMMA are elevated under pathological conditions, reaching concentrations sufficient to impair eNOS derived vascular relaxation whereas physiological concentrations had only a modest effect on NOS activity (Cardounel *et al.* 2007).

Under pathological conditions, the superoxide production and subsequent oxidative stress can be initiated and mediated by several mechanisms within the cells before the NOS derived superoxide begins to amplify the pathological process (Wilcox. 2012). For example, the formation of ROS derived oxidative stress in the vasculature was hypothesised to be initiated by risk factors for cardiovascular and kidney diseases such as hypertension, aging, hypercholesterolemia, obesity, diabetes mellitus, insulin resistance, hyperhomocysteinemia and smoking by inducing the expression of initiators of risk such as angiotensin II, endothelin-1 and activation of the sympathetic nervous system. In the next step, these initiators activate NADPH oxidase and the generation of ROS which tends to amplify the expression of initiators forming a positive feedback loop. Oxidative stress generates more cellular ADMA and SDMA by inhibiting the ADMA degrading enzyme dimethylarginine dimethylaminohydrolase (DDAH) and cationic amino acid transporters further amplifying superoxide generation by uncoupling NOS. In addition, oxidative stress-induced breakdown of the NOS stabilising cofactor tetrahydrobiobeterin (BH_4) to the $BH_3^{\bullet}$ radical and BH_2 leads to an increased ratio of superoxide to NO generation (Vasquez-Vivar *et al.* 1998). As a result, increased ROS and diminished NO leads to adverse changes in vascular and organ functions translating cardiovascular risk into overt disease (Wilcox. 2012).

2.1.2 Effects of ADMA, SDMA and L-arginine administration into animals and humans

The fast-acting nitrodilator nitroglycerin has been used from the 19th century to relieve angina pectoris in ischemic heart diseases. Aside from the therapeutic use of nitrodilators that release NO either directly (e.g. sodium nitroprusside) or indirectly after enzymatic action (organic nitrates) (Klabunde. 2012), the effects of other molecules interfering the L-arginine/NO/cGMP pathway have also been tested in healthy and diseased animals and humans.

2.1.2.1 Administration into healthy animals and humans

In the anesthetised rabbit *in vivo*, L-NMMA induced a dose-dependent increase in mean systemic arterial blood pressure and inhibited the hypotensive action of acetylcholine which were both reversed by L-arginine (Rees *et al.* 1989). The L-arginine alone had no direct effect on blood pressure and the investigators suggested that, under normal conditions, there is sufficient L-arginine to saturate NOS. In a prospective, double-blind, randomised crossover trial of 12 healthy young men, an arginine dose of 7 g three times daily for three days did not have any effect on systemic hemodynamic parameters or endothelium-dependent vasodilation of the brachial artery compared to placebo (Adams *et al.* 1995). However, oral arginine inhibited platelet aggregation in response to adenosine diphosphate *ex vivo* by way of the NO/cGMP pathway.

In a study of healthy humans, the effects of intravenous infusion of ADMA on systemic cardiovascular parameters were investigated using invasive methods (Kielstein *et al.* 2004). At ADMA concentrations seen in pathological conditions (2–10 µM), a reduction in cGMP levels was observed with parallel increases in systemic vascular resistance and blood pressure as well as a reduction in cardiac output in a dose-dependent manner. In another clinical trial, the effects of intravenous low-dose ADMA and placebo on heart rate, blood pressure, cardiac output and systemic vascular resistance were investigated in 12 healthy men (Achan *et al.* 2003). A low-dose ADMA bolus reduced heart rate and cardiac output whereas ADMA increased mean blood pressure and systemic vascular resistance. Urine dimethylamine, a product from ADMA degradation by DDAHs, was significantly increased after ADMA injection and the investigators estimated that ~80% of ADMA is metabolised by DDAHs.

A study tested the effects of long-term (four weeks) SDMA infusion in healthy mice by minipumps (Veldink *et al.* 2013) after increasing *in vitro* evidence had shown that SDMA induced ROS production of monocytes by stimulating Ca^{2+} entry via store-operated Ca^{2+}-channels (Schepers *et al.* 2009) and stimulated cytokine expression in monocytes (Schepers *et al.* 2011). Despite a significant 10-fold increase in SDMA levels, neither a change in mean systolic blood pressure nor differences in renal histology nor renal eNOS expression were observed. Interestingly, left ventricular endsystolic diameter increased significantly, an effect that did not occur in the placebo group.

It has been proposed that oral administration of L-citrulline could be a more elegant way of increasing circulating levels of L-arginine (Schwedhelm *et al.* 2008) than oral L-arginine supplementation. High dose(s) of oral arginine is required to result in a sufficient increase in blood concentrations because, unlike L-citrulline, L-arginine is extensively metabolised before reaching systemic circulation. L-citrulline is systematically converted into arginine in different tissues. Therefore, in a placebo controlled, blinded, cross-over trial of 20 healthy volunteers, the pharmacokinetics and pharmacodynamics of L-arginine plus L-citrulline versus L-arginine were studied (Schwedhelm *et al.* 2008). Oral L-citrulline supplementation resulted in increased circulating L-arginine levels as well as urinary nitrates and cGMP indicating enhanced NO/cGMP-dependent signalling. However, no treatment improved endothelium-dependent vasodilation over placebo, although L-arginine levels were positively correlated with flow-mediated dilation of the brachial artery when all data were combined.

2.1.2.2 L-arginine administration into diseased animals and humans

The therapeutic potential of L-arginine has been studied under pathological conditions in animals and humans. A study in the hypercholesteraemic rabbit demonstrated that L-arginine-supplemented animals had improved endothelium-dependent vasorelaxation compared to untreated animals which was also associated with a reduction in atherogenesis (Cooke *et al.* 1992). Another study, in normal subjects and hypercholesterolemic humans, reported that endothelium-dependent vasodilation was impaired in hypercholesteremic patients and that vasorelaxation could be improved acutely by administration of L-arginine (Creager *et al.* 1992). Again, L-arginine did not augment the forearm blood flow response to methacholine in the healthy control subjects. Moreover, L-arginine prevented the intima thickening in the coronary arteries of hypercholesterolemic

rabbits but did not change cholesterol levels in line with the hypothesis that NO has antiatherogenic properties (Wang *et al.* 1994). In a double-blind, randomised and placebo controlled trial, a long-term (six months) L-arginine supplementation improved the small-vessel coronary function and patients' symptoms scores in patients with non-obstructive coronary artery disease compared to placebo (Lerman *et al.* 1998). Another study, in patients with normal coronary angiograms and cardiac syndrome X, reported increased ADMA levels and abnormal vascular reactivity compared to control subject, and a L-arginine bolus restored a physiological profile of all endothelial variables measured (Piatti *et al.* 2003). In a randomised placebo controlled trial of healthy elderly people aged over 70 years, 14 days oral L-arginine significantly improved flow-mediated dilation of the brachial artery but did not have any effect on circulating ADMA levels (Bode-Böger *et al.* 2003). Clinical trials on the effects of L-arginine in patients with peripheral artery occlusive disease and intermittent claudication are not conclusive, as one reported improved function of the femoral artery and alleviated patients' symptoms (Boger *et al.* 1998) while another study reported that L-arginine treatment was associated with no benefit and possible harm (Wilson *et al.* 2007). Finally, in patients with acute coronary syndrome (ACS), no harm or benefit from L-arginine therapy was observed in a clinical trial of 792 patients with acute myocardial infarction (MI) (Bednarz *et al.* 2005), while another small trial of 153 patients with a first ST-segment elevation MI was terminated due to excess mortality and safety concerns (Schulman *et al.* 2006). For obvious reasons, no experiments have been performed using ADMA or SDMA infusion into diseased humans.

2.1.3 Formation and clearance of ADMA and SDMA

2.1.3.1 Synthesis: protein break down by PRMTs

ADMA, SDMA and L-NMMA are continuously formed during proteolysis within all mammalian cells. Arginine residues of the intracellular proteins are constitutively methylated by a family of proteins called protein arginine methyltransferases (PRMTs) and subsequently released as free methylarginines into cytosol after hydrolysis of the arginine-methylated proteins (Morales *et al.* 2016). There are six known type I PRMTs capable of forming L-NMMA and ADMA on target proteins. Two type II PRMTs catalyse monomethylation

forming L-NMMA and, in the second round of turnover, result in the formation of SDMA. Type III PRMTs such as PRMT7 are capable of forming only L-NMMA residues on proteins. Although constitutively acting on cellular proteins, the fine-tuning of their activity and substrate recognition of each PRMT is poorly understood (Morales *et al.* 2016). Recent experimental data shows that the ADMA producing PRMT1 is under redox control and that oxidised PRMT1 displays reduced activity, challenging the paradigm that increased protein expression of PRMTs under oxidative stress always leads to increased methylarginine synthesis (Morales *et al.* 2015). In **Figure 3**, the chemical structures of the amino acids L-arginine and hArg as well as the three free endogenous methylarginines are shown.

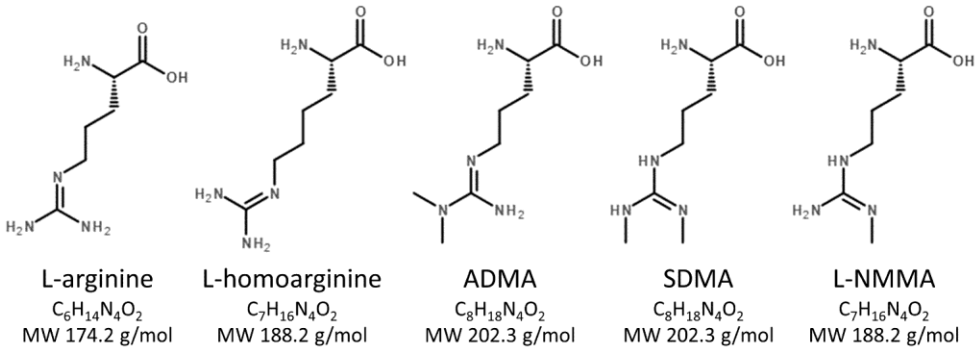

Figure 3. Chemical structures and molecular weights (MW) of the five naturally occurring arginine derivatives.

2.1.3.2 Renal clearance of ADMA and SDMA

ADMA is primarily eliminated from the circulation by the enzyme DDAH and it has been estimated that, in humans, only ~20% of ADMA is filtered through the kidneys and excreted in urine (Achan *et al.* 2003). By contrast, SDMA is primarily eliminated from the systemic circulation by renal excretion and the enzymatic catabolism of SDMA plays a minor part. Due the close correlation between SDMA and other markers of renal function such as creatinine, SDMA is considered to be a useful marker for detection of patients in very early stages of chronic kidney disease (Bode-Böger *et al.* 2006).

2.1.3.3 ADMA elimination by dimethylarginine dimethylaminohydrolases (DDAHs)

ADMA and L-NMMA, but not SDMA, are primarily degraded by the two isoforms of the enzyme dimethylarginine dimethylaminohydrolase (DDAH1 and DDAH2) which catalyse the conversion of ADMA to dimethylamine and L-citrulline (Leiper *et al.* 1999, Ogawa *et al.* 1987a, Ogawa *et al.* 1989). DDAH(1) was shown to be widely distributed in post-mortem human tissues including the pancreas, kidney and liver (Kimoto *et al.* 1995).

The effects of native low-density lipoprotein (LDL), oxidised LDL (oxLDL) and tumor necrosis factor-α (TNF-α) on the dysregulation of DDAH were investigated in human umbilical vein endothelial cells (Ito *et al.* 1999). The addition of oxLDL or TNF-α into the conditioned medium resulted in ADMA accumulation, which was not due to a change in DDAH expression but rather the reduction of DDAH activity. In the same study, it was investigated whether the dysregulation occurred also *in vivo* by using rabbits on high-fat and normal diet. Hypercholesterolemia significantly reduced DDAH activity in aortic, renal and hepatic tissues. Therefore, the impaired endothelium-dependent vasorelaxation seen in hypercholesterolemia may be due to reduced activity of DDAH leading to ADMA accumulation. The overexpression of DDAH1 was studied *in vitro* and *in vivo* by gene transfer and transgenic approaches (Dayoub *et al.* 2003). In line with the results seen *in vitro*, in the hDDAH-1 transgenic mice, they observed a 2-fold increase in tissue NOS activity and a 2-fold reduction in plasma ADMA. Blood pressure was ~13 mmHg lower than in wild-type controls with parallel reductions in systemic vascular resistance and cardiac contractility in response to the increased NO generation.

In another study, which examined the cardiovascular effects of ADMA, methylarginine metabolism was disturbed by deleting the *Ddah1* gene in mice and by DDAH-specific inhibitors (Leiper *et al.* 2007). The investigators found that loss of DDAH1 activity led to impaired NO signalling and accumulation of ADMA. The mice exhibited increased systemic vascular resistance and elevated systemic and pulmonary blood pressure. Other global DDAH1 gene-deficient mice were independently developed and investigated (Hu *et al.* 2011). Plasma and tissue ADMA and L-NMMA levels were several fold higher than in wild-type mice. The DDAH1-deficient animals exhibited ~20 mmHg higher blood pressure while other cardiovascular parameters were unchanged in unstressed conditions compared to wild-type animals. In contrast to the $DDAH1^{-/-}$ mice that died *in utero* (Leiper *et al.* 2007), these $DDAH1^{-/-}$ mice were viable indicating that the DDAH1 gene was not required for embryogenic development in their

mice strain. Furthermore, the experiments with DDAH2 silenced endothelial cells and the lack of DDAH activity in tissues despite highly abundant DDAH2 expression, implying that DDAH2 does not contribute to the degradation of ADMA and L-NMMA. Finally, two endothelium-specific DDAH1 knockout mice have also been generated. The first studies in such mice showed that ADMA levels and blood pressure were elevated in response to the endothelium-specific DDAH1 deletion (Hu *et al.* 2009). In contrast, the hemodynamic and vascular reactivity profile of the other endothelium-specific DDAH1-deficient mouse was unchanged but the angiogenesis was severely impaired compared to controls (Dowsett *et al.* 2015). The results of the second study are in line with the wide distribution of DDAH1 in different tissues and organs and implies that DDAH1 in endothelial cells has little role in the regulation of the systemic ADMA levels or vascular reactivity.

Little attention has been paid to the second isoform DDAH2 until recently. A global DDAH2-knockout mouse was generated and studied under physiological conditions as well as in a model of severe polymicrobial sepsis (Lambden *et al.* 2015). DDAH2-deficient mice exhibited elevated blood pressure during periods of activity and increased myocardial ADMA concentrations compared to their wild-type littermates. It was also shown that, in a model of sepsis, the DDAH2-deficient mice had significantly worse 120-hour survival rates compared to the controls. By monocyte-specific deletion of DDAH2, it was demonstrated that the poor outcome in mice globally deficient in DDAH2 was mediated through impairment of NO-mediated macrophage-dependent immune response.

Several candidate gene studies have studied the associations of the *DDAH1* and *DDAH2* gene variants with circulating methylarginine levels and different cardiovascular phenotypes. Among 1609 Finnish men, a rare *DDAH1* mutation was found: there were only 16 heterozygote and no homozygote genotype carriers (Valkonen *et al.* 2005). This variant was associated with markedly increased risks of coronary artery disease (CAD) and hypertension. Both *DDAH1* and *DDAH2* variants have been linked to circulating ADMA levels using a candidate gene approach (Abhary *et al.* 2010). A novel *DDAH1* promoter -396 4N insertion polymorphism was associated with reduced messenger ribonucleic acid (mRNA) levels and increased ADMA levels *in vivo* (Ding *et al.* 2010). In addition, this polymorphism was associated with an increased risk of both thrombosis stroke and CAD in two independent populations.

2.1.3.4 ADMA and SDMA elimination by alanine-glyoxylate aminotransferase 2 (AGXT2)

The enzyme alanine-glyoxylate aminotransferase 2 (AGXT2) was purified from the rat kidney followed by detailed characterisation in the late 1980s (Ogawa *et al.* 1987b, Ogawa *et al.* 1990). In these experiments, it was shown that rat AGXT2 was capable of metabolising both dimethylarginines and forming corresponding α-keto acid analogues, i.e. α-keto-δ-(N^G,N^G-dimethylguanidino) valeric acid (DMGV) from ADMA and α-keto-δ-(N^G,N'^G-dimethylguanidino) valeric acid (DM'GV) from SDMA. Little attention was paid to AGXT2 until 2010, when it was shown that an overexpression of human AGXT2 in mice livers resulted in significant decreases of plasma and liver ADMA levels (Rodionov *et al.* 2010). Moreover, it was shown that hAGXT2 was localised to mitochondria and that overexpression of hAGXT2 protected endothelial cells from ADMA-mediated inhibition of NO production. In another study, AGXT2-deficient mice were developed and investigated under normal physiological conditions (Caplin *et al.* 2012). AGXT2-knockout mice had elevated plasma ADMA, SDMA and DMGV levels compared to wild-type littermates and they exhibited a hypertensive phenotype. In addition, *ex vivo* aortas from $AGXT2^{-/-}$ mice were hypersensitive to acetylcholine, indicating enhanced endothelium-dependent responses compared to controls. In a study using C57/BL6 mice, the role of AGXT2 in dimethylarginine metabolism was studied by chronic (seven days) infusion of β-aminoisobutyrate (BAIB), another substrate of AGXT2, and by measuring ADMA, SDMA and DMGV concentrations from plasma and urine (Kittel *et al.* 2013). BAIB concentrations were elevated by a factor of 25 in both plasma and urine, plasma ADMA and SDMA increased by ~30%, plasma DMGV decrease by 24% while the tissue mRNA and protein expression of *Agxt2* did not change. The investigators concluded that endogenous AGXT2 was involved in the regulation of systemic ADMA, SDMA and DMGV levels.

2.1.4 Circulating dimethylarginines, cardiometabolic diseases and mortality

2.1.4.1 ADMA, SDMA and atherosclerotic diseases

Clinical manifestations of atherosclerosis are leading causes of morbidity and mortality around the world. In European countries, the proportion of all deaths attributable to cardiovascular diseases (over 4 million deaths per year) is 51% and

42% among women and men, respectively, whereas coronary artery disease accounts for ~20% of all deaths in both sexes in Europe annually (Nichols *et al.* 2014). Atherosclerosis is a chronic disease that affects the entire artery tree while its major clinical manifestations include CAD, ischemic stroke and peripheral arterial disease.

Endothelial dysfunction, vascular production of ROS and lipid oxidation are thought be the initiators in the development of atherosclerosis (Ellulu *et al.* 2016). ADMA and SDMA contribute to these pathological processes by reducing the NO bioavailability and promoting oxidative stress, as discussed in chapter 2.1.1. The second initiator of atherosclerosis is the oxidation of native LDL particles within the artery wall, leading to foam cell formation and inflammation. These initial fatty streaks gradually develop into atheroma and advanced plaques over the course of decades. Acute rupture of the fibrous cap of an atherosclerotic plaque leads to local thrombosis and a partial or total occlusion of the affected artery (Herrington *et al.* 2016). Given that NO inhibits several proatherogenic mechanisms such as platelet activation and aggregation, VSMC proliferation/migration and leucocyte adhesion and infiltration into the artery wall (Kessler *et al.* 2016), it is plausible that reduced NO bioavailability promotes atherosclerosis during the progression of the disease.

Interestingly, variants in both the *eNOS* and *GUCY1A3* (encodes the α-subunit of soluble guanylyl cyclase 1, a receptor for NO, **Figure 1**) genes have been identified to be associated with CAD by using the hypothesis-free GWAS approach involving ~180 000 individuals (Nikpay *et al.* 2015). Both of these genes have also been linked to blood pressure traits in large-scale GWASs (Ehret *et al.* 2016, Salvi *et al.* 2012); however, their associations with CAD cannot solely be explained by their association with blood pressure (Kessler *et al.* 2016). Indeed, a loss-of-function variant of *GUCY1A3* was identified in an extended family with a high prevalence of early-onset CAD and MI (Erdmann *et al.* 2013). This genetic data together provide compelling evidence to support the causal involvement of NO/cGMP signalling in atherosclerosis pathogenesis.

In a prospective study involving middle-aged men from Eastern Finland, it was shown that ADMA levels were associated with acute coronary events in those who did not smoke (n=150): those in the highest quartile for ADMA had a 3.9-fold risk of acute coronary events compared with the other quartiles (Valkonen *et al.* 2001). In a prospective study including 1,874 consecutive patients with coronary artery disease, the highest tertile of baseline ADMA was associated with future cardiovascular mortality or non-fatal MI with a hazard ratio (HR) of ~2.5

compared with the first ADMA tertile (Schnabel *et al.* 2005). The ADMA concentrations were similar in those who had ACS compared with stable angina patients at baseline. Some case-control studies have detected an association between elevated ADMA levels and prevalent CAD (Lu *et al.* 2011, Schulze *et al.* 2006), although others have not (Bode-Böger *et al.* 2006, Meinitzer *et al.* 2007b). Moreover, SDMA, but not ADMA, was associated with a stenosis score in 147 patients who underwent elective coronary angiography (Bode-Böger *et al.* 2006). Finally, in 281 patients with suspected CAD undergoing coronary angiography, serum ADMA was found to be independent predictor of coronary atherosclerosis extent and functional significance of CAD as assessed by fractional flow reserve, whereas associations with SDMA were not investigated (Mangiacapra *et al.* 2016).

2.1.4.2 ADMA, SDMA, atrial fibrillation and ischemic stroke

Atrial fibrillation (AF) is the most common sustained arrhythmia and remains one of the major causes of stroke, HF, cardiovascular morbidity and mortality worldwide (Chugh *et al.* 2014, Kirchhof *et al.* 2016). One in four middle-aged adults will develop AF during their lifetime in Europe and the number of patients with AF is predicted to rise steeply in the coming years. AF can be classified based on the AF pattern into five mutually exclusive classes: first diagnosed AF, paroxysmal AF (may continue for up to 7 days), persistent AF (lasts longer than 7 days), long-standing persistent AF (continuous AF lasting ≥1 year) and permanent AF.

In experimental models of AF, very similar pathophysiological mechanisms have been observed than those in vascular pathology in the context of NOS, ADMA and SDMA. In a pig model of AF, AF was induced with rapid atrial pacing for one week and NO production was measured from freshly isolated tissues by a NO-specific microelectrode (Cai *et al.* 2002). Left atrial NO production was reduced by ~70% in AF compared to control animals. Although NO concentration was reduced in left atrial appendage, endocardial eNOS expression was not. It was concluded that the loss of this antithrombotic enzyme likely contributes to the thromboembolic complications in AF. Because human AF has been associated with oxidative stress, the sources of superoxide production from right atrial tissues from patients undergoing cardiac surgery were studied (Kim *et al.* 2005). The investigators found that NADPH and dysfunctional NOS contributed significantly to atrial superoxide production and suggested that

this phenomenon contributes to the atrial oxidative injury and electrophysiological remodelling observed in human AF.

In dogs with AF, circulating ADMA levels significantly increased and NOx levels decreased (Liu *et al.* 2008). The DDAH activity and eNOS expression were significantly decreased in the fibrillating atria, while the expression of PRMT1 increased in the left atrial appendage. Therefore, it was concluded that the DDAH-PRMT-ADMA system may play a pivotal role in regulating endothelial function in AF. In pigs, rapid atrial pacing for seven hours increased ADMA and troponin T levels and reduced mRNA expression of ventricular and aortic eNOS (Goette *et al.* 2012).

In addition, increasing evidence from clinical studies has implicated ADMA in AF pathogenesis, although only a few studies have investigated the role of SDMA in this arrhythmia. In a study of 363 patients with acute cerebrovascular disease and 48 controls, high SDMA, but not ADMA, was independently associated with cardioembolic stroke with an OR of 9.2 in the fourth SDMA quartile compared to the first SDMA quartile (Wanby *et al.* 2006). SDMA was not associated with other types of cerebral infarction or transient ischemic attack. Cardioembolic infarction was equivalent to AF in this study, suggesting that SDMA levels at admission were associated with prevalent AF in acute cerebrovascular disease patients. The positive association between SDMA and AF was also present in a study investigating the predictive value of ADMA and SDMA in acute ischemic stroke patients (Schulze *et al.* 2010). The association between ADMA and AF was first reported as a side result in a study investigating the relation between ADMA and aortic stenosis (Ngo *et al.* 2007). In a large population-based study of 5000 individuals, SDMA was related to left atrial diameter and deceleration time whereas ADMA and L-NMMA were associated with left ventricular mass (Ramuschkat *et al.* 2016). ADMA and L-NMMA, but not SDMA, were related to prevalent AF. Using data from a community-based study, investigators tested the hypothesis of whether ADMA or SDMA were associated with incident AF over a follow-up time of ten years in 3,310 individuals and 247 incident AF cases (Schnabel *et al.* 2016). Neither dimethylarginine was associated with incident AF after accounting for several risk factors.

2.1.4.3 ADMA, SDMA and heart failure

Heart failure (HF) is a clinical syndrome (not a disease) characterised by typical symptoms such as breathlessness, ankle swelling and fatigue caused by structural and/or functional cardiac abnormality resulting in reduced cardiac output or intracardiac pressures at rest or during stress (Ponikowski *et al.* 2016). HF due to a cardiac abnormality results in either systolic or diastolic dysfunction. It can also be classified based on left ventricular ejection fraction (LVEF) to HF with normal LVEF (HF with preserved EF, HFpEF) and to those with reduced LVEF (HF with reduced EF, HFrEF). Diastolic dysfunction is generally accepted to be the likely cause of HFpEF which is characterised by impaired LV filling and LV that is not dilated. The New York Heart Association's (NYHA) functional classification system is commonly used to grade the severity of functional limitations in patients with HF. In the context of ADMA, SDMA and NOS, oxidative stress and impaired NO signalling are the hallmarks of HF that contribute to the cardiac abnormality causing this syndrome.

In 84 patients hospitalised due to HF, both ADMA and NOx levels were observed to be elevated in patients with severe HF (NYHA class III and IV) compared with patients with mild HF (NYHA class I and II) (Usui *et al.* 1998). In 285 patients with ischemic HF, ADMA levels were associated with NYHA functional classes and elevated N-terminal pro-B-type natriuretic peptide (NT-proBNP) level and was an independent predictor of long-term adverse clinical outcomes (Hsu *et al.* 2012). Similarly, in a study of 341 patients with chronic HF due to dilated (n=226) or ischemic (n=115) cardiomyopathy, both ADMA and SDMA levels were increased in patients with severe forms of HF (NYHA class III and IV) compared with milder forms of HF (NYHA class I and II) while no differences were observed between the two ethnologies (Anderssohn *et al.* 2012). In a multicentre study of primary care patients at cardiovascular risk, among 1,396 patients diastolic dysfunction was assessed by using standardised echocardiography, 900 had mild and 261 moderate/severe diastolic dysfunction, while in 261 patients diastolic dysfunction was ruled out (Pilz *et al.* 2014a). Higher ADMA and SDMA were independently associated with the severity of diastolic dysfunction. In a prospective study of 2,224 apparently healthy middle-aged German men and women, 195 participants developed HF during a mean follow-up of 8.3 years (Wirth *et al.* 2017). ADMA was positively associated with incident HF risk whereas a U-shaped association was observed between baseline SDMA and HF risk.

2.1.4.4 ADMA, SDMA and mortality

In the LURIC study, both ADMA and SDMA independently predicted total and cardiovascular mortality in ~3500 patients referred to coronary angiography (Meinitzer *et al.* 2011). In 394 patients with acute ischemic stroke, SDMA independently predicted total mortality irrespective of renal function during 7.4 years of follow-up (HR of 2.41, 95% CI 1.55 to 3.72, p<0.001) (Schulze *et al.* 2010). ADMA and SDMA are also predictive of overall mortality in population samples. In a multi-ethnic United States population, SDMA, but not ADMA, was independently associated with all-cause and cardiovascular mortality (Gore *et al.* 2013). In another community-based study, ADMA was associated with all-cause mortality, particularly in non-diabetic individuals (Boger *et al.* 2009). A recent systematic review and meta-analysis of prospective studies found that high ADMA levels were associated with all-cause mortality (relative risk [RR] of 1.52, 95% CI 1.37 to 1.68, 34 studies) and cardiovascular outcomes (RR of 1.33, 95% CI 1.22 to 1.45, 30 studies) compared to low ADMA levels (Schlesinger *et al.* 2016). Likewise, high SDMA levels were associated with all-cause mortality (RR of 1.31, 95% CI 1.18 to 1.46, 17 studies) and cardiovascular outcomes (RR of 1.36, 95% CI 1.10 to 1.68, 13 studies) compared to low SDMA levels.

2.2 L-homoarginine

2.2.1 Formation and clearance of hArg

In humans, L-homoarginine (hArg) and the creatine precursor guanidino acetic acid (GAA) are synthesised by the enzyme L-arginine:glycine amidinotransferase (AGAT), while the urea cycle plays a minor role in the homoarginine synthesis (Davids *et al.* 2012). AGAT utilises arginine and glycine to form ornithine and GAA while hArg and ornithine are synthesised from lysine and arginine by the same enzyme. In the second step of creatine synthesis, GAA is methylated by guanidinoacetate-*N*-methyltransferase to form creatine, which is used as an energy buffer in organs with high energy demand, e.g. in skeletal muscle, brain and heart. Creatine is non-enzymatically converted to creatinine, which is excreted to urine and is clinically used as a marker of renal function. In AGAT-deficient mice, plasma hArg concentrations were barely detectable while GAA

and creatine were also deficient in the AGAT$^{-/-}$ mice compared to wild-type littermates (Choe *et al.* 2013).

In *in vitro* and *in vivo* experiments, it was shown that hArg is utilised as a substrate in the NO synthesis by NOS instead of L-arginine (Chen & Sanders. 1993, Hecker *et al.* 1991). However, hArg is present at much lower concentrations in the circulation than L-arginine (~2 versus ~100 µM in humans) likely limiting the role of endogenously formed hArg in the NO synthesis under physiological conditions. In line with this view, ~95% of hArg supplemented to pigs and rats were excreted into urine and numerous tissues did not degrade hArg in these animals (Hou *et al.* 2016). However, significant increases in hArg plasma concentrations can easily be achieved in humans. In a double-blind, randomised, placebo-controlled crossover trial, hArg supplementation to 20 young volunteers resulted in several-fold increases in plasma hArg concentrations while any of the studied hemodynamic parameters or flow-mediated dilatation did not change with the hArg supplementation (Atzler *et al.* 2016c). Moreover, no adverse systemic effects resulted from the hArg supplementation despite a moderate increase in plasma glucose. The therapeutic potential of oral hArg remains to be tested in different pathological conditions in humans.

2.2.2 hArg and cardiometabolic risk factors

2.2.2.1 hArg and body mass index

A strong positive association between circulating hArg and body mass index (BMI) has been observed in several population-based studies (Atzler *et al.* 2014, van der Zwan *et al.* 2013) as well as in different patient populations (Atzler *et al.* 2016a, Drechsler *et al.* 2011, März *et al.* 2010). It has been of interest to investigate whether hArg is a marker of obesity or whether it plays a causal role in the development of excess body weight. Studies in AGAT-deficient mice exhibiting creatine and hArg deficiencies showed that the animals were completely protected from obesity and metabolic syndrome (Choe *et al.* 2013). However, these changes were completely reversed by oral creatine supplementation, indicating that the effect of AGAT deficiency on body weight is hArg-independent. Moreover, effects of bariatric surgery induced dramatic weight loss in four morbid obese individuals while plasma and tissue concentrations remained unchanged (May *et al.* 2015). Although the sample size

is small, this data suggests that a change in body weight within the same individual does not have an effect on hArg levels either. Moreover, oral hArg supplementation to animals or humans did not change body weight despite a several-fold increase in plasma hArg for several weeks (Atzler *et al.* 2016c, Stockebrand *et al.* 2015). This data suggests that hArg could be a biomarker for the predisposition for weight gain and obesity rather than affected by weight changes or plays a causal mediator role in the weight gain process itself.

2.2.2.2 hArg and other risk factors

hArg has also been consistently and positively associated with active smoking; however, the molecular mechanisms underlying this association remain unknown (Sobczak *et al.* 2014). hArg was reported to be associated with blood pressure in a population sample of older adults (van der Zwan *et al.* 2013). In the same study, hArg was found to be positively and independently associated with fasting glucose. In mice fed a high-fat diet, chronic oral hArg supplementation resulted in a 6-fold increase in plasma hArg, increased insulin secretion and reduced blood glucose, effects that were not seen in mice on a normal diet (Stockebrand *et al.* 2015). It has also been suggested that low hArg could be an early marker of decreased renal function and associated with the progression of chronic kidney disease (Drechsler *et al.* 2013, Ravani *et al.* 2013, Tomaschitz *et al.* 2014).

2.2.3 hArg and cardiometabolic diseases

2.2.3.1 hArg and atherosclerosis

The effects of hArg on vascular pathology have recently been studied in experimental settings. It was shown that hArg promotes vascular calcification by augmenting the osteo-/chondrogenic transformation of human aortic VSMCs (Alesutan *et al.* 2016). Another study using a model of vascular injury demonstrated that hArg had a beneficial role in balloon-injured rat carotids via inhibition of neointimal formation (Dellera *et al.* 2016).

There is little evidence from clinical studies to support the effects seen in these experimental studies. In the population-based Dallas Heart Study, there was a lack of cross-sectional association between hArg and coronary artery calcium

measured by electron beam-computed tomography (Atzler *et al.* 2014). Moreover, hArg was not cross-sectionally associated with prevalent CAD in a cohort of ~3000 patients referred for coronary angiography (Pilz *et al.* 2011a) or with peripheral arterial disease (Vogl *et al.* 2015). Neither did baseline hArg predict future cardiovascular events or MI in haemodialysis patients (Drechsler *et al.* 2011). Therefore, it is currently unclear whether endogenously synthetised hArg plays any role in the development of cardiovascular diseases in humans.

2.2.3.2 hArg and ischemic stroke

In an experimental ischemic stroke model, AGAT-deficient mice exhibited more severe cerebral damage and neurological deficits compared to controls (Choe *et al.* 2013). The effects of AGAT-deficiency in experimental ischemic stroke were attenuated by hArg supplementation. hArg plasma concentrations were negatively correlated with infarct size and neurological score after 24 hours in mice after 30 minutes' temporary middle cerebral artery occlusion. Plasma hArg was also negatively associated with the National Institutes of Health Stroke Scale+age score and 30-day mortality in 137 acute ischemic stroke patients (Choe *et al.* 2013).

2.2.3.3 hArg and mortality

Low levels of circulating hArg predict increased mortality from different causes and in different patient groups and population-based cohorts. In patient populations, low hArg was associated with an increased risk of death from cardiovascular diseases, including fatal strokes, sudden cardiac death (SCD), HF and treatment-naïve pulmonary hypertension (Atzler *et al.* 2013, Atzler *et al.* 2016a, Atzler *et al.* 2016b, Drechsler *et al.* 2011, März *et al.* 2010, Pilz *et al.* 2011b, Vogl *et al.* 2015). Associations with mortality endpoint have also been observed in a population-based study of older adults as well as in a multi-ethnic general United States population (Atzler *et al.* 2014, Pilz *et al.* 2014b). Interestingly, hArg was exclusively associated with death due to HF in subjects with reduced kidney function (Tomaschitz *et al.* 2014). One explanation for this observation is that low hArg is a marker of reduced creatine synthesis and associated energy metabolism in the myocardium of the failing hearts.

3 AIMS OF THE STUDY

High ADMA, high SDMA and low hArg predict an increased risk of cardiovascular and overall mortality. However, their genetic determinants and the underlying molecular mechanisms are poorly understood. Genetic variation affecting concentrations of these endogenous arginine derivatives in circulation might also have an effect on the risk of CAD, MI, ischaemic stroke or mortality, although this has not been previously studied.

The specific aims of the present study were to:

1. Reveal novel genome-wide significant associations between common genetic variants and serum ADMA, SDMA and hArg levels by means of GWAS (studies I and II).
2. Identify associations of newly identified functional *AGXT2* variants (rs37369, Val140Ile; rs16899974, Val498Leu) with heart rate variability, sudden cardiac death and overall mortality in healthy young adults and patients referred for coronary angiography, respectively (I).
3. Identify associations between functional *AGXT2* variants (rs37369 and rs16899974) and atrial fibrillation and its age-related thromboembolic complications, i.e. ischemic stroke, in various patient populations (III).
4. Characterise the biomarker and causal roles of serum hArg in the development of cardiometabolic diseases using Mendelian randomisation (IV).

4 MATERIALS AND METHODS

4.1 Study cohorts and data sources

4.1.1 The Cardiovascular Risk in Young Finns Study (YFS) (I, II & IV)

The YFS is a longitudinal, ongoing prospective Finnish population study on the evolution of cardiovascular risk factors from childhood to adulthood (Raitakari *et al.* 2008). The study began in 1980, when 3,596 children and adolescents aged 3–18 years were randomly selected from five university hospital catchment areas in Finland. In 2001, 2,288 participants aged 24–39 years attended the 21-year follow-up. Thereafter, the subjects have been followed up in 2007 and 2011 with comprehensive risk factor assessments and examinations including physical measurements, blood tests and questionnaires.

In the GWASs on ADMA, SDMA (I) and hArg (II), we used the data collected in 2001. For the GWAS on dimethylarginines, simultaneous genotype, covariate, and phenotype data were available for 2,024 subjects, whereas the data was complete for 2,102 individuals in the hArg GWAS.

In study IV, 2,106 participants contributed to the cross-sectional association analysis of hArg and cardiometabolic risk factors at baseline in 2001 after excluding 33 pregnant women, 11 subjects who had type 1 diabetes mellitus at baseline and one participant who had an exceptionally high hArg serum concentration (20.2 µmol/L). For the longitudinal analyses, we included individuals who attended the 2001 follow-up and at least once later in 2007 or 2011, and who had hArg and covariate data at baseline (in 2001) and cardiometabolic outcome data at the 2007 and/or 2011 follow-up and who were not pregnant in 2001 (n=1,801).

4.1.2 The Ludwigshafen Risk and Cardiovascular Health (LURIC) study (I-III)

The LURIC study consists of 3,316 Caucasian patients referred for coronary angiography at a tertiary care centre in Southwestern Germany due to chest pain

or suggestive myocardial ischemia in a stress test during the years 1997 to 2000 (Winkelmann *et al.* 2001). Patients suffering from chronic non-cardiac diseases, including a malignancy within the five past years or acute illnesses other than ACS, were excluded to limit clinical heterogeneity.

For the GWAS on ADMA and SDMA (I), all the necessary genotype, phenotype and covariate data were available for 3,027 LURIC patients. For the subsequent association analyses of cardiovascular outcomes, all the necessary genotype, covariate and cause of death data were available for 2,756 individuals. For the hArg GWAS (II), homoarginine levels, genotypes and covariates were available for 3,041 LURIC participants. In study III, 2,923 LURIC patients had both genotype and AF status data available and were included.

4.1.3 The Finnish Cardiovascular Study (FINCAVAS) (III)

The FINCAVAS study consists of all consecutive patients referred for a clinically indicated exercise test using a bicycle ergometer at Tampere University Hospital between October 2001 and the end of 2008 and willing to participate (Nieminen *et al.* 2006). A total of 4,068 participants had a technically successful exercise test. The main indications for the exercise test were suspicion of CAD (46%), evaluation of work capacity (26%), testing vulnerability to arrhythmia during exercise (25%), and adequacy of the CAD treatment (13%); some patients had more than one indication.

4.1.4 Other cohorts (III)

The Corogene study included 5,295 consecutive Finnish patients assigned to coronary angiogram in four hospitals servicing 1.5 million people in the Hospital District of Helsinki and Uusimaa (Vaara *et al.* 2012). Of the Corogene study, 2,500 patients with ACS (ICD-10: I20–I25) were genotyped on GWAS arrays. The ischemic stroke GWAS by Wellcome Trust Case Control Consortium 2 (WTCCC2) included samples from the United Kingdom (UK) and Germany, with a total of 3,548 cases and 5,972 controls (Bellenguez *et al.* 2012).

4.1.5 Selection of genetic instruments and publicly available summary-level data from large-scale GWASs (IV)

In study IV, we selected three SNPs associated with serum hArg at genome-wide significance in study II to be used as genetic instruments in a Mendelian randomisation (MR) analysis – *GATM* (glycine amidinotransferase) rs1153858, *CPS1* (carbamoyl-phosphate synthase 1) rs1047891 (formerly rs7422339), and *AGXT2* (alanine-glyoxylate aminotransferase 2) rs37369. The summary statistics for the SNP-outcome associations were extracted from the summary-level data available in the public domain from large GWASs investigating the associations of genetic variants with cardiometabolic risk factors, serum metabolites and diseases, including BMI (Locke *et al.* 2015, Winkler *et al.* 2015), waist circumference (Shungin *et al.* 2015), fasting glucose (Dupuis *et al.* 2010), fasting insulin (Dupuis *et al.* 2010), HbA1c (Soranzo *et al.* 2010), proinsulin (Strawbridge *et al.* 2011), total cholesterol (Willer *et al.* 2013), high-density lipoprotein (HDL) cholesterol (Willer *et al.* 2013), triglycerides (Willer *et al.* 2013), low-density lipoprotein (LDL) cholesterol (Willer *et al.* 2013), 122 nuclear magnetic resonance (NMR)-method-based serum metabolites (Kettunen *et al.* 2016) as well as CAD (Nikpay *et al.* 2015, Schunkert *et al.* 2011) and T2DM (Mahajan *et al.* 2014). For more detail, see online Supplementary Information of study IV.

4.2 Genotyping and imputation methods

4.2.1 YFS (I-IV)

As previously described (I-IV), genomic deoxyribonucleic acid (DNA) was extracted from peripheral blood leukocytes using a commercially available kit and the Qiagen BioRobot M48 Workstation according to the manufacturer's instructions (Qiagen, Hilden, Germany). Genotyping was performed on 2,556 samples using a custom-built Illumina Human 670k BeadChip at the Welcome Trust Sanger Institute. Genotypes were called using the Illuminus clustering algorithm. Fifty-six samples failed to meet the Sanger genotyping pipeline quality control (QC) criteria, one failed the gender check and three were removed due to low genotyping call rate (<0.95) and 54 for possible relatedness (pi-hat >0.2). Based on the Hardy-Weinberg equilibrium (HWE) test, 11,766 SNPs were

excluded ($p \leq 10^{-6}$), and 7,746 SNPs failed the missingness test (call rate <0.95) and another 34,596 SNPs failed the frequency test (minor allele frequency, MAF <0.01). Following QC, 2,442 samples and 546,677 genotyped SNPs were available for further analysis (Smith *et al.* 2010).

In study I, genotype imputation was performed using IMPUTE2 (Howie *et al.* 2012) and 1000 Genomes Phase I Integrated Release Version 3 (March 2012) samples as a reference. We excluded imputed SNPs and insertion/deletion polymorphisms (indel) with low imputation quality (info <0.4) and allele frequency of <0.1% leaving 10,085,758 imputed genetic variants for analysis.

In study II, genotype imputation was performed using MACH 1.0 (Li *et al.* 2010) and HapMap II CEU (release 22, National Centre for Biotechnology Information build 36, dbSNP 126) samples as a reference. Imputed 2,543,887 SNPs with a squared correlation of ≥0.3 between imputed and true genotypes were used in the GWAS.

4.2.2 LURIC (I-III)

As previously described (I-III), genomic DNA was prepared from EDTA-anticoagulated peripheral blood using a common salting-out procedure. Genotyping was accomplished by using the Affymetrix Genome-Wide Human SNP Array 6.0. The following QC filters were applied: individual call rate <0.95, SNP call rate <0.98, HWE p-value $<10^{-4}$, and MAF <0.01. Following QC, 3,061 samples and 686,195 genotyped SNPs were available for further analysis.

In study I, genotype imputation was performed using minimac (Howie *et al.* 2012) and 1000 Genomes (March 2012) samples as a reference. After excluding variants with low imputation quality (minimac Rsq <0.3) and low MAF (<0.1%), 10,085,758 imputed genetic variants were available in both the YFS and LURIC.

In study II, genotype imputation was performed using MACH 1.0 (Li *et al.* 2010) and HapMap II CEU (release 22, NCBI build 36) samples as a reference. The same post-imputation filtering criterion (Rsq <0.3) was used as in the YFS leaving 2,543,887 SNPs for analysis.

In study III, genotypes were imputed using the IMPUTE2 software and the 1000 Genomes March 2012 haplotypes as a reference. Genotyped data was used for rs37369 and imputed data for rs16899974 with an excellent imputation quality (info ~0.935).

4.2.3 FINCAVAS (III)

We genotyped rs16899974 successfully for 3,889 participants using Taqman@SNP Genotyping Assay C__25742181_10 and ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). No evidence of deviation from the HWE was observed ($\chi_1^2 = 0.21$, p = 0.65). The data for rs37369 and rs6817105 were obtained for 3,195 individuals by genotyping using the Illumina HumanCardio-Metabo BeadChip or HumanCoreExome chip arrays and imputation using the IMPUTE2 software and 1000 Genomes March 2012 haplotypes as a reference.

4.2.4 Other cohorts (III)

The Corogene cohort was genotyped with Illumina 660 K BeadChip array at the Sanger Institute (Hixton, Cambridge, UK) and imputed using the 1000 Genomes April 2012 reference panel as a reference. For the WTCCC2 samples, Illumina BeadChips were used for genome-wide genotyping and genotype imputation was performed using MACH based on HapMap Phase 2 European (CEU) reference data. In Study II, we used the Tampere Vascular Study (TVS) to study whether the lead variant at the *GATM* locus was also an expression quantitative locus in arterial tissues, peripheral blood and monocytes.

4.3 Biochemical methods

4.3.1 Quantification of serum ADMA, SDMA and L-homoarginine (I-IV)

For both YFS and LURIC participants, venous blood samples were drawn after an overnight fast. Blood glucose, cholesterol, and triglycerides were measured by standard laboratory procedures. In YFS and LURIC (I and III), ADMA and SDMA were measured from frozen serum (−80 °C) using reversed-phase high-performance liquid chromatography (HPLC) method (Teerlink *et al.* 2002), with slight modifications (Meinitzer *et al.* 2007a). The within-day CVs for ADMA were 3.1% (0.62 μmol/L) and 1.0% (2.0 μmol/L), and the between-day CVs 9% (0.62 μmol/L) and 1.5% (2.0 μmol/L). The within-day CVs for SDMA were 4.6%

(0.60 μmol/L) and 1.9% (1.0 μmol/L), and the between-day CVs were 9.8% (0.60 μmol/L) and 6.1% (1.0 μmol/L).

In studies II and IV, hArg was measured from frozen serum (−80 °C) using the same reversed phase HPLC method as ADMA and SDMA. Intraday coefficients of variation at different concentrations (mean levels) were 4.7% (1.21 μmol/L) and 2.2% (3.53 μmol/L), and between-day coefficients of variation were 7.9% (1.25 μmol/L) and 6.8% (3.66 μmol/L) (Meinitzer *et al.* 2007a). All quantifications of the arginine derivatives were performed centrally at the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz.

4.3.2 Metabolic profiling (IV)

High-throughput NMR spectroscopy was used for the absolute quantification of serum metabolites. The metabolite set (includes 228 quantified metabolites) covers multiple metabolic pathways, including amino acids and glycolysis precursors, lipoprotein lipids and subclasses as well as fatty acids and fatty acid compositions. All molecular measures are quantified in a single experimental setup. This NMR-based metabolite profiling has previously been used in various epidemiological (Wang *et al.* 2015, Wang *et al.* 2016) and genetic studies (Kettunen *et al.* 2016) and has been recently reviewed (Soininen *et al.* 2015). Details of the experimentation have been described elsewhere (Soininen *et al.* 2009, Soininen *et al.* 2015).

4.4 Definition of subclinical and clinical cardiometabolic outcomes

4.4.1 Heart rate variability (I)

In study I, spectral short-term heart rate variability (HRV) components were measured in the YFS participants as described previously (Koskinen *et al.* 2009a, Koskinen *et al.* 2009b). In brief, a single-channel chest-lead electrocardiogram (ECG) was recorded during a 3-min period of metronome-controlled breathing at a frequency of 0.25 Hz. The ECG signal was collected after the participants had remained comfortably in a supine position for at least 15 min during a vascular ultrasound scan. Frequency domain components were analysed using the

commercial WinCPRS program (Absolute Aliens, Turku, Finland), a program for the general analysis of physiological data. The HRV spectrum was computed using the non-parametric fast Fourier transformation method.

4.4.2 Sudden cardiac death (I)

The LURIC cohort was followed for mortality and no patients were lost to follow-up. Causes of death were independently classified as cardiovascular deaths and others by three experienced clinicians blinded to any data on the study patients. Cardiovascular deaths included the following categories: sudden death, fatal MI, death due to congestive HF, death immediately after intervention to treat CAD, fatal stroke and other causes of death due to CAD. During a median follow-up period of 8.0 years, 694 patients with available genetic data died. Of the total deaths, 430 were due to cardiovascular causes, including 173 SCDs, 100 from congestive HF, 77 from fatal MI and 40 from fatal strokes. SCD was defined as sudden unexpected death either within 1 h of symptom onset or within 24 h of having been observed alive and symptom-free. Patients whose death was not unexpected (any non-cardiac chronic and terminal disease) or whose sudden death was most likely attributed to a non-cardiac cause were not classified as SCDs. (Pilz *et al.* 2007)

4.4.3 Atrial fibrillation and Ischemic stroke (III)

4.4.3.1 Classification of atrial fibrillation cases (III)

In LURIC, 2,923 patients had both genotype and AF status data available. Of the 2,923 participants, 360 had a history of AF at baseline. In addition, 161 individuals were in AF rhythm during the index coronary angiography, of which 21 were not included in the 360 cases previously diagnosed. Therefore, a total of 381 individuals were classified as any AF. Of the 381 AF cases, 348 were further classified as having either paroxysmal AF (n = 175) or chronic AF (n=173).

For the Corogene study, potential prevalent AF cases were screened from the baseline database. For these patients, we ascertained the AF status and its subtype from their medical records. Of the 2,208 patients included in this study, 265, 141 and 107 had any AF, paroxysmal AF and chronic AF, respectively.

For the FINCAVAS participants, clinical AF was ascertained from the central university hospital discharge diagnostic codes (ICD-9 427.3, 427.31, or 427.32; or ICD-10 I48) from 1987 to 2015 (1179 incident AF cases), and study population area-wide electrical ECG recordings from 2005 onward (16 additional incident AF cases). In addition, to further ascertain the AF status at the index exercise stress test, we utilised the data from the baseline examination. According to the FINCAVAS baseline database, 13 patients had a history of AF/flutter or developed one during the exercise stress test but did not have any previous discharge diagnosis of AF with the date of diagnosis and were therefore excluded from all analyses. Both the genotype and phenotype data were available for 1,188 incident AF cases and 2,674 censored controls.

4.4.3.2 Classification of ischemic stroke cases (III)

For the WTCCC2 ischemic stroke cohorts, Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification (Adams *et al.* 1993) was performed by an in-house neurologist and all stroke cases were classified into mutually exclusive etiologic subtypes: large-artery atherosclerosis, small-vessel disease, cardioembolic stroke, other aetiology, or unknown aetiology (Bellenguez *et al.* 2012).

For the FINCAVAS cohort, age at the first ischemic stroke was ascertained from the Central University Hospital discharge diagnostic codes (ICD-9 433.x1, 434 (excluding 434.x0), or 436; or ICD-10 I63.0–I63.9). Of the 3,862 individuals with both genotype and phenotype data available, there were 360 incident ischemic stroke cases diagnosed between 1987 and 2015. In the majority of the cases, 233 (65%), the aetiology was uncertain at the time of the diagnosis and was therefore diagnosed as a cryptogenic ischemic stroke (I63.9).

4.4.4 Markers of preclinical atherosclerosis (IV)

In 2008, cardiac computed tomography was performed to measure coronary artery calcification (CAC) for a subsample of 589 participants then aged 40 to 46 years (Hartiala *et al.* 2012). The absence of CAC was defined as an Agatston score of 0, and individuals with an Agatston score of 1 or greater were classified as having CAC. In 2001 and 2007, carotid artery intima-media thickness (IMT) and distensibility were measured, as previously described in detail (Juonala *et al.*

2005, Raitakari *et al.* 2003). As previously (Laitinen *et al.* 2015), high-risk carotid IMT and distensibility were defined as falling within the age- and sex-specific ≥90 percentile and ≤10 percentile, respectively.

4.4.5 Incident metabolic syndrome components, obesity and type 2 diabetes mellitus (IV)

In the YFS participants, the metabolic syndrome components were defined according to harmonised criteria (Alberti *et al.* 2009): waist circumference ≥102 cm in males and ≥88 cm in females (1), hypertriglyceridemia >1.7 mmol/l (2), HDL cholesterol <1.0 mmol/l in males and <1.3 mmol/l in females (3), blood pressure ≥130/85 mmHg or treatment for blood pressure (4), and fasting plasma glucose ≥5.6 mmol/l or previously diagnosed T2DM. High insulin was defined as a 10-year fasting insulin in the >90th percentile using sex-specific values corresponding with a fasting insulin of ≥18.8 IU/L for men and ≥15.2 IU/L for women. Obesity was defined as BMI ≥30 kg/m^2. Participants were classified as having T2DM if they had HbA1c ≥6.5% (48 mmol/mol), a fasting plasma glucose level of ≥7.0 mmol/l, reported the use of oral glucose-lowering medication or insulin but had not reported having type 1 diabetes mellitus or reported a diagnosis of T2DM by a physician.

4.5 Statistical Methods

4.5.1 GWASs of ADMA and SDMA (I)

To correct for potential population stratification, we performed multidimensional scaling (MDS) analyses as implemented in PLINK version 1.07 (Purcell *et al.* 2007) for the YFS and LURIC samples. In YFS and LURIC, ADMA, SDMA and ADMA/SDMA ratio were adjusted for sex, age, BMI and serum creatinine to account for variation in renal function, as well as the first three MDS components to control for population stratification after excluding extreme outliers of ≥4 standard deviations (SD) from the mean by using the R statistical software. As circulating ADMA is a major determinant of SDMA levels and vice versa (Meinitzer *et al.* 2011), the model for ADMA was additionally adjusted for

SDMA and, for SDMA, was adjusted for ADMA. Residuals were normalised to have a mean of 0 and SD of 1 using inverse-normal transformation.

The GWASs, assuming an additive genetic model, were conducted utilising the SNPTEST (version 2.4.1) and ProbABEL (version 0.3.0) (Aulchenko *et al.* 2010) softwares in YFS and LURIC, respectively. The results were visualised using quantile-quantile and Manhattan plots. The calibration of the distribution of p-values was estimated by calculating the genomic inflation factor (λ) for each phenotype in both studies and ranged from 0.993 to 1.012, indicating that the test statistics were not inflated. Regional association plots were crated with LocusZoom (Pruim *et al.* 2010). The random-effects meta-analysis method implemented in GWAMA was used to meta-analyse the summary results from both cohorts (Magi & Morris. 2010). A genome-wide significance level of 1.6×10^{-8} was set to account for multiple testing.

Fine-mapping of the *AGXT2* locus was carried out using a series of sequential conditional analyses. We added the most strongly associated SNP into the regression model as a covariate and used the inverse-normal transformed residuals as an outcome variable to test all remaining regional SNPs for association. The results from both cohorts were meta-analysed and the procedure was repeated until the strongest SNP showed a conditional $P>10^{-4}$.

4.5.2 GWAS of hArg (II)

Raw hArg serum levels were adjusted for principle components to control for population stratification and additionally for sex, age, and BMI followed by inverse normal transformation to obtain a normal distribution. The residuals from the linear regression models were used as outcome variables in the GWAS using the QUICKTEST software in LURIC and ProbABEL (Aulchenko *et al.* 2010) in YFS. The results were visualised using quantile-quantile and Manhattan plots. Regional plots were drawn using Locuszoom (Pruim *et al.* 2010). The usual threshold of $P <5\times10^{-8}$ was used for genome-wide significance. A fixed-effect, effective sample-weighted Z-score meta-analysis method implemented in the software METAL was used to combine the results from both studies (Willer *et al.* 2010).

4.5.3 Associations of functional *AGXT2* variants with HRV parameters and SCD (I)

To test whether the *AGXT2* and *SLC25A45* genotypes were associated with mortality endpoints, a Cox regression proportional hazards analysis was conducted. Schoenfeld's residuals were studied to check the proportional hazards assumption was met for all models. Additive genetic models were adjusted for age and sex and, additionally, for well-established SCD risk factors. We additionally stratified the analyses for SCD according to the median value of left ventricular systolic pressure to study the effect modification by cardiac systolic function. Linear regression analysis was used to test the associations with HRV parameters.

4.5.4 Associations of functional *AGXT2* variants with AF and Ischemic stroke (III)

Statistical analyses were performed under the R statistical environment. The associations of the *AGXT2* and 4q25 variants with AF and its subtypes were tested using multivariable logistic regression analyses assuming an additive genetic model. In addition, we tested the interactions of the studied polymorphisms with established AF risk factors on any AF in LURIC. For the WTCCC2 cohorts, associations of the *AGXT2* variants were tested with unadjusted logistic regression using PLINK (Purcell *et al.* 2007) under an additive genetic model and the results were meta-analysed with inverse-variance-weighted method implemented in the METAL software (Willer *et al.* 2010).

Survival analyses were used to assess associations with incident AF/stroke and differences in age at AF/stroke onset as a function of the studied genetic variants. In addition, for the age of onset analyses, we applied linear regression considering the age of onset as a quantitative trait. Survival curves of time to AF/stroke onset were estimated using the Kaplan–Meier method. We used a Cox regression analysis to examine the effect of the SNPs together with covariates on the survival functions of incident AF and ischemic stroke. We used chronological age as the fundamental time scale in all analyses. All patients were followed to a fixed date (April 2015). Those with no incident AF or ischemic stroke during the observation period were right censored at their last visit at the central hospital or at their last electrical ECG recording in the community, whichever came later. Significance was accepted at $P < 0.05$ in all analyses.

4.5.5 Cross-sectional and longitudinal associations of hArg with cardiometabolic outcomes in YFS (IV)

All statistical analyses were conducted with R version >3.1.2. We used linear regression analysis to explore the associations of hArg with common cardiovascular risk factors at baseline (age, sex, LDL cholesterol, HDL cholesterol, triglycerides, systolic and diastolic blood pressure, CRP, glucose, insulin, BMI, waist circumference, smoking and family history of CAD) as well as serum sex hormone-binding globulin (SHBG) and hormonal oral contraceptive use in women. Stepwise model selection by Akaike's information criterion was performed to determine the most important determinants of hArg levels using the stepAIC R function.

To facilitate the log-transformation, zero values in NMR-based metabolic measures were replaced by half of the minimum positive value found for each metabolite, assuming this to be the best estimation of detection limit. Prior to statistical analyses, NMR-based metabolic measures were log-transformed and scaled to SDs to facilitate comparisons across metabolites. Association magnitudes are reported in SD units of metabolite concentration per 1-SD increment in log-transformed hArg. Sex-specific scaling was applied for metabolites in cross-sectional analyses. Due to the correlated nature of the systemic metabolite measures, over 95% of the variation in the metabolic data was explained by 25 principle components, and a P of 0.05/25=0.002 was thus required after multiple testing correction (Wang *et al.* 2015).

Cross-sectional and longitudinal associations of baseline hArg with circulating metabolites at baseline and at the follow-ups were analysed using linear regression models, stratified by sex, with each metabolic measure as the outcome and hArg as the explanatory variable. The regression models were adjusted for age, BMI, daily smoking, serum SHBG and oral contraceptive use (in women). For each metabolic measure, the sex-related differences in observational estimates were tested by using the Z-statistic. Interactions between BMI and each metabolite measure in relation to hArg levels were tested in men and women separately by adding the interaction term to the linear regression models.

The prospective associations of hArg with 6- or 10-year incident Mets components, high insulin and obesity (BMI >30 kg/m^2) were assessed using logistic regression models with scaled log-transformed baseline hArg as an explanatory variable. Incident T2DM during the 10-year follow-up was used as an outcome variable to assess the prospective association of hArg with T2DM. The follow-up period for carotid artery ultrasound parameters was six years. CAC

was assessed once, seven years after the baseline in 2008. Those with the condition at baseline were excluded from the analysis. We fitted unadjusted models (Model 1) and models that were adjusted for various cardiometabolic risk factors (Model 2) and, further, for serum SHBG and oral contraceptive use (in women, Model 3).

4.6 Mendelian randomisation analysis using summary-level data for hArg related genetic variants (IV)

The strengths of the genetic instruments used for hArg were assessed based on the F-statistic derived from the summary statistics of the hArg GWAS. All F-statistics for the instruments were well over 10 (between 120 and 370), indicating that all three instruments separately are sufficiently strong to be used in MR studies. We then used these instruments in the MR analyses to quantify the strengths of the causal associations of hArg with metabolites, cardiometabolic traits, T2DM and CAD. As previously (Surendran *et al.* 2016), to evaluate combined causal estimates with 95% confidence intervals (95% CI) from summary statistics of the three independent SNPs, we performed a weighted linear regression of the genetic associations with each outcome variable on the genetic associations with hArg using first-order weights described in more detail elsewhere with an R code implementation (Burgess & Bowden. 2015). This multiplicative random-effects model was used for combining causal estimates to obtain the same point estimate as from a fixed-effects model while avoiding overly precise estimates in the case of heterogeneity in the causal effects obtained from different genetic variants. The combined MR estimates are in units of 1-unit increments of a continuous outcome (or odds ratio of disease risk) per 1-µmol/L increment in hArg. We performed heterogeneity tests based on Cochran's Q statistic to detect potential dissimilarity between the causal effects across the hArg SNPs (instrumental variables).

4.7 Ethics (I-IV)

All the studies were conducted according to the Declaration of Helsinki principles and written informed consent was obtained from all the participants. YFS has been approved by the 1st Ethical Committee of the Hospital District of Southwest Finland and by local ethical committees. The LURIC study was approved by the

ethics committee at the Ärztekammer Rheinland-Pfalz. The FINCAVAS study was approved by the Ethical Committee of the Hospital District of Pirkanmaa, Finland. The Corogene study was approved by appropriate Ethics Committees of the Helsinki and Uusimaa Hospital region. The recruitment of patients for the WTCCC2 ischemic stroke cohorts was approved by the relevant local ethics committees from all the participating centres.

5 RESULTS

5.1 GWAS on serum ADMA and SDMA levels (I)

The characteristics of the study populations used in studies I and II are shown in **Table 1**.

Table 1. Characteristics of the study populations in studies I and II.

	YFS	LURIC
Study I	n = 2,083	n = 3,027
Clinical characteristics		
Age, years	32 ± 5	63 ± 11
Men, %	45	70
Body mass index, kg/m^2	25 ± 4	27 ± 4
Hypertension, %	1.9	59
Current smokers, %	24	19
Diabetes mellitus, %	0.6	40
Coronary artery disease, %	-	69
History of MI, %	-	41
Systolic BP, mm Hg	117 ± 13	141 ± 24
Diastolic BP, mm Hg	71 ± 11	81 ± 11
Laboratory measures		
ADMA, µmol/L	0.60 ± 0.08	0.82 ± 0.15
SDMA, µmol/L	0.59 ± 0.10	0.58 ± 0.19
ADMA/SDMA	1.04 ± 0.18	1.52 ± 0.37
HDL cholesterol, mmol/L	1.29 ± 0.32	0.99 ± 0.28
LDL cholesterol, mmol/L	3.26 ± 0.84	2.99 ± 0.88
Triglycerides, mmol/L	1.33 ± 0.86	1.93 ± 1.38
Study II	n = 2,102	n = 3,041
Age, years	32 ± 5	63 ± 11
Men, %	45	70
Body mass index, kg/m^2	25 ± 4	28 ± 4
hArg, µmol/L	1.8 (1.4-2.2)	2.4 (1.8-3.1)

Statistics: Values are means ± SDs in cases of continuous variables and percentages in cases of categorical variables. HArg is presented as median (25th and 75th percentile).
Abbreviations: MI, myocardial infarction; BP, blood pressure; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

The YFS participants were much younger (mean age 32 years) and more often women (45% men) than the patients undergoing coronary angiography in the LURIC study (63 years, 70% men). The YFS participants were free of clinical CAD whereas, in contrast, 69% and 41% of the LURIC patients had a history of CAD and MI. ADMA and hArg levels were higher in LURIC than in YFS.

To gain an insight into the genetics of circulating ADMA and SDMA levels in a hypothesis-free manner, we conducted GWASs using the data from the YFS and LURIC cohorts and combined the genome-wide results by using a random-effects meta-analysis. We used ADMA, SDMA and ADMA/SDMA ratio as outcome variables in separate GWASs. The results from the meta-analyses of the three dimethylarginine traits are depicted in **Table 2**.

For ADMA, a genome-wide significant signal ($P < 5 \times 10^{-8}$) was detected in an intron of the *DDAH1* gene. Three independent signals from different chromosomes were detected for SDMA in or near *DDAH1*, *AGXT2* and *SLC25A45*. The analysis with ADMA/SDMA ratio did not reveal any additional independent signals at genome-wide significance.

Following this, we fine-mapped the novel *AGXT2* locus for independent association signals by using SDMA as an outcome variable. We used 1000 Genomes imputed data sets in both studies that contained much denser sets of SNPs and indels than the HapMap II CEU reference panel widely used for genotype imputation at the time of the study. The results from the two first rounds of our fine-mapping procedure are shown as regional plots in **Figure 4**. The results from the GWAS meta-analysis for SDMA are shown in the panel A. The most significant variant in the *AGXT2* region is the coding sequence variant rs37369 (Val140Ile) which changes valine to isoleucine at position 140 of the AGXT2 protein. When further adjusting the analysis for rs37369, another missense variant rs16899974 (Val498Leu) showed an independent effect at genome-wide significance on the residual SDMA values.

Table 2. Genome-wide significant associations from GWASs on ADMA, SDMA and ADMA/SDMA ratio.

Trait	Variant	Locus	Chr.	Position	EA	NEA	EAF (%)	n	β (SE)	P-value
ADMA	rs28489187	Intron of *DDAH1*	1	85 797 110	G	A	21.6	5105	0.37 (0.028)	1.39×10^{-40}
SDMA	rs37369	CDS of *AGXT2* (V140I)	5	35 037 115	T	C	8.8	5103	0.47 (0.035)	1.42×10^{-40}
SDMA	rs1884139	Intron of *DDAH1*	1	85 845 998	T	G	37.1	5103	−0.18 (0.020)	9.65×10^{-19}
SDMA	rs34400381	CDS of *SLC25A45* (R285C)	11	65 143 892	A	G	4.3	5102	0.36 (0.056)	2.48×10^{-10}
ADMA/SDMA	rs37369	CDS of *AGXT2* (V140I)	5	35 037 115	T	C	8.8	5102	−0.41 (0.035)	2.08×10^{-32}
ADMA/SDMA	chr1:85897175:D	Intron of *DDAH1*	1	85 897 175	A	ATCC	17.4	5102	0.27 (0.027)	3.85×10^{-24}

Statistics: Values are means ± SDs in cases of continuous variables and percentages in cases of categorical variables. hArg is presented as median (25th and 75th percentile).

Abbreviations: Chr., chromosome; EA, effect allele; NEA, non-effect allele; EAF, effect allele frequency; β, beta-coefficient; SE, standard error; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; CDS, coding sequence.

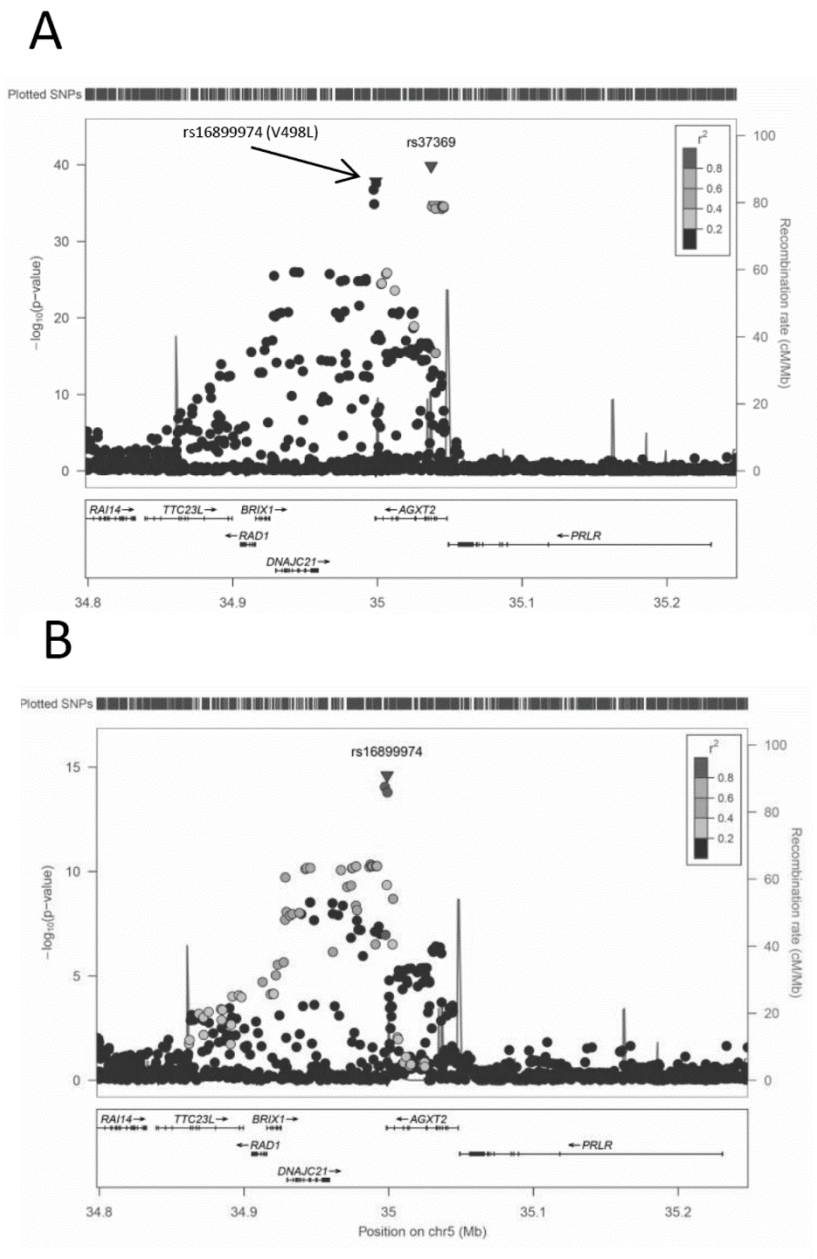

Figure 4. Fine-mapping results of the *AGXT2* gene region for multiple independent signals. Shown are the two first rounds of the fine-mapping procedure. Panel A shows the regional association results for SDMA from the original GWAS, panel B illustrates the association results after further adjusting the analysis for the lead SNP rs37369 from panel A. Modified from study I.

5.2 GWAS on serum hArg levels (II)

The study populations in the hArg GWAS (study II) were essentially the same as in the ADMA/SDMA GWAS (study I) (**Table 1**). **Figure 5** shows the genome-wide meta-analysis results from the hArg GWAS as a Manhattan plot. The association results for the lead variants at three genome-wide significant and one suggestive loci are shown in **Table 3**.

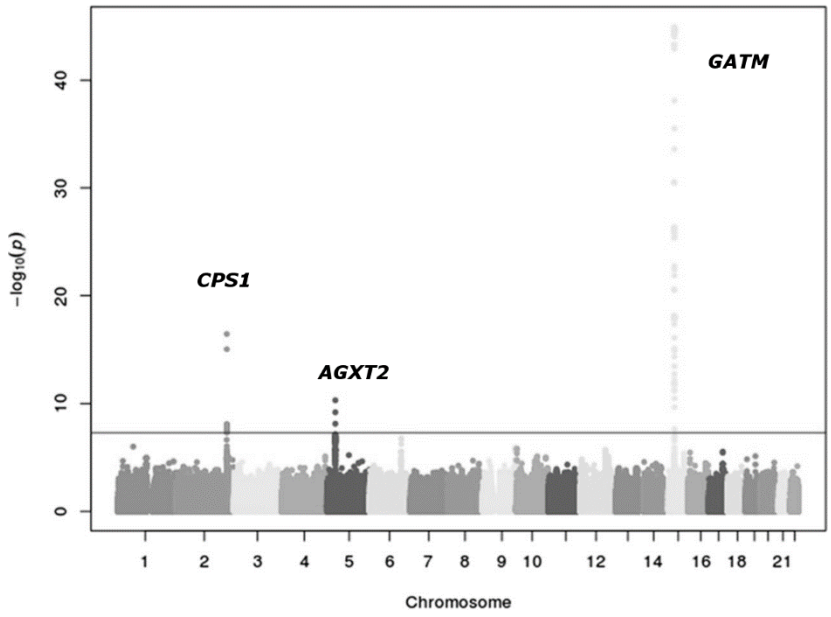

Figure 5. Manhattan plot from the results of genome-wide meta-analysis of serum hArg. Each dot represents 1 SNP present in the meta-analysis dataset, and its *y* axis coordinate indicates significance level in association with homoarginine. Modified from study II.

We identified three genome-wide significant loci near the *CPS1*, *AGXT2* and *GATM* genes on chromosomes 2, 5 and 15, respectively. A suggestive association signal was detected near the *MED23* and *ARG1* genes on chromosome 6. The lead variants in *CPS1* and *AGXT2* are amino acid changing missense variants. The *AGXT2* rs37369 SNP is the same variant as identified in the ADMA/SDMA GWAS for SDMA. The lead variant at the *GATM* gene region is located in an intron of the *GATM* gene.

Table 3. Genome-wide significant associations from GWASs on hArg in YFS, LURIC and combined meta-analysis.

				EAF		β		P-value			
SNP	**Chr.**	**Position**	**Alleles**	**YFS**	**LURIC**	**YFS**	**LURIC**	**YFS**	**LURIC**	**Combined**	**Candidate gene**
rs7422339	2	211248752	C/A	0.64	0.69	0.148	0.189	3.2×10^{-14}	1.2×10^{-9}	3.6×10^{-17}	*CPS1*
rs37369	5	35072872	C/T	0.90	0.92	−0.222	−0.220	1.7×10^{-9}	4.4×10^{-6}	5.0×10^{-11}	*AGXT2*
rs17060430	6	131960430	G/A	0.94	0.97	−0.154	−0.308	1.1×10^{-3}	4.0×10^{-5}	1.8×10^{-7}	*MED23/ARG1*
rs1153858	15	43439995	C/T	0.70	0.72	−0.253	−0.271	6.8×10^{-27}	1.0×10^{-21}	1.3×10^{-45}	*GATM*

Notes: Alleles indicate effect allele/reference allele**.**
Abbreviations: Chr, chromosome; EAF, effect allele frequency; β, beta-coefficient.

We studied the function of the *GATM* intron variant utilising genome-wide expression and genotype data from the Tampere Vascular Study (TVS) series. The results from the expression quantitative trait loci (eQTL) analysis for the proxy SNP rs1346268 in three different tissues are illustrated in **Figure 6**. In all three tissues studied, the hArg increasing allele at rs1346268 was associated with higher *GATM* mRNA levels.

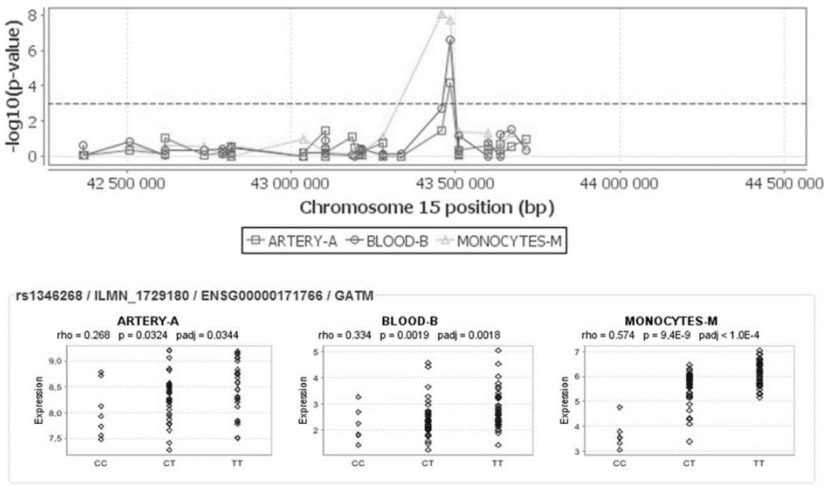

Figure 6. eQTL analysis in Tampere Vascular Study. eQTL expression analysis of the association of rs1346268 (proxy of rs1153858) with the expression of glycine amidinotransferase and nearby genes in artery tissue, cells from whole blood, and monocytes in Tampere Vascular Study (TVS). Modified from study II.

5.3 Associations of functional *AGXT2* variants with cardiovascular outcomes (I & III)

5.3.1 Heart rate variability and sudden cardiac death (I)

We tested the hypothesis that the two newly identified likely functional *AGXT2* SNPs would associate with: **a)** resting heart rate and HRV in young apparently healthy adults; and **b)** with cardiovascular outcome and SCD in particular in patients undergoing coronary angiography. The results from these association tests are shown in **Table 4** and **Table 5**.

Table 4. Associations of missense *AGXT2* and *SLC25A45* variants with SDMA, HRV and resting heart rate.

SNP	EA	EAF (%)	SDMA GWAS n=5,110		lnLF/HF (YFS) n=1,723		Mean HR (YFS) n=1,723	
			β (SE)	P	β (SE)	P	β (SE)	P
rs37369	C	91.2	-0.47 (0.035)	1.4×10^{-40}	0.10 (0.052)	0.045	-0.54 (0.58)	0.36
rs16899974	C	77.2	-0.32 (0.024)	1.5×10^{-38}	0.13 (0.036)	0.00047	-0.73 (0.41)	0.073
rs34400381	G	95.7	-0.36 (0.056)	2.5×10^{-10}	0.055 (0.064)	0.39	-2.02 (0.71)	0.0046

Notes: Effect alleles are serum SDMA levels increasing alleles as identified in the GWAS.
Abbreviations: SDMA, symmetric dimethylarginine; HRV, heart rate variability; EA, effect allele; EAF, effect allele frequency; LF/HF, low-frequency to high-frequency ratio of spectral HRV; HR, heart rate; β, beta-coefficient; SE, standard error.

Table 5. Associations of functional *AGXT2* variants with sudden cardiac death in LURIC.

SNP	EA	EAF (%)	All (n=2,748; 173 SCDs)		LVSP <145 mmHg (n=1,220; 66 SCDs)		LVSP ≥145 mmHg (n=1,199; 83 SCDs)		P for interaction
			HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	
rs37369	C	91.2	1.04 (0.70-1.55)	0.83	1.98 (0.88-4.45)	0.10	0.72 (0.44-1.19)	0.20	0.041
rs16899974	C	77.2	1.17 (0.87-1.55)	0.30	1.84 (1.10-3.09)	0.021	0.81 (0.55-1.21)	0.31	0.035

Notes: Effect alleles are serum SDMA levels increasing alleles as identified in the GWAS.
Abbreviations: EA, effect allele; EAF, effect allele frequency; SCD, sudden cardiac death; LVSP, left ventricular systolic pressure; HR, hazard ratio; CI, confidence interval.

In YFS, the major alleles of all three independent SNPs associated with decreased SDMA levels in the GWAS were also consistently associated with increased values of a frequency domain HRV-parameter (the LF/HF ratio, an estimate of sympathovagal balance) and decreased values of resting heart rate during the HRV measurements. In LURIC, the major alleles of the two *AGXT2* variants showed suggestive associations with incident SCD in patients with low left ventricle systolic pressure (LVSP <145 mmHg, below the median value) in the baseline coronary angiography whereas no associations were observed within the whole study population or in the high LVSP (≥145 mmHg) group. The interaction between the LVSP groups and two AGXT2 SNPs on future SCD were statistically significant (Table 5). Further, the SDMA increasing allele (G) of the SLC25A45 SNP rs34400381 was associated with SCD in the whole study population with per allele HR of 13.0 (95% CI 1.54-110, P=0.018). It was not possible to perform any meaningful LVSP subgroups analysis with this SNP due to the relatively small MAF of 4.3% and limited number of incident SCD cases.

5.3.2 Atrial fibrillation (III)

For study III, we screened the LURIC database for associations between the two functional *AGXT2* variants and novel SNP-disease associations. A cross-sectional association with a history of atrial fibrillation (AF) at baseline appeared to be a promising one and we started to gather evidence to support this observation. We used a SNP on chromosome 4q25, known to be strongly associated with AF, as a positive control in some of the analyses to increase the credibility of our novel results. The associations of the *AGXT2* variants and the 4q25 control variant with AF and its subtypes in LURIC is shown in **Figure 7**. The analyses were adjusted with a myriad of known AF risk factors, which tended to strengthen the associations between *AGXT2* variants and AF. We also studied whether some conditions known to be risk factors for AF modified the association between the *AGXT2* variants and AF. Valvular heart disease and cardiomyopathy showed significant interactions between the *AGXT2* variants and AF with p-values of 0.00043 and 0.043, respectively. As shown in the lower part of **Figure 7**, when analysing patients without these structural heart diseases, the effect estimates for associations between the *AGXT2* variants and AF increased even further compared to the analyses with all patients. In patients without structural heart disease, the association with AF was seen separately with paroxysmal and chronic AF (**Figure 7**).

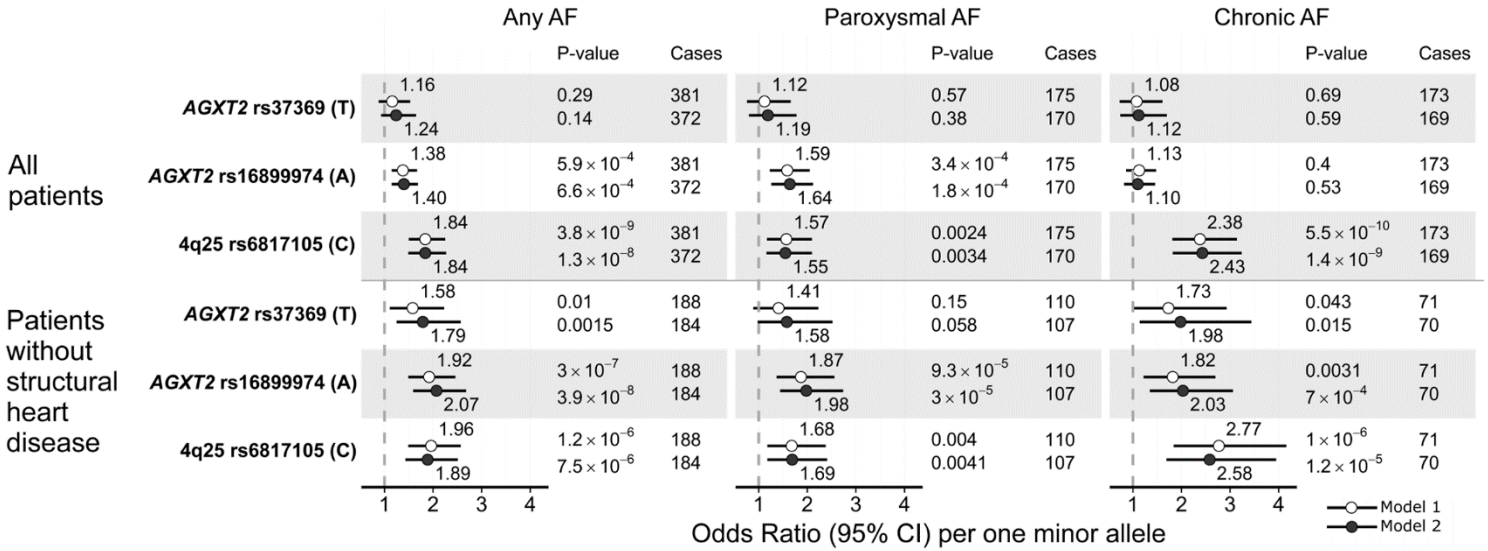

Figure 7. Associations of the *AGXT2* and 4q25 variants with prevalent AF and its subtypes in LURIC. ORs per one minor allele increase from logistic regression models assuming additive genetic effect are shown. Model 1: adjusted for age, sex and BMI. Model 2: Model 1 further adjusted for arterial hypertension, diabetes, coronary artery disease (> 50% stenosis), serum NT-proBNP and eGFR. Structural heart disease refers to cardiomyopathy or valvular heart disease. Modified from study III.

Next, we sought to replicate the AF association in independent study cohorts. To this end, we utilised prospective diagnostic code data of the FINCAVAS study participants and cross-sectional data from the Corogene study with an additional look-up of patients' electronic health records to further classify the AF cases into paroxysmal and chronic subtypes. **Figure 8** illustrates the univariate Kaplan-Meier curves as a function of the *AGXT2* rs16899974 SNP in all patients (A) and in patients with AF (B) (case-only analysis).

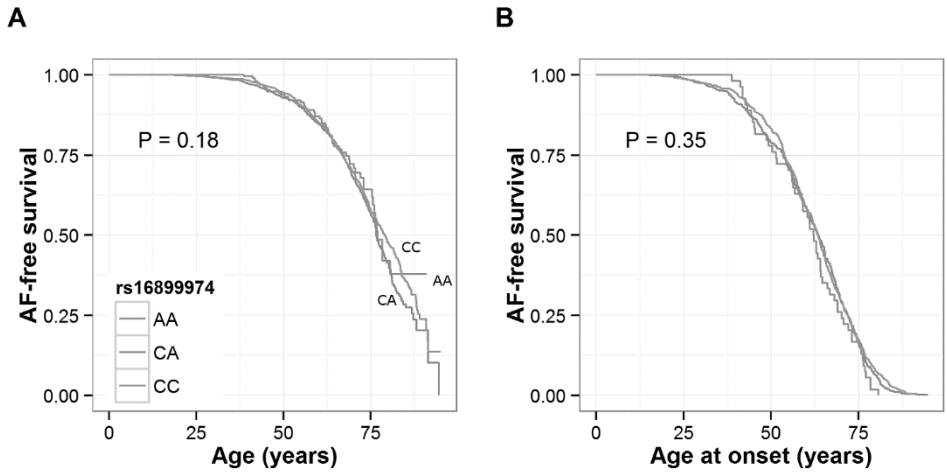

Figure 8. Survival curves are shown as a function of the *AGXT2* rs16899974 genotype groups for incident AF (**A**) and for cases of incident AF (**B**) only (age of onset analysis). Modified from study III.

No evidence for associations between *AGXT2* rs16899974 and AF was observed in these analyses. In panel A, however, the survival curves for the three genotypes start to separate after the age of around 75 years, suggesting that there might be an association with AF risk in patients aged over 75. Therefore, we conducted Cox regression analyses in the overall study population as well as in the two age groups (using age 75 years as a cut-off) separately. The results are shown in **Table 6**. The control 4q25 variant shows a strong association with AF in the younger age group whereas the effect size is reduced in the older age group. Of the *AGXT2* variants, rs16899974 shows a moderate association with AF in the older age group (per allele OR=1.38, P=0.0068), but not among those aged <75 years. In Corogene, the *AGXT2* variants were not associated with any AF, paroxysmal AF or chronic AF in any consistent or statistically significant manner. However, a moderate association was observed between the 4q25 rs6817105 control variant and any AF with per allele OR of 1.50 (95% CI, 1.18–1.91; P=0.001) when 265 patients with AF and 1,943 control subjects were analysed.

Table 6. Associations of *AGXT2* and 4q25 variants with incident clinical AF in FINCAVAS.

Locus	SNP	cases/N	HR	(95% CI)	P
All					
AGXT2	rs37369	972/3,122	1.02	(0.87, 1.19)	0.83
AGXT2	rs16899974	1188/3,862	1.05	(0.96, 1.16)	0.28
4q25	rs6817105	972/3,122	1.51	(1.36, 1.69)	9.7×10^{-14}
Age <75 years					
AGXT2	rs37369	802/2,562	0.95	(0.80, 1.12)	0.54
AGXT2	rs16899974	991/3,210	0.95	(0.85, 1.05)	0.32
4q25	rs6817105	808/2,587	1.55	(1.37, 1.74)	6.1×10^{-13}
Age ≥75 years					
AGXT2	rs37369	170/560	1.07	(0.71, 1.61)	0.75
AGXT2	rs16899974	197/662	1.38	(1.10, 1.74)	0.0063
4q25	rs6817105	170/560	1.26	(0.95, 1.67)	0.11

Statistics: HRs per one minor allele increase are from Cox regression models, assuming an additive genetic effect. The baseline hazard function of Cox models are stratified by sex.
Notes: Age is used as the time scale in Cox models. Analyses are stratified based on the age at the end of the follow-up.

5.3.3 Ischaemic stroke and age at ischemic stroke onset (III)

Following this, we investigated whether the *AGXT2* variants were associated with incident ischemic stroke in FINCAVAS and a history of ischemic stroke in the WTCCC2 ischaemic stroke case-control cohorts. For the FINCAVAS patients, the Kaplan-Meier survival curves for incident ischemic stroke (ICD-10 code I63) as a function of the three *AGXT2* rs16899974 genotypes and chronological age as the time-scale are shown in **Figure 9**. Rs16899974 was not associated with ischaemic stroke risk (panel A, P=0.082) whereas the association with incident ischaemic stroke in the case-only analysis (panel B) was statistically significant (P=0.0018). In **Table 7**, the results from Cox regression models and for the age at onset analysis additionally using linear regression are shown. The minor allele A (also AF risk allele in LURIC) of rs16899974 was associated with 3.2 (95% CI, 1.5–6.0; P=0.0015) and 3.8 (95% CI, 1.3–5.2; P=0.0009) years earlier age at first-ever ischaemic stroke and cryptogenic ischemic stroke (I63.9) diagnosis. The *AGXT2* SNPs were not associated with incident IS in the whole FINCAVAS population; however, we observed a nominally significant association between

rs16899974 and any ischaemic stroke in the WTCCC2 ischaemic stroke case-control cohorts with per allele OR of 1.04 (95% CI, 1.00-1.08, P=0.032).

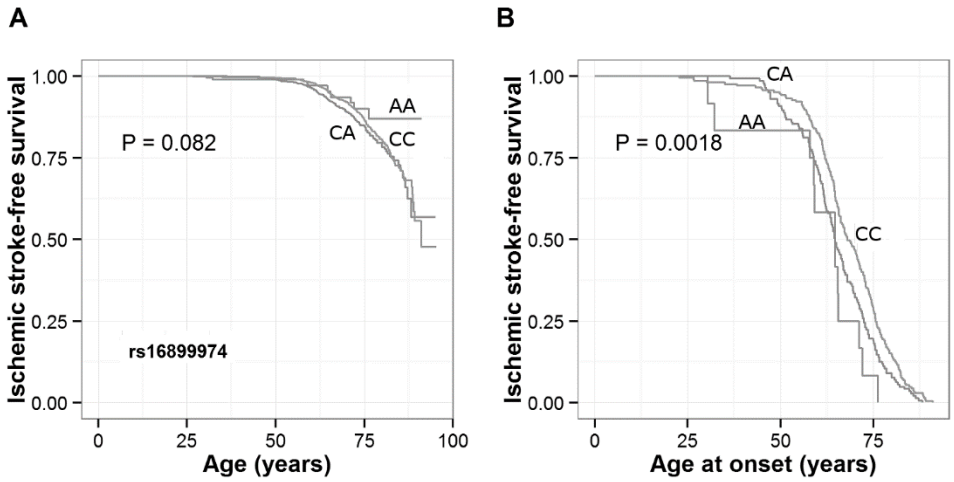

Figure 9. Survival curves are shown as a function of the *AGXT2* rs16899974 genotype groups for incident ischaemic stroke (**A**) and for cases of incident ischaemic stroke (**B**) only (age of onset analysis). Modified from study III.

Table 7. Associations of the AGXT2 and 4q25 variants with: (a) incident ischemic stroke and; (b) age at ischemic stroke onset in FINCAVAS.

Locus	**SNP**	**EA**	**EAF**	**cases/N**	**HR**	**(95% CI)**	**P**
a) incident first ever ischemic stroke							
AGXT2	rs37369	T	0.094	295/3,122	0.97	(0.73, 1.30)	0.86
AGXT2	rs16899974	A	0.24	360/3,862	1.05	(0.88, 1.24)	0.61
4q25	rs6817105	C	0.156	295/3,122	1.22	(0.90, 1.40)	0.96
b) age at the first ischemic stroke diagnosis							
Linear regression							
Locus	**SNP**	**EA**	**EAF**	**N**	**ß**	**(95% CI)**	**P**
AGXT2	rs37369	T	0.086	295	-2.6	(-5.75, 0.55)	0.11
AGXT2	rs16899974	A	0.232	360	-3.24	(-5.24, -1.25)	0.0015
4q25	rs6817105	C	0.151	295	-2.6	(-5.14, -0.074)	0.044
Cox model							
Locus	**SNP**	**EA**	**EAF**	**N**	**HR**	**(95% CI)**	**P**
AGXT2	rs37369	T	0.086	295	1.34	(0.99, 1,81)	0.062
AGXT2	rs16899974	A	0.232	360	1.44	(1.19, 1.75)	0.00018
4q25	rs6817105	C	0.151	295	1.18	(0.93, 1.51)	0.18

Statistics: HRs and βs (in years) per one minor allele increase are from Cox and linear regression models, respectively, assuming an additive genetic effect. Models are controlled for sex and a history of clinical atrial fibrillation.

5.4 The biomarker and causal roles of serum hArg in the development of cardiometabolic diseases (IV)

5.4.1 Cross-sectional determinants of circulating hArg in young men and women

We studied the cross-sectional determinants of serum hArg as well as the predictive value of hArg on incident Mets components, obesity, high insulin, markers of preclinical atherosclerosis and T2DM during a 10-year follow-up among the YFS participants. The baseline characteristics of the YFS population by sex at baseline in 2001 are shown in **Table 8**. We used all the cardiometabolic risk factors shown in **Table 8** as covariates in subsequent multivariable modelling of the above mentioned outcomes.

Table 8. Baseline descriptive data for the YFS cohort in 2001.

	All	Men	Women
Number of subjects (%)	2,106	957 (45.4)	1,149 (54.6)
Age (years)	31.7 (5.0)	31.6 (5.0)	31.7 (5.0)
Homoarginine (hArg) (μmol/L)	1.85 (0.65)	1.93 (0.61)	1.79 (0.68)
*ln*hArg (μmol/L)	0.56 (0.34)	0.61 (0.30)	0.52 (0.36)
LDL cholesterol (mmol/L)	3.27 (0.84)	3.42 (0.90)	3.14 (0.77)
HDL cholesterol (mmol/L)	1.29 (0.31)	1.17 (0.27)	1.39 (0.30)
Triglycerides (mmol/L)	1.26 (0.64)	1.41 (0.70)	1.13 (0.55)
Systolic blood pressure (mm Hg)	117 (13)	121 (12)	113 (12)
Diastolic blood pressure (mm Hg)	72 (11)	73 (11)	69 (10)
C-reactive protein (mg/L)	1.9 (4.0)	1.5 (3.4)	2.1 (4.4)
Glucose (mmol/L)	5.1 (0.85)	5.2 (0.93)	4.9 (0.75)
Insulin (IU/L)	7.7 (5.7)	7.6 (5.8)	7.8 (5.7)
Body mass index (kg/m^2)	25.0 (4.4)	25.6 (4.1)	24.4 (4.5)
Waist circumference (cm)	84 (12)	90 (11)	79 (11)
Daily smokers (%)	520 (24.7)	288 (30.1)	232 (20.2)
Family history of CAD (%)	281 (13.3)	123 (12.9)	158 (13.8)

Statistics: values are mean (SD) or n (%); *ln*hArg is natural log-transformed.

The results from a bi-directional stepwise model selection procedure for *ln*hArg as an outcome variable and all the risk factors in **Table 8** and serum SHBG as explanatory variables are shown in **Table 9**. The selected variables explained ~11.4% of the variance in serum *ln*hArg. When adding the three SNPs associated at genome-wide significance with hArg to the model selection procedure, all the same variables in **Table 9** were selected in addition to the genetic variants and the R^2 of the model increased to a value of 22.4%.

Table 9. Cross-sectional stepwise multivariable linear regression modelling for hArg (n=2057).

Explanatory variable	β	95% CI	P-value
Male sex	0.14	[0.10, 0.17]	4.1×10^{-13}
Body mass index (kg/m^2)	0.014	[0.0093, 0.018]	1.2×10^{-9}
Daily smoking	-0.089	[-0.12, -0.056]	1.3×10^{-7}
Age (years)	-0.0067	[-0.0097, -0.0038]	7.8×10^{-6}
*ln*SHBG (nmol/L)	0.061	[0.032, 0.089]	3.2×10^{-5}
*ln*Triglycerides (mmol/L)	0.045	[0.019, 0.072]	8.2×10^{-4}
LDL cholesterol (mmol/L)	0.022	[0.0045, 0.040]	0.014
*ln*CRP (mg/L)	0.016	[0.0023, 0.029]	0.021

Statistics: In the bi-directional stepwise regression modelling applied, serum hArg was used as a dependent variable and all the variables shown in Table 1 and *ln*SHBG as explanatory variables. Those variables that were selected by Akaike's information criterion (AIC) using the stepAIC R function with the default settings and had a p-value <0.05 are shown above. HArg, SHBG, triglycerides and C-reactive protein (CRP) were natural log-transformed. For the continuous variables, β (95% CI) are shown for each 1-unit change in the variable.

Next, we studied the cross-sectional and longitudinal associations between hArg and 228 NMR metabolites. The results for 73 selected metabolites adjusted for age, BMI, daily smoking, serum SHBG and oral contraceptive use (in women) are illustrated in **Figure 10** for men and women separately. In this multivariable model, only the positive associations of amino acids and docosahexaenoic acid with hArg in women were seen. For both sexes, glycine showed a strong negative association with hArg, whereas the negative association of HDL subclasses and hArg was seen only in men. Significant men-specific interactions were observed between hArg and BMI on glucose, valine and saturated fatty acids (%).

5.4.2 Longitudinal associations of hArg with metabolic and atherosclerotic outcomes in young adults

In our 6- to 10-year follow-up (depending on the endpoints used) in unadjusted analyses, hArg was significantly and directly associated with incident obesity (BMI $\geq$30 kg/m^2), abdominal obesity (high waist circumference), hyperglycaemia, high insulin, high-risk carotid IMT and distensibility in men, and with incident T2DM in women (**Figure 11**). In our 10-year follow-up analysis, after further adjustments with other cardiovascular risk factors, hArg served as a significant biomarker for future hyperglycaemia (OR 1.31, 95% CI 1.06–1.63) and abdominal obesity (OR 1.60, 95% 1.14–2.30) in men.

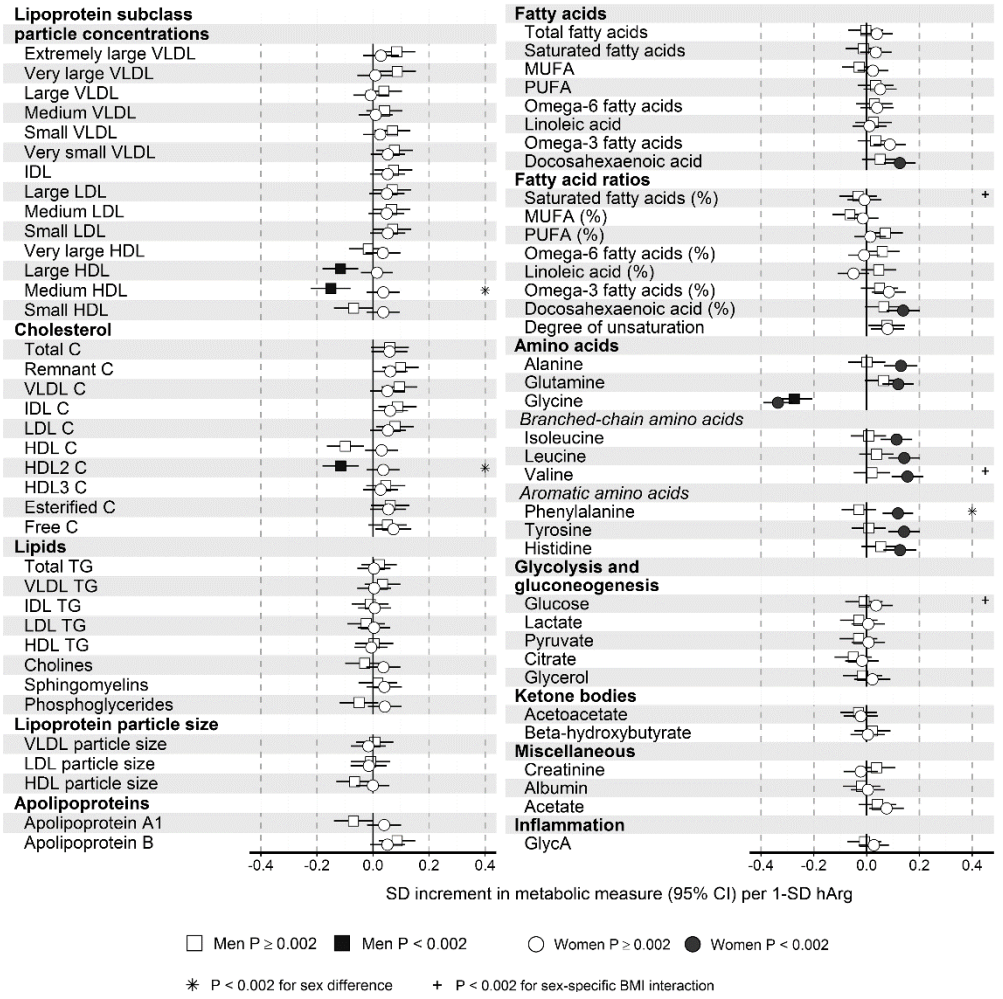

Figure 10. Sex-specific cross-sectional associations of baseline hArg with 73 NMR-based serum metabolites, adjusted for age, BMI, daily smoking, serum SHBG and oral contraceptive use (in women). The analyses were conducted for 867 men and 1097 women. Squares indicate men, and circles represent women. Open and closed symbols indicate P ≥0.002 and P <0.002, respectively. Sex differences with P <0.002 are marked by asterisks. BMI interactions with P <0.002 are marked by the plus or minus sign, depending on the direction of the estimated interaction effect. Modified from study IV.

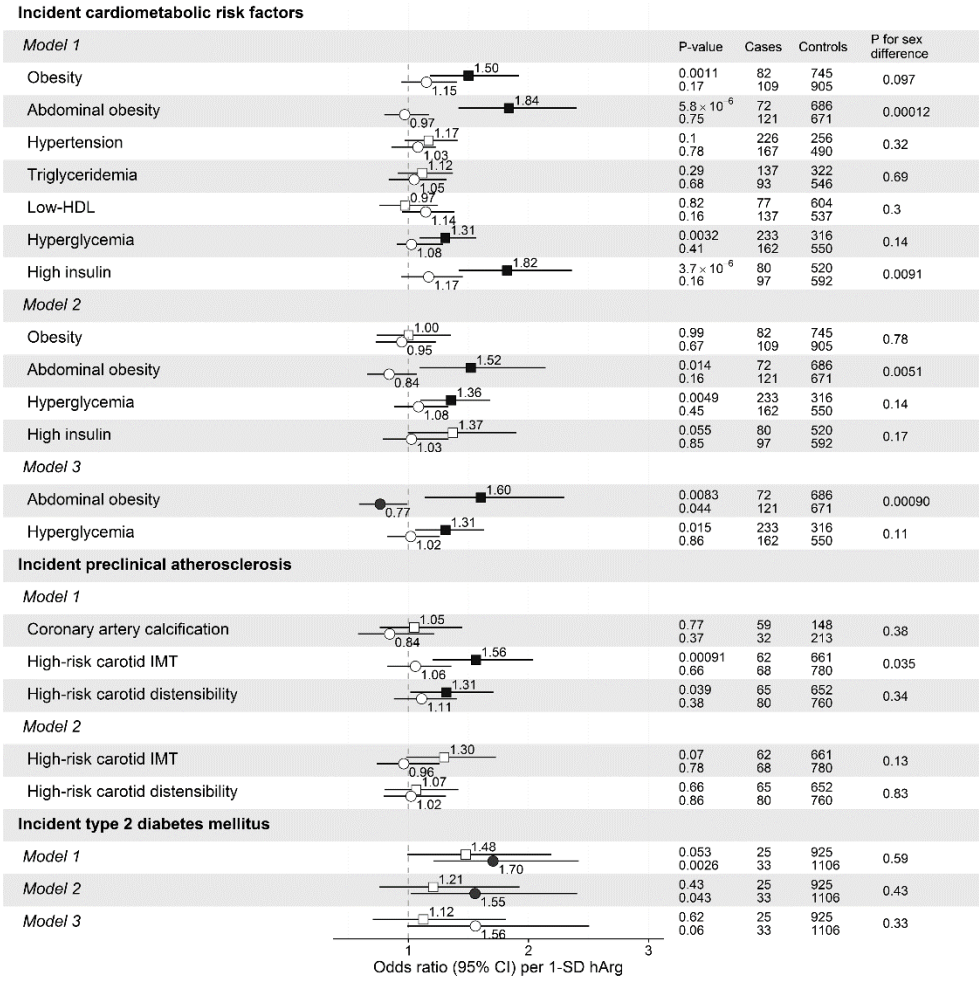

Figure 11. Sex-specific prospective (observational) associations of baseline hArg (in 2001) and cardiometabolic risk factors, preclinical atherosclerosis and T2DM during a 10-year follow-up in YFS. The prospective associations are shown as unadjusted (Model 1), adjusted for all baseline cardiometabolic risk factors shown in Table 1 (Model 2), and further adjusted for baseline serum SHBG and oral contraceptive use in women. Open and closed squares and circles indicate P ≥0.05 and P <0.05, respectively. Modified from study IV.

5.4.3 Associations of genetically determined serum hArg with cardiometabolic risk factors and diseases

When utilising summary-level data from different GWAS meta-analyses available in the public domain, no evidence was found that serum hArg was causally associated with any of the studied obesity traits (BMI or BMI-adjusted waist circumference), glycaemic or lipid traits, or T2DM or CAD (**Figure 12**). However, a strong cross-sectional association was observed between hArg and BMI (for men $P=5.7\times10^{-23}$, for women $P=8.9\times10^{-8}$ and for all subjects combined $P=1.4\times10^{-26}$), but not with BMI-adjusted waist circumference, in the observational analyses using the YFS data (**Figure 12**).

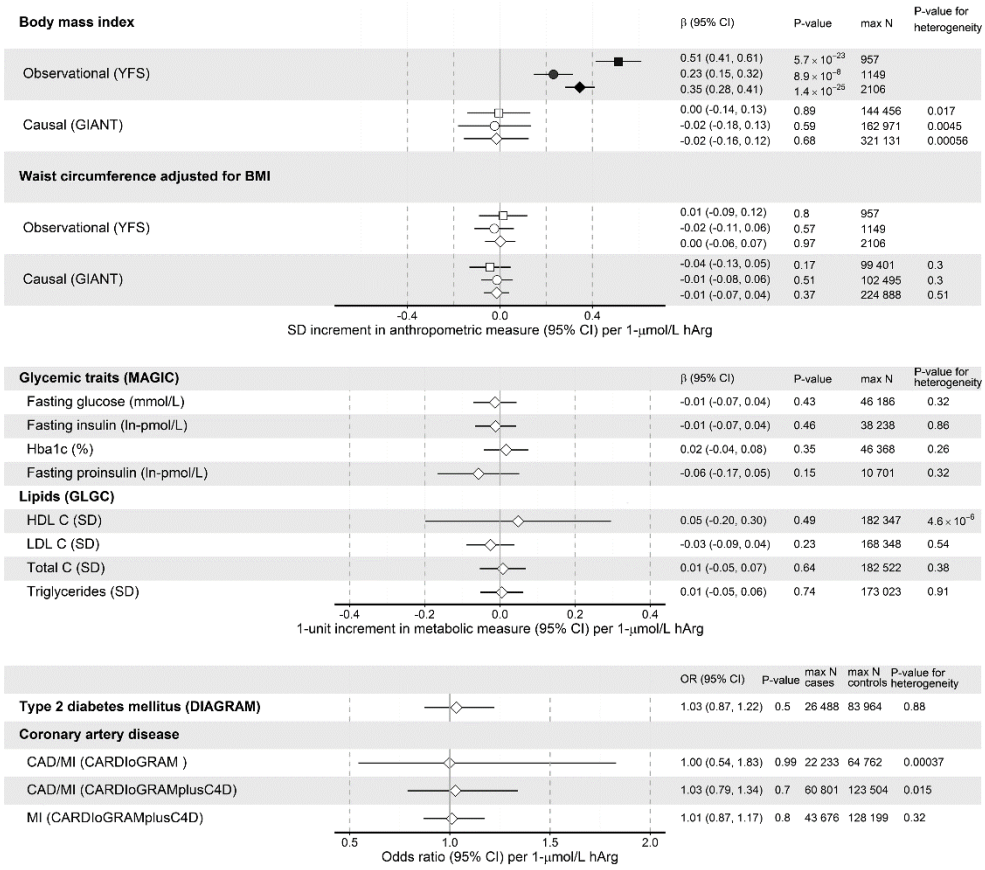

Figure 12. Combined causal effect estimates (β=beta, OR=odds ratio and 95% CI confidence intervals) of hArg with cardiometabolic risk factors, T2DM and CAD. For each metabolite, the summary-level data across the three hArg-associated SNPs (*GATM* rs1153858, *CPS1* rs1047891 and *AGXT2* rs37369) was combined using weighted linear regression, and the heterogeneity in the causal effects from different individual variants was tested by Cochran's Q statistic. All p-values for combined causal effects >0.05. A p-value of >0.05 from Cochran's Q statistic indicates that there is no more heterogeneity between causal effects estimated using the variants individually than would be expected by chance. Modified from study IV.

6 DISCUSSION

6.1 Genetics of ADMA and SDMA (I)

6.1.1 DDAH1 and *DDAH2*

Intron variants in the *DDAH1* gene were identified to be associated with serum ADMA and SDMA levels after adjusting for the other dimethylarginine, sex, age, BMI and serum creatinine to account for variation in renal function. The associations between *DDAH1* variants and ADMA are expected, as the enzyme DDAH1 catalyses the breakdown of ADMA to citrulline and dimethylamine. Moreover, of the two DDAH isoforms, DDAH1 is the isoentzyme that is shown to regulate the circulating ADMA levels whereas DDAH2 has a different tissue distribution and plays a minor role in the regulation of systemic ADMA levels as previously discussed in chapter 2.1.3.

Several candidate gene studies have shown associations between common SNPs in *DDAH1* and *DDAH2* genes and circulating ADMA levels. In a study of 343 Australian patients with T2DM, several *DDAH1* and *DDAH2* SNPs were significantly associated with serum ADMA levels after correcting for multiple testing (Abhary *et al.* 2010). Twenty-six *DDAH1* and 10 *DDAH2* tag SNPs studied captured all common alleles (MAF >5%) with an r^2 (a measure of linkage disequilibrium, LD) of at least 0.8. In another study of 1,016 Swedish 70-year-old individuals from the general population, 55 SNPs in the *DDAH1* and *DDAH2* genes genotyped tagged >94% of the common variation in these genes (Lind *et al.* 2013). Several *DDAH1* tag SNPs were significantly associated with ADMA, while none of the 11 tag SNPs in the *DDAH2* gene were associated with ADMA. Using a stepwise linear regression model selection procedure, of the 16 highly significantly associated *DDAH1* SNPs, only three remained significant and were not in high LD (rs233109, rs12140935 and rs6669293). The first SNP rs233109 is in a high and moderate LD with rs1884139 and rs28489187 (r^2~0.88 and r^2~0.62 in the European populations of the 1000 Genomes project, n=1,006) identified to be associated with the dimethylarginine traits at genome-wide

significance in our GWAS (**Table 2**) and also with *DDAH1* rs18582 (r^2~0.92) which was the lead variant associated with ADMA levels in another GWAS (Lüneburg *et al.* 2014). The second independent *DDAH1* rs12140935 SNP identified in the study of Swedish older people, is in a perfect LD with the 1:85897175 (TCC/-) deletion polymorphism (r~1) which was associated with ADMA and ADMA/SDMA ratio at genome-wide significance (**Table 2**). Taken together, there are likely to be several common variants that independently regulate circulating ADMA levels by affecting *DDAH1* mRNA expression in relevant tissues as has been shown to be the case in renal tissue (Caplin *et al.* 2010). In contrast, it is unlikely that common variants in the *DDAH2* gene region have major effects on circulating ADMA levels under normal conditions as GWASs by ourselves and others have failed to show such associations.

The associations between intronic variants in *DDAH1* and SDMA adjusted for ADMA are likely to be due to the indirect effects of DDAH1 on serum SDMA levels by ADMA elimination because DDAH1 do not degrade SDMA. The effect estimates were consistently in the opposite direction for SDMA compared with those for ADMA, thus supporting this reasoning. It should be noted that serum levels do not necessarily reflect the intracellular concentrations where the enzymatic reactions take place, complicating the interpretation of potential indirect effects of DDAH1 on serum SDMA levels. On the other hand, another GWAS on ADMA, SDMA and L-arginine did not report any associations between *DDAH1* variants and SDMA levels (Lüneburg *et al.* 2014). The major difference between their GWAS and ours was that they did not adjust ADMA for SDMA and *vice versa* to increase statistical power by taking account for the fact that serum ADMA is a major determinant of serum SDMA and *vice versa* (Meinitzer *et al.* 2011). It is possible that our statistical models resulted in sufficient statistical power to detect the indirect effects of *DDAH1* SNPs on the SDMA traits at genome-wide significance. Nevertheless, our adjustment strategy followed by a random-effects meta-analysis, although complicating the interpretation of the results from the *DDAH1* locus, turned out to be an effective one in the fine-mapping of the *AGXT2* gene region for independent functional variants associated with SDMA levels.

6.1.2 AGXT2

We observed a strong association signal for SDMA and ADMA/SDMA ratio at rs37369 on chromosome 5p13 causing valine to isoleucine substitution at the

position 140 (Val140Ile) of the nuclear-encoded mitochondrial enzyme AGXT2. Our fine-mapping procedure revealed another independent coding sequence variant *AGXT2* rs16899974 (Val498Leu) causing an amino acid change in AGXT2 at position 498. The associations of these two *AGXT2* SNPs with plasma and urinary SDMA and BAIB, another substrate of AGXT2, were studied in 400 healthy volunteers followed by functional studies of the rs37369 variant (Kittel *et al.* 2014). Both *AGXT2* SNPs were independently associated with BAIB in plasma and urine validating our fine-mapping results. While *AGXT2* rs37369 was associated with plasma SDMA in this study, *AGXT2* rs16899974, probably due to the limited sample size, was not associated with SDMA in plasma or urine. Moreover, functional studies of rs37369 *in vitro* revealed that mutant (Val140Ile) AGXT2 protein resulted in reduced enzyme activity compared with wild-type AGXT2. In another study involving 935 men and women with T2DM, tag SNPs in the *DDAH1*, *DDAH2* and *AGXT2* genes were genotyped followed by association analyses with plasma ADMA and SDMA (Anderssohn *et al.* 2014). In this study, both *AGXT2* rs37369 and rs16899974 were associated with plasma SDMA levels, further confirming our fine-mapping results. In the same study, *DDAH1* variants, but not *DDAH2* variants, were associated with plasma ADMA.

In a GWAS of plasma ADMA, SDMA and L-arginine, rs37369 was the SNP most strongly associated with plasma SDMA while rs16899974 was not reported to be associated with SDMA (Lüneburg *et al.* 2014). One possible explanation for the lack of genome-wide significant association of rs16899974 with SDMA in that study is that rs16899974 is not directly genotyped on the GWAS arrays (Affymetrix 50K, 500K and 6.0) used any of the discovery cohorts. Moreover, we used a 1000 Genomes reference for genotype imputation, which contains a denser set of genetic variants than the HapMap II reference (~2.2 versus ~10 million variants after post imputation QC filtering) used in the other GWAS, likely resulting in better imputation quality for rs16899974 in the LURIC study genotyped using the Affymetrix 6.0 array.

AGXT2 rs37369 was first reported to be associated with NMR-measured BAIB levels in the urine and is a likely candidate for the causative SNP of hyper-β-aminoisobutyric aciduria, which is probably the most common Mendelian metabolic variant in humans [Online Mendelian Inheritance in Man (OMIM) no. 210100] (Suhre *et al.* 2011). In another GWAS on plasma metabolites, the *AGXT2* rs37370 missense variant was identified to be strongly associated with plasma BAIB and accounting for ~36% of its estimated heritability (Rhee *et al.* 2013). In individuals of European ancestry, rs37369 and rs37370 are in a moderately high

LD (r^2~0.60), making the estimation of their independent effects by bioinformatic means challenging. However, this is not the case in East Asian populations in which rs37369 and rs37370 are in a low LD (r^2~0.10 in the East Asian populations of the 1000 Genomes project, n=1,008). Indeed, in the Japanese, it has been reported that four *AGXT2* missense variants (rs37370, rs37369, rs180749, rs16899974) have independent effects on AGXT2 activity using multivariable linear regression modelling and urinary BAIB as a dependent variable (Yoshino *et al.* 2014a, Yoshino *et al.* 2014b). The rs180749 mutation is very rare in the European populations (MAF~0.002, n=1,006). Therefore, it is likely that at least these four missense variants, rather than any single variant alone, contribute to the large differences in the prevalence of hyper-β-aminoisobutyric aciduria observed across populations, e.g. up to 10% in whites and ~36% in Japanese (Yanai *et al.* 1969).

Although the *AGXT2* variants are not associated with circulating ADMA levels with directions of effect similar than with SDMA and BAIB, AGXT2 may play a significant role in systemic and/or local ADMA metabolism in humans under pathological conditions when the elimination by DDAHs is abrogated (Rodionov *et al.* 2010). One way to test this hypothesis is to study whether the functional *AGXT2* variants are associated with the risk and prognosis in various disease states. There is experimental evidence in mice showing that AGXT2 plays a role in methylarginine metabolism under physiological conditions *in vivo*. In $AGXT2^{-/-}$ mice, both ADMA and SDMA levels are elevated compared with wild-type littermates under normal conditions (Caplin *et al.* 2012). In the same study, it was shown that allograft tissue levels of *AGXT2* mRNA were correlated directly with urinary DMGV (the AGXT2-specific ADMA breakdown product) and inversely with plasma ADMA and SDMA levels in 35 renal transplant patients. Moreover, the association with ADMA was independent of tissue *DDAH1* and *DDAH2* gene expression, supporting the hypothesis that AGXT2 contributes significantly to endogenous methylarginine turnover in humans. In healthy humans, it is likely that DDAHs can compensate for any differences in the AGXT2 activity caused by the functional *AGXT2* SNPs, explaining the lack of associations between *AGXT2* SNPs and circulating ADMA levels. Nevertheless, a three-day ADMA infusion into mice, mimicking ADMA accumulation observed in certain human diseases, resulted in a ~3- to 4-fold increase in plasma and urine ADMA and DMGV (Rodionov *et al.* 2014a). Interestingly, after a bilateral nephrectomy ADMA levels did not increase at all but DMGV was elevated 32-fold while AGXT2 expression was not changed indicating that

AGXT2 was not saturated in these conditions and the authors concluded that AGXT2 may play an important role in ADMA homeostasis under conditions associated with elevated ADMA levels. Finally, an experiment using chronic BAIB infusion to mice demonstrated that endogenous Agxt2 plays a role in basal ADMA and SDMA metabolism because the plasma levels of dimethylarginines elevated ~30% after saturating the enzyme with BAIB (Kittel *et al.* 2013).

6.1.3 SLC25A45

The missense mutation rs34400381 (R285C/R243C/etc. depending on the splice variant) in the solute carrier family 25 member 45 (*SLC25A45*) gene was associated with serum SDMA levels in the combined analysis of the YFS and LURIC cohorts. SLC25A45 belongs to the SLC25 family of mitochondrial carrier proteins that catalyse the transport of solutes across the inner mitochondrial membrane and has several splice variants (Haitina *et al.* 2006). Although the substrates and transport properties of this nuclear-encoded inner mitochondrial membrane transporter remain to be biochemically characterised (Palmieri. 2013), it is possible that SLC25A45 imports the substrates for mitochondrial metabolism by AGXT2. Interestingly, in addition to its known substrates lysine and arginine, another mitochondrial carrier for cationic amino acids (SLC25A2) was shown to be an efficient mitochondrial transporter for ADMA, whereas SDMA was not transported at all (Porcelli *et al.* 2016). The two other studied carriers for cationic amino acids, SLC25A15 and SLC25A29, did not transport ADMA or SDMA. Therefore, the SLC25 family member that translocates SDMA across the mitochondrial membrane to be catabolised by AGXT2 remains to be identified. SLC25A45 is a potential candidate for importing SDMA into mitochondria because SLC25A45 is clustered with the other cationic amino acid transports in a phylogenetic tree analysis of human mitochondrial carriers (Palmieri. 2013) and we observed a strong association between the *SLC25A45* missense mutation and serum SDMA levels in young healthy adults. Finally, a GWAS on serum creatinine involving 81,656 Icelanders and 112,630 relatives identified the same *SLC25A45* rs34400381 variant to be associated with serum creatinine but not with the risk of chronic kidney disease in 15,594 cases and 291,428 controls (Sveinbjornsson *et al.* 2014). The authors postulated that SLC25A45 could be involved in the biosynthesis of arginine due to its similarity with the ornithine/citrulline transporters SLC25A2 and SLC25A15 and may thus play a role in the creatine synthesis. I rather speculate that SLC25A45 imports the

arginine derivatives SDMA and arginine for the mitochondrial enzymes AGXT2 and AGAT which catalyse the SDMA breakdown and the biosynthesis of the creatine precursor GAA, respectively. This hypothesis is supported by a positive association of mutated SLC25A45 with serum SDMA (**Table 2**) and a negative association with serum creatinine (a waste product of creatine) in Icelanders (Sveinbjornsson *et al.* 2014).

6.2 Associations of the functional *AGXT2* variants with preclinical and clinical cardiovascular outcomes (I & III)

6.2.1 Associations with heart rate variability and sudden cardiac death

In study I, we found the SDMA decreasing alleles of the two *AGXT2* missense SNPs and one in *SLC25A45* to be associated with increased sympathovagal balance (LF/HF ratio) of spectral HRV in YFS and with increased risk of incident SCD in patients with low LVSP (<145 mmHg, below the median value) in LURIC. The SDMA decreasing alleles showed consistent direction of associations with HRV and SCD across the two almost independent *AGXT2* variants and the entirely independent *SLC25A45* variant. Although the variants were strongly associated with SDMA levels in our GWAS, the associations seen with HRV and resting heart rate under metronome controlled breathing in young healthy adults and with incident SCD in patients with low LVSP in coronary angiography do not need to be mediated by SDMA or even by the same substrate of AGXT2. Indeed, AGXT2 is a relatively promiscuous mitochondrial aminotransferase with numerous potential substrates according to *in vitro* and *in vivo* evidence (Rodionov *et al.* 2014b). It has recently been shown that, *in vivo*, AGXT2 is capable of not only metabolising BAIB, ADMA and SDMA but also hArg (Rodionov *et al.* 2016) and β-alanine (Blancquaert *et al.* 2016). Therefore, the molecular mechanisms underlying the observed associations of the functional *AGXT2* and *SLC25A45* variants with clinical outcomes remain elusive without additional experimental data.

Nevertheless, it is tempting to speculate about the potential mechanisms underlying the associations of the *AGXT2* variants with SCD and HRV. In a prospective analysis of 140 Chinese HF patients, the GG (or CC) genotype carriers of *AGXT2* rs37369 tended to have worse survival compared with the A (or T) allele carriers (Hu *et al.* 2016). Although, in this study, the *in vitro* data on

the effects of *AGXT2* rs37369 on the enzyme activity is in the opposite direction compared with experimental data reported by others (Kittel *et al.* 2014, Lüneburg *et al.* 2014), the direction of effect of the association between rs37369 and mortality in HF patients is the same as observed in LURIC, i.e. the SDMA decreasing C (or G) allele carriers have increased risk of death. Therefore, the effects of the *AGXT2* variants on mortality in HF patients cannot be explained by AGXT2-dependent increases of ADMA and SDMA levels resulting in decreased NO bioavailability and oxidative stress.

One alternative explanation is the potential effects of the *AGXT2* variants on β-alanine transamination by AGXT2. Indeed, the effects of AGXT2 inhibition by aminooxyacetic acid (AOA) in mice placed on a diet rich in β-alanine resulted in 3-fold increase in circulating β-alanine and 10-fold higher levels of histidine-containing dipeptides (carnosine and anserine) in the heart under normal conditions (Blancquaert *et al.* 2016). These results are in line with the observation that β-alanine is the rate limiting amino acid in the synthesis of carnosine (β-alanyl-L-histidine) from L-histidine and β-alanine. Interestingly, carnosine has several cardioprotective effects including anti-ischaemic, antioxidant and inotropic properties (Baye *et al.* 2016, McCarty & DiNicolantonio. 2014) all of which can contribute to the devolvement of HF and clinical outcomes in patients with HF. Therefore, mutated AGXT2 in humans, mimicking global AGXT2-specific inhibition, may have beneficial effects via decreased transamination of β-alanine and accumulation of carnosine into the failing heart and thus outweigh any detrimental effects of increased ADMA and SDMA levels due to decreased activity of AGXT2. It has also been reported in several studies using animals that carnosine is capable of inhibiting sympathic nervous system activity (Baye *et al.* 2016) providing one possible explanation for the associations of the two functional *AGXT2* variants with HRV in YFS and also with SCD in LURIC via reduced inhibition of sustained cardiac sympathic activation and associated fatal arrhythmia.

6.2.2 Associations with atrial fibrillation and ischaemic stroke

The SDMA increasing alleles of the two *AGXT2* missense variants were strongly associated with prevalent AF and its subtypes in LURIC. The associations were strengthened when patients with structural heart disease were excluded and after adjusting the analyses with several known risk factors for AF, including the cardiac marker NT-proBNP, increased in HF. These associations with AF were

not replicated in an independent cohort of patients with ACS although the association with a control variant on chromosome 4q25 previously found to be associated with AF was present. In a prospective analysis of patients undergoing a clinical exercise stress test, the *AGXT2* rs16899974 SDMA increasing A allele was associated with increased risk of AF in patients aged over 75 only. Nevertheless, in line with experimental and clinical data indicating that ADMA and SDMA levels are elevated in AF, our results suggest that mutated AGXT2 might increase the risk of AF at least in selected patient populations with ischemic heart disease and in older individuals by potentially contributing to the accumulation of ADMA and SDMA into cardiac tissues and to the oxidative stress observed in AF.

The same AF risk increasing A allele of *AGXT2* rs16899974 was also nominally associated with the risk of any ischemic stroke in the WTCCC2 ischemic stroke cohorts and also with earlier age at ischemic stroke diagnosis in the FINCAVAS cohort. The associations of the *AGXT2* variants with ischemic stroke may be mediated via the atherosclerosis promoting effects of ADMA and SDMA accumulation and reduced NO bioavailability in the vasculature resulting in increased risk of large-artery atherosclerosis stroke. An alternative mechanism behind the association with ischemic stroke is the cardioembolic aetiology via ADMA and SDMA induced endocardial dysfunction, reduced NO bioavailability and subsequent thrombus formation in the left atrium. Interestingly, circulating ADMA levels were independently associated with the presence of left atrial appendage thrombus in patients with non-valvular atrial fibrillation, further supporting the hypothesis that endocardial dysfunction contributes to the intra-atrial thrombus formation in AF (Xia *et al.* 2015).

6.3 Genetics of hArg (II)

In study II, three independent association signals were identified in the GWAS on serum hArg in the *GATM* (encodes AGAT), *CPS1* and *AGXT2* genes. Moreover, a suggestive association at the *MED23/ARG1* locus was found. In another GWAS of 2,806 German individuals, the signals in *GATM* and *CPS1*, but not the one in *AGXT2*, were independently identified (Choe *et al.* 2013). In the same study, hArg was absent in AGAT-deficient mice in line with an earlier study showing that hArg is synthesised by AGAT in humans (Davids *et al.* 2012). A missense variant in *CPS1* probably exhibits its effects on serum hArg levels by

regulating glycine levels and thus sifting the balance from the hArg synthesis to the creatine precursor GAA synthesis. The association between *AGXT2* rs37369 and hArg is explained by a recently identified reaction of hArg breakdown to 6-guanidino-2-oxocaproic acid (GOCA) catalysed by AGXT2 (Rodionov *et al.* 2016).

6.4 Serum hArg, cardiometabolic risk factors and diseases (IV)

In study IV, we studied the cross-sectional determinants of serum hArg and its predictive value in the development of cardiometabolic diseases in young healthy adults. No prior longitudinal studies have been conducted to investigate the predictive value of hArg in the development of metabolic syndrome components, preclinical atherosclerosis or T2DM in early adulthood and middle age. We replicated several cross-sectional associations reported before, including positive associations with several cardiometabolic risk factors such as BMI and male sex as well as the negative association with daily smoking and age.

The sex-specific association with serum NMR-based metabolite profile revealed a strong positive association between oestrogen-containing oral contraceptives and serum hArg levels. This association is supported by a previous observation that hArg levels are elevated during normal pregnancy and that hArg levels correlate positively with gestational age. The molecular explanation for these associations is provided by an experimental study showing that renal AGAT protein expression and creatine synthesis is increased in pregnant spiny mice at term compared to non-pregnant animals due to increased levels of oestrogen (Ellery *et al.* 2015).

In prospective analyses, baseline hArg predicted the development of abdominal obesity and hyperglycaemia in young men, but not in women. No associations were observed with incident preclinical atherosclerosis or T2DM after adjusting the analyses for numerous baseline risk factors.

The MR analyses revealed that it is unlikely that serum levels of hArg play any significant causal role in the development of cardiometabolic diseases due to the lack of causal effects on any of the studied clinical or biochemical risk factors, CAD and T2DM despite sufficient statistical power through utilising summary-level data based on GWASs of tens to hundreds of thousands individuals.

6.5 Limitations and strengths of the study

The strengths of study I include large well-characterised cohorts with a dense set of genetic markers to discover novel associations between serum methylarginines and common genetic variants. In the same study, we were able to study genetic associations with previously described and validated data on HRV measured under controlled breathing. In addition, long-term follow-up data were available for genetic association analyses on the prospectively adjudicated SCD cases in patients undergoing coronary angiography. Some degree of misclassification is possible in the cause of death data; however, in the LURIC cohort, the causes of death were carefully classified by several independent specialists. Moreover, any misclassification would rather make any true genetic association weaker than create false positive finding. The association results with SCD are not necessarily generalizable to a general population as the LURIC study consists of patients with suspected ischemic heart disease who underwent coronary angiography. Furthermore, the individuals in the YFS and LURIC studies are almost entirely white people of European decent and thus the results are not directly generalizable to other ethnic groups with different genetic and environmental backgrounds.

In study II, the genetics of serum hArg was studied using the same study cohorts, i.e. YFS and LURIC, than in study I and thus similar strengths and limitations apply to also to this GWAS study. In addition, the function of the lead intronic *GATM* variant was investigated using the tissue expression data of the TVS study to support the functional role of the lead SNP in the regulation of *GATM* mRNA expression in different tissues. The limitation in this study and also in all other studies is that we did not provide any experimental *in vitro or in vivo* evidence to support our association results. Nevertheless, such experimental evidence have been reported by others after our inspiring original communications, e.g. the breakdown of hArg by AGXT2 (Rodionov *et al.* 2016) and the effect of *AGXT2* rs37369 on AGXT2 activity (Kittel *et al.* 2014).

In study III, the strengths included the classification of the AF cases to the paroxysmal and chronic AF subtypes in the LURIC and Corogene studies as well as up to 28 years of diagnostic code data for the FINCAVAS participants to study the associations of the two functional *AGXT2* variants with incident AF and ischemic stroke. In FINCAVAS, it was also possible to not only investigate the associations with the disease risk but also with age at diagnosis as a continuous trait. The limitations include the uncertainty in the classification of AF cases due to the often silent and paroxysmal nature of the arrhythmia. However, this

limitation is common to all clinical data sets that do not utilise prolonged ambulatory ECG monitoring to detect paroxysmal asymptomatic AF among the controls. As a result, more symptomatic and sustained AF cases are likely overrepresented among AF cases in study III. By contrast, underdiagnosing ischemic stroke cases is less likely to occur because patients with acute ischemic stroke have clear sustained neurological symptoms and are thus less likely to be left without a diagnosis.

The strengths of study IV include the simultaneous prospective analyses of baseline hArg with incident cardiometabolic outcomes in young adults and the utilisation of publically available summary-level data from large-scale GWASs to study the causal effects of serum hArg on different cardiometabolic risk factors and diseases. Despite careful adjustment for possible confounding factors, the observational cross-sectional and longitudinal association analyses are subject to residual confounding and reverse causation that may bias the results. The MR analyses used to study causal effects of hArg on outcomes are based on several assumptions that are not all testable. One issue in all MR analyses is horizontal pleiotropy (i.e. the genetic variants affect the outcome through pathways not mediated by hArg levels). We could minimise any effects of single-variant pleiotropy on the combined causal effect estimates by using a random effects meta-analysis method and also formally testing the presence of heterogeneity between the causal estimates using Cochran's Q statistic. Although we observed single-variant pleiotropy in some SNP–outcome associations, the other two independent variants did not show even a weak association with the outcome making any true causal effects between serum hArg and any of the studied cardiometabolic risk factors and diseases unlikely.

6.6 Future prospects and clinical implications

It is likely that, by using larger sample sizes and denser sets of genetic variants, novel associations between the arginine derivatives and genetic variants could be identified. The identified ADMA, SDMA and hArg related SNPs could be used in phenome-wide association studies utilising electronic diagnostic data to reveal novel SNP-disease associations in a hypothesis-free manner. Further MR studies investigating causal effects of serum ADMA, SDMA and hArg on various phenotypes are now also possible due to an ever increasing amount of summary-level data from large-scale GWASs in the public domain.

The associations of the functional *AGXT2* variants with clinical outcomes leave open questions about the molecular underpinnings behind these associations. Therefore, the gaps in our knowledge on molecular mechanisms underlying the SNP-outcome associations should be tested by using AGXT2-deficient or overexpressing mice or by injecting AGXT2-spesific inhibitors into animals in different experimental settings and measuring plasma and tissue levels of the AGXT2 substrates, carnosine and its derivatives. Moreover, whether the functions of the autonomic nervous system could be modulated by disturbing the AGXT2/β-alanine/carnosine or AGXT2/ADMA/NO pathways warrants further investigation. By closing these gaps it might be possible to develop novel pharmacological treatments for the ever increasing burden of the studied age-dependent common diseases.

Although our results do not provide any support that interventions aimed at modifying serum hArg levels will improve the metabolic profiles or risk of T2DM or CAD, hArg might be a useful biomarker predicting future abdominal obesity in young men independent of baseline BMI or waist circumference. Additional research is warranted to replicate and validate this finding. If proved to be a useful biomarker for abdominal obesity, it could be used for early detection of individuals at high risk that would benefit from preventive interventions.

7 SUMMARY AND CONCLUSIONS

The present study was performed to analyse the genetic background of cardiometabolic disease risk by focusing on novel genetic variants associated with circulating dimethylarginines (ADMA and SDMA) and hArg levels and to investigate their role in the pathogenesis of AF, ischeamic stroke, CAD, MI and overall mortality. Further, we studied the predictive value of serum hArg in the prediction of incident cardiometabolic risk factors and diseases in young adults as well as the causal effects of serum hArg on numerous cardiometabolic risk factors, T2DM and CAD.

The main findings of the study were:

1. Several intronic *DDAH1* (rs28489187 as the lead variant) and two *AGXT2* missense variants (rs37369, Val140Ile; rs16899974, Val498Leu) were strongly associated with serum ADMA and SDMA levels, respectively. In addition, a missense variant in the mitochondrial solute carrier *SLC25A45* (rs34400381, Arg285Cys/Arg243Cys/etc. depending on the splice variant) gene showed a strong association with serum SDMA levels especially in young healthy adults from the general population.

2. Variants at the *GATM* (rs1153858), *CPS1* rs1047891 (Thr1406Asn, formerly rs7422339) and *AGXT2* (rs37369, Val140Ile) loci were associated with serum hArg at genome-wide significance. A suggestive association at the *MED23/ARG1* locus (rs17060430) for hArg was also identified.

3. The two functional *AGXT2* variants (rs37369 and rs16899974) were associated with the LF/HF ratio of spectral HRV in YFS, AF in LURIC and age at ischemic stroke onset in FINCAVAS.

4. Serum hArg predicted 10-year hyperglycemia and abdominal obesity in young men and was cross-sectionally associated with the use of

oestrogen-containing oral contraceptives in women at reproductive age. No causal effects were detected on any of the studied cardiometabolic risk factors or diseases in Mendelian randomisation analyses.

Figure 13 summarises the molecular pathways of the ADMA, SDMA and hArg metabolism and associated SNPs identified in this study.

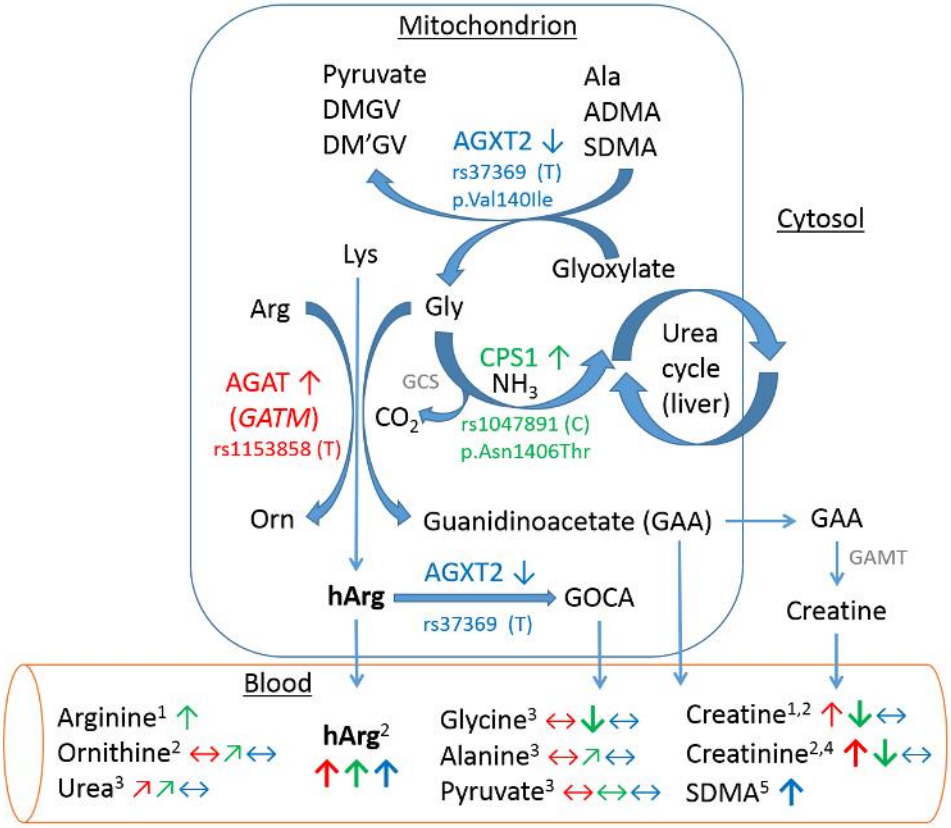

Figure 13. A model of hArg and creatine synthesis via the AGAT enzyme (encoded by the *GATM* gene) and its intra mitochondrial substrate bioavailability based on GWAS identified single-nucleotide polymorphisms associated with circulating hArg levels (study II). AGAT catalyses the formation of GAA and ornithine from arginine and glycine as well as the formation of hArg and ornithine from arginine and lysine as discussed in chapter 2.2.1. The decreased bioavailability of glycine for the GAA synthesis due to the decreased and increased activities of AGXT2 and CPS1, respectively, may shift the production of GAA by AGAT towards hArg explaining the associations of the CPS1 and AGXT2 missense variants with circulating hArg levels. In addition, hArg is directly metabolised to 6-guanidino-2-oxocaproic acid (GOCA) by AGXT2 (Rodionov *et al.* 2016). The direction of association of *GATM* rs1153858 with *GATM* mRNA expression is tissue-specific as illustrated in the supplementary material of study IV. Directions of the effects of the *CPS1*, *AGXT2* and *GATM*

variants on the enzyme activities or mRNA expression are presented by arrows. Directions and p-values of associations of the hArg associated genetic variants (marked by different colours) with blood metabolites are presented by arrows and the reference additionally marked: ↑ or ↓ = $P<5\times10^{-8}$; ↑ or ↓ = $P<0.001$; ↗ or ↘ = $P<0.05$; ↔ = $P>0.05$; 1 = (Shin *et al.* 2014), 2 = Kleber et al. (study II), 3 = (Kettunen *et al.* 2016), 4 = Pattaro et al. (Pattaro *et al.* 2016), 5 = Seppälä et al. (study I). GATM or AGAT, glycine amidinotransferase or L-arginine:glycine amidinotransferase; AGXT2, alanine-glyoxylate aminotransferase 2; CPS1, carbamoyl-phosphate synthase 1; GCS, glycine cleavage system; GAMT, guanidinoacetate N-methyltransferase; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; DM'GV, α-keto-δ-(N^G,N'^G-dimethylguanidino) valeric acid; DMGV, α-keto-δ-(N^G,N^G-dimethylguanidino) valeric acid. Modified from study IV.

These novel findings improve our understanding of the genetic regulation of the studied arginine derivatives. Moreover, the identified genetic variants may help to identify and prioritise common disease conditions and mortality end points that could be potentially treated and/or prevented by pharmacologically modifying the activity of the identified enzymes and pathways.

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Genome-wide association study on dimethylarginines reveals novel *AGXT2* variants associated with heart rate variability but not with overall mortality

AtheroRemo Consortium

Ilkka Seppälä (MSc, BM)*[1], Marcus E. Kleber (PhD)*[2,3], Leo-Pekka Lyytikäinen (MD)[1], Jussi A. Hernesniemi (MD, PhD)[1,4], Kari-Matti Mäkelä (MSc, BM)[1], Niku Oksala (MD, PhD, DSc)[1,5], Reijo Laaksonen (MD, PhD)[1], Stefan Pilz (MD, PhD)[6,7], Andreas Tomaschitz (MD)[8], Günther Silbernagel (MD)[9], Bernhard O. Boehm (MD)[10,11], Tanja B. Grammer (MD)[3], Tuomas Koskinen (MD)[12], Markus Juonala (MD, PhD)[12,15], Nina Hutri-Kähönen (MD, PhD)[13], Georg Alfthan (PhD)[14], Jorma S.A. Viikari (MD, PhD)[15], Mika Kähonen (MD, PhD)[16], Olli T. Raitakari (MD, PhD)[12,17], Winfried März (MD)[3,18,19], Andreas Meinitzer (PhD)†[19], Terho Lehtimäki (MD, PhD)†[1]

*Equally contributed. †These authors share senior authorship.

[1]Department of Clinical Chemistry, Fimlab Laboratories, Tampere University School of Medicine, Tampere, Finland; [2]Department of Internal Medicine II – Cardiology, University of Ulm Medical Centre, Ulm, Germany; [3]Institute of Public Health, Social and Preventive Medicine, Mannheim Medical Faculty, University of Heidelberg, Mannheim, Germany; [4]Department of internal medicine, North Karelia Central Hospital, Joensuu, Finland; [5]Division of Vascular Surgery, Department of Surgery, Tampere University Hospital, Tampere, Finland; [6]Department of Epidemiology and Biostatistics, EMGO Institute for Health and Care Research, VU University Medical Center, Amsterdam, the Netherlands; [7]Department of Internal Medicine, Division of Endocrinology and Nuclear Medicine, Medical University of Graz, Graz, Austria; [8]Department of Cardiology, Medical University of Graz, Graz, Austria; [9]Division of Endocrinology, Diabetology, Nephrology, Vascular Disease, and Clinical Chemistry, Department of Internal Medicine, Eberhard-Karls-University Tübingen, Tübingen, Germany; [10]Division of Endocrinology and Diabetes, Department of Internal

Medicine, Ulm University, Ulm, Germany; [11]LKC School of Medicine, Imperial College London and NTU, Singapore; [12]Research Centre of Applied and Preventive Cardiovascular Medicine, University of Turku, Turku, Finland; [13]Department of Paediatrics, University of Tampere and Tampere University Hospital, Tampere, Finland; [14]Department of Chronic Disease Prevention, National Institute for Health and Welfare, Helsinki, Finland; [15]Department of Medicine, Turku University Hospital and University of Turku, Turku, Finland; [16]Department of Clinical Physiology, Tampere University Hospital and University of Tampere, Tampere, Finland; [17]Department of Clinical Physiology and Nuclear Medicine, Turku University Hospital, Turku, Finland; [18]Synlab Services GmbH, Mannheim, Germany; [19]Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Graz, Austria.

Address for correspondence:

Ilkka Seppälä

Department of Clinical Chemistry

Finn-Medi 2, 3rd floor

P.O. Box 2000, FI-33521 Tampere, FINLAND

Email: ilkka.seppala@uta.fi

Aims: The purpose of this study was to identify novel genetic variants influencing circulating asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) levels and to evaluate whether they have prognostic value on cardiovascular mortality.

Methods and Results: We conducted a genome-wide association study on the methylarginine traits and investigated the predictive value of the new discovered variants on mortality. Our meta-analyses replicated the previously known locus for ADMA levels in *DDAH1* (rs997251; $P=1.4\times10^{-40}$), identified two nonsynomyous polymorphisms for SDMA levels in *AGXT2* (rs37369; $P=1.4\times10^{-40}$ and rs16899974; $P=1.5\times10^{-38}$) and one in *SLC25A45* (rs34400381; $P=2.5\times10^{-10}$). We also fine-mapped the *AGXT2* locus for further independent association signals. The two nonsynonymous *AGXT2* variants independently associated with SDMA levels were also significantly related with short-term heart rate variability (HRV) indices in young adults. The major allele (C) of the novel nonsynonymous rs16899974 (V498L) variant associated with decreased SDMA levels and an increase in the ratio between the low frequency (LF) and high frequency (HF) spectral components of HRV (P=0.00047). Furthermore, the SDMA decreasing allele (G) of the nonsynomyous SLC25A45 (R285C) variant was associated with lower resting mean heart rate during the HRV measurements (P=0.0046) but not with the HRV indices. None of the studied genome-wide significant variants had any major effect on cardiovascular or total mortality in patients referred for coronary angiography.

Conclusions: *AGXT2* has an important role in SDMA metabolism in humans. *AGXT2* may additionally have an unanticipated role in the autonomic nervous system regulation of cardiac function.

Keywords: genome-wide association study; asymmetric dimethylarginine; symmetric dimethylarginine; genetics; heart rate variability; mortality; sudden cardiac death

Introduction

Increasing evidence shows that both ADMA and SDMA might have independent roles in cardiovascular diseases (1) and the prediction of cardiovascular events (2,3). Elevated circulating SDMA levels have been reported to be associated with increased mortality in stroke patients (4), increased risk of cardiac death in patients with non-ST-elevation myocardial infarction (5) and worse prognosis in patients referred for coronary angiography (3). Indeed, increasing *in vitro* and *in vivo* evidence support the concept that SDMA may have an independent role in the pathogenesis of cardiovascular diseases. SDMA induces reactive oxygen species (ROS) production of monocytes (6) and promotes inflammation in chronic kidney disease (7). Moreover, recent data show that SDMA may promote endothelial dysfunction (8).

Previous candidate gene studies have identified a limited number of single-nucleotide polymorphisms (SNPs) associated with plasma ADMA levels in the dimethylarginine dimethylaminohydrolase-1 and -2 (*DDAH1* and *DDAH2*) loci (9), whereas the genetic variants regulating SDMA levels are not known. There is evidence that AGXT2 plays a role in both ADMA and SDMA metabolisms *in vivo* (10), but association data between genetic variants and circulating methylarginine levels in humans is currently lacking. Furthermore, AGXT2 knockout mice show a hypertensive phenotype, but the underlying mechanisms behind this observation are not currently well understood (11). To investigate the role of AGXT2 in health and disease in humans, the identification of the functional genetic variants affecting the enzymatic activity of the AGXT2 protein and the regulation of its expression in different human tissues is of major importance.

Therefore, to identify additional new loci for ADMA, and especially for SDMA, we performed the first meta-analyses of genome-wide association (GWA) studies for the methylarginines using data from 5,110 participants from the Young Finns Study (YFS) and the Ludwigshafen Risk and Cardiovascular Health (LURIC) study. Secondly, we fine-mapped the *AGXT2* locus, that showed

the strongest association with SDMA, for variants independently affecting circulating SDMA levels. Finally, we tested the association of these variants with mortality end-points in LURIC and heart rate variability parameters in YFS to be able to characterize the pathophysiological role of these new *AGXT2* variants.

Methods

The study flow is summarized in **Figure 1**.

Study populations

We conducted a meta-analysis of GWA data on 5,110 individuals of European descent drawn from two large cohorts: the Young Finns Study (YFS) and the Ludwigshafen Risk and Cardiovascular Health study (LURIC). The YFS cohort is a Finnish longitudinal population study sample on the evolution of cardiovascular risk factors from childhood to adulthood (12). In the present study, we used the variables measured in 2001. The LURIC study consists of 3,316 Caucasian patients hospitalized for coronary angiography between 1997 and 2000 at a tertiary care center in Southwestern Germany (13). Clinical indications for angiography were chest pain or a positive non-invasive stress test suggestive of myocardial ischemia. To limit clinical heterogeneity, individuals suffering from acute illnesses other than acute coronary syndrome (ACS), chronic non-cardiac diseases and a history of malignancy within the five past years were excluded. See further description and characteristics in **Supplementary Methods 1** and **Table S1**.

Biochemical

ADMA and SDMA were measured from frozen serum using the same reversed-phase HPLC method in both studies. For details and other biochemical measurements, see **Supplementary Methods 2**.

Genotyping and quality control

In LURIC, genotyping was done by using the Affymetrix Human SNP Array 6.0 at the LURIC Study facility. In YFS genotyping was done using a custom-built Illumina Human 670k BeadChip at the Welcome Trust Sanger Institute (**Supplementary Methods 3**). Genotype imputation was performed using minimac (14) and IMPUTE2 (15) in LURIC and YFS, respectively. Genotype imputation in both studies was performed using 1000 Genomes Phase I Integrated Release Version 3 (Mar 2012) samples as a reference. We excluded imputed SNPs and insertion-deletion polymorphisms with low imputation quality (Rsq<0.3 or info<0.4) and allele frequency <0.1%. After the exclusions, 10,085,758 imputed genetic variants were available in both studies.

Short-term heart rate variability

We used the ratio between low and high frequency components (LF/HF) of spectral HRV to estimate sympathovagal balance in the YFS participants. Mean heart rate during HRV measurement was used as an estimate of resting heart rate. Breathing was metronome controlled at the frequency of 0.25 Hz. We excluded subjects with current antihypertensive medications, type 1 diabetes, pregnant women, and breast-feeding women. After these exclusions genotype data was available for 1,723 subjects and were included in the association analyses. For details, see **Supplementary Methods 4.**

Classification of SCD cases

In LURIC, SCD was defined as sudden unexpected death either within 1 h of symptom onset or within 24 h of having been observed alive and symptom-free. Patients who suffered from any non-cardiac chronic and terminal disease (e.g., cancer) so that their death was not unexpected and those whose sudden death was most likely attributed to a non-cardiac cause were not classified as SCD cases. See

Supplementary Methods 4 for more details about the cause of death classifications.

Statistical analyses

To identify and correct for population stratification, we performed an MDS analysis as implemented in PLINK 1.07 (16). In both studies, residual ADMA and SDMA concentrations or residuals of ADMA/SDMA ratio were determined after regression adjustment using R software. Outliers (≥4 SD from the mean) were removed from the dimethylarginine phenotype values before covariate adjustment to avoid spurious associations (typically 5–20 data points for any one outcome). Residuals were obtained using linear regression analysis in which the dimethylarginine phenotypes were adjusted for sex, age, body mass index and serum creatinine to account for variation in renal function, as well as the first three MDS components to control for population stratification. As circulating ADMA is a major determinant of SDMA levels and vice versa (3), the model for ADMA was additionally adjusted for SDMA and the model for SDMA was adjusted for ADMA. Residuals were normalized to have a mean of 0 and SD of 1 using inverse normal transformation. The association analysis of the imputed SNPs and indels assuming an additive genetic model was carried out utilizing the SNPTEST v2.4.1 (17) and ProbABEL v. 0.3.0 (18) softwares in YFS and LURIC, respectively. QQ and Manhattan plots were drawn for the analysis of the results. To estimate how well the distribution was calibrated, for each phenotype in both studies, we calculated the genomic inflation factor (λ) from all imputed variants. The λ for each trait in both studies ranged from 0.993 to 1.012, suggesting that there was no major residual confounding by population stratification, and little evidence of cryptic relatedness. We created the regional association plots using LocusZoom (19). The summary results from both cohorts were meta-analyzed with the random effects meta-analysis method implemented in GWAMA (20). A stringent genome-wide significance level of 1.66×10^{-8} was set to correct for multiple testing of the 3 phenotypes.

We fine-mapped the *AGXT2* locus using a series of sequential conditional analyses by adding the most strongly associated SNP into the regression model as a covariate and using the inverse-normal transformed residuals as an outcome variable to test all remaining regional SNPs for association. The results from both studies were combined using random-effects meta-analysis. We repeated this procedure until the strongest SNP showed a conditional *P* value $>10^{-4}$.

Further statistical analyses were performed using R 3.0.0 (http://www.r-project.org). A Cox regression proportional hazards analysis was performed to test whether the *AGXT2* and *SLC25A45* genotypes were associated with mortality end-points. The risk of mortality was quantified as hazard ratios (HRs). The proportional hazards assumption was checked with Schoenfeld's residuals and was met for all models. The screening tests were conducted using age and sex as covariates and additive genetic model. Further analyses were performed adjusting for well-established SCD risk factors. Secondly, to investigate the possible mechanism underlying the associations with SCD, analyses were stratified according to the median value of left ventricular systolic pressure. Finally, the associations with heart rate variability variables were tested using linear regression analysis. Genotyped data was used for rs37369 in LURIC. For all other SNPs and analyses, imputed data was used. Imputation quality parameters and minor allele frequencies for *AGXT2* and *SLC25A45* SNPs are provided in **Table S2**.

Results

GWA studies for dimethylarginines and their ratio

We carried out GWA meta-analyses on ADMA, SDMA, and their ratio using data from the YFS and LURIC populations involving a total of 5,110 individuals of European ancestry. The strongest associations for each locus from the meta-analyses are shown in **Table 1** with an index SNP representing the most significant association labeled for each independent signal. Across the genome,

the most significant association for ADMA levels ($P=1.4\times10^{-40}$) was on chromosome 1p22 harboring *DDAH1*, which encodes the key regulatory enzyme of ADMA metabolism dimethylarginine dimethylamiohydrolase (DDAH). The most significant signal for SDMA levels ($P=1.4\times10^{-40}$) was on chromosome 5p13 at rs37369 (V140I) in the coding sequence of alanine-glyoxylate aminotransferase 2 (*AGXT2*). Another genome-wide significant signal for SDMA levels ($P=2.5\times10^{-10}$) was identified in the coding sequence of a mitochondrial carrier protein *SLC25A45* at rs34400381 (R285C). A list of suggestive signals for each trait not reaching genome-wide significance is provided in **Tables S4-S5**.

Fine-mapping of the *AGXT2* region

Next, we fine-mapped the newly identified *AGXT2* region showing the strongest association with SDMA for additional independent signals using sequential conditional analysis. Of the methylarginine traits, SDMA was used as an outcome variable in this analysis because it showed the strongest association with *AGXT2* variants in the original meta-analysis. Interestingly, another independent genome-wide significant association was pinpointed at rs16899974 (V498L) when conditioning on the top SNP at rs37369 (V140I) **(Figure 2A-B)**. In the next step, when adjusting for the two missense mutations (rs37369 and rs16899974), a number of intronic variants showed significant association ($P<10^{-4}$) with SDMA after correcting for multiple testing in the *AGXT2* region (**Figure 2C**). No other informative ($P<10^{-4}$) intronic or coding sequence *AGXT2* variants were identified when further adjusting for the top intronic variant rs13165070 (**Figure 2D**).

Association of nonsynonymous *AGXT2* variants with heart rate variability in young adults (YFS)

The SDMA decreasing alleles of the studied *AGXT2* coding variants were associated with an increase in the ratio between low frequency (LF) and high frequency (HF) spectral components of the short-term heart rate variability in YFS (**Table 2**). The association was especially marked between the novel nonsynonymous rs16899974 (V498L) variant and the LF/HF ratio (P=0.00047). None of the variants showed an association with LF component (always P>0.20). Instead, rs16899974 (V498L) variant showed a significant association (P=0.028) with the HF component (estimate of vagal-tone). The nonsynonymous *SLC25A45* rs34400381 (R285C) variant was not associated with the LF/HF component (P=0.39) but the SDMA decreasing allele (G) was associated with a decrease in resting mean heart rate during the HRV measurements independent of the LF/HF ratio (**Table 2**). Further adjustment for serum ADMA or SDMA levels did not change our results.

Associations of nonsynonymous *AGXT2* and *SLC25A45* variants with mortality in LURIC

Finally, we investigated whether the nonsynonymous *AGXT2* and *SLC25A45* coding variants independently associated with AGXT2 substrate levels could also predict mortality among patients referred for coronary angiography. None of the studied nonsynonymous *AGXT2* or *SLC25A45* SNPs were associated with all-cause or cardiovascular mortality (always P>0.05). However, the SDMA decreasing major allele (G) of the *SLC25A45* polymorphism (rs34400381, R285C) was associated with increased risk for SCD with per allele hazard ratio of 13.0 (95% CI 1.54-110, P=0.018). The two nonsynonymous *AGXT2* variants were not associated with SCD in the whole study population. However, both variants showed a significant interaction with left ventricular systolic pressure (LVSP) on SCD (**Table 3**). *AGXT2* rs16899974 (V498L) showed a nominally significant associated with SCD in the low LVSP group (P=0.021) whereas there was a trend toward an association for rs37369 (P=0.10). Analogously to the association for the *SLC25A45* variant (R285C), the SDMA decreasing

alleles were associated with increased risk for SCD. No associations were observed between the AGXT2 variants and SCD in the high LVSP group (all P>0.20) and there was no significant interaction between *SLC25A45* rs34400381 and the LVSP status on SCD (P=0.63). Additional adjustments for circulating ADMA or SDMA did not materially change our results.

Discussion

We report the first GWAS results on circulating ADMA and SDMA levels based on meta-analyses of 5,110 individuals of European ancestry and provide strong evidence that two common non-synonymous coding variants in alanine-glyoxylate aminotransferase 2 (*AGXT2*) and one non-synonymous coding variant in *SLC25A45* (R285C) are independently associated with circulating SDMA levels. Moreover, we document that the nonsynonymous *AGXT2* and *SLC25A45* variants showed associations with the LF/HF component of spectral heart rate variability (HRV) and resting heart rate in young adults, respectively.

The polymorphism (rs37369) with the strongest association with circulating SDMA codes for a non-synonymous valine-to-isoleucine (V140I) substitution in the AGXT2 protein. Another nonsynomyous *AGXT2* rs16899974 (V498L) SNP and a number of intronic variants were also associated independently with SDMA levels. The lead intronic variant (rs13165070) identified in the conditional analysis is in a high LD (r^2~0.9) with intronic rs10521021 that has been previously associated with *AGXT2* mRNA levels in human liver (21). The T allele of rs37369 is associated with both increased circulating SDMA levels in the present study and increased levels of urinary 3-aminoisobutyrate (BAIB) metabolite (22). These results together refer to the possibility that the two novel nonsynomyous *AGXT2* variants may contribute to plasma SDMA levels through the enzymatic decomposition of SDMA in the kidneys. Indeed, AGXT2 expression in the kidney has recently been

shown to be inversely correlated with both circulating ADMA and SDMA levels in humans (11). SDMA is transaminated to α-keto-δ-(*N*,*N'*-dimethylguanidino)valeric acid (DMGV') by AGXT2 (23). Our findings support the hypothesis that this mitochondrial (24) AGXT2-related pathway can effectively metabolize SDMA in humans. Moreover, measurable quantities of α-keto-δ-(*N*,*N*-dimethylguanidino)valeric acid (DMGV), the transamination product of ADMA by AGXT2, was recently reported being present in human plasma and urine (25).

We observed a genome wide significant association for SDMA at rs34400381 (R285C) in *SLC25A45* encoding a mitochondrial inner membrane transporter protein. Interestingly, *SLC25A45* has the same intracellular localization than *AGXT2* although its function is not well known (26). The association is highly genome-wide significant in YFS ($P=2.4\times10^{-9}$) but only nominally significant in LURIC (P=0.026). This association clearly needs replication in an independent population.

Our data suggests that nonsynonymous *AGXT2* variants may have an effect on the autonomic balance mainly by modulating the vagal tone because *AGXT2* rs16899974 was associated with both the LF/HF ratio and the vagal component HF of HRV alone. The physiological interpretation of LF/HF is not straightforward. The LF component was observed to be correlated with baroreflex function (27). Moreover, both HF and LF have been shown to be reduced by atropine administration indicating that both spectral components of HRV are under vagal control (28), in line with the idea that under resting conditions variations in heart rate are largely dependent on vagal modulation (29). Nevertheless, increased LF/HF and decreased HF have been previously associated with increased prevalence of the metabolic syndrome in the YFS cohort (30). Moreover, decreased HRV appears to be associated with hypertension, dyslipidemia, impaired glucose metabolism, increased ventricular arrhythmias and mortality (31-35). In the present study, we also showed that a nonsynonymous *SLC25A45* variant (R285C) was associated with decreased resting heart rate during the HRV measurements but not with HRV indices. The observation that the adjustment for circulating

methylarginine levels did not change the results suggests that the associations between the *AGXT2* and *SLC25A45* coding variants and heart rate regulation could be mediated through transamination of other AGXT2 substrates than methylarginines. Although further studies are needed to understand the molecular mechanisms underlying the associations between heart rate regulation and nonsynonymous variants within *AGXT2* and *SLC25A45*, it is tempting to speculate that transamination activity of AGXT2 on GABA isomers glycine and/or β-aminoisobuturic acid (BAIB), a glycine receptor agonist (37), could play a role explaining the associations. The striking association of *AGXT2* rs37369 with circulating BAIB levels (22) and the presence glycinergic synaptic inputs to cardiac vagal neurons in the brainstem nuclei (36) support this hypothesis.

None of the genome-wide significant variants were associated with cardiovascular or overall mortality. Interestingly, the AGXT2 substrate (SDMA) decreasing major allele (G) of the nonsynonymous *SLC25A45* (R285C) variant was associated with increased risk for SCD in the whole LURIC population independent of circulation methylarginine levels. However, this association should be interpreted with conscious due to the moderate imputation quality for the variant (r^2~0.43). Moreover, we observed significant interactions between the pressure creation capacity of the left ventricle and the two nonsynonymous *AGXT2* variants on SCD. We used left ventricular systolic pressure (LVSP) to quantify the degree of the systolic dysfunction and heart failure. Both the increased sympathic drive (38) and the parasympathetic withdrawal (39) are integral components of congestive heart failure. It has been known for decades that sympathetic activation can trigger fatal ventricular arrhythmias, whereas vagal activity may exert a protective effect (38). Interestingly, the HF HRV decreasing major allele (C) of rs16899974 was associated with increased risk for SCD in patients with impaired systolic function but not in patients with preserved LVSP. Although the AGXT2 substrate levels decreasing alleles of these variants are consistently associated with increased risk for SCD, these results should be considered as explanatory and a replication in an independent

population is warranted.

Based on our results it may be worth investigating whether AGXT2-/- animals have altered vagal tone compared to wild-type animals and whether they are protected against ventricular arrhythmia in an experimental model of MI-induced systolic heart failure. The associations with the HRV parameters and SCD should also be investigated in an independent population of MI and heart failure patients.

The present study has several strengths and limitations that warrant consideration. The strengths include large, well-characterized cohorts, the prospective design, and long-term follow-up with prospectively adjudicated SCD cases. The potential limitations include the possibility of some degree of misclassification of SCD cases. However, this kind of bias is likely to lead to an underestimation rather than an overestimation of the true genetic effects. Moreover, the generalizability of the SCD association to the general population and other ethnic groups may be limited. Finally, although we attempted to adjust for all relevant covariates, we could not rule out the possibility of residual confounding.

In summary, our data clearly indicate that two coding sequence SNPs rs37369 (V140I) and rs16899974 (V498L) in *AGXT2* are associated with circulating SDMA levels in two large cohorts of healthy adults and patients referred to coronary angiography, supporting the idea that AGXT2 has a physiological role in the SDMA metabolism in humans. The two nonsynomyous *AGXT2* variants showed associations with the LF/HF component of spectral HRV whereas the novel nonsynomyous *SLC25A45* variant was associated with resting heart rate in young adults. AGXT2 and SLC25A45 may have unanticipated roles in the regulation of autonomic balance and heart rate in health and disease.

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Figure legends

Figure 1. Study Population and Flow.

Flow diagram depicting the cohorts and numbers of participants. The associations of the *AGXT2* variants identified in the conditional analysis with heart rate variability and SCD were investigated in the YFS and LURIC cohorts, respectively.

Figure 2. Regional plots for fine-mapping results in the *AGXT2* region.

Fine mapping of the *AGXT2* region for independent association signals using sequential conditional analysis and circulating SDMA levels as an outcome variable. Original meta-analysis **(A)**, adjusted for rs37369 (V140I) **(B)**, additionally adjusted for rs16899974 (V498L) **(C)** and additionally adjusted for rs13165070 **(D).** Signals above the red line ($P<1\times10^{-4}$) were considered to exhibit evidence of association in the *AGXT2* region.

Tables

Table 1. Genome-wide significant associations for each methylarginine trait in the meta-analyses.

Trait	Variant	Locus	Chr.	Position	EA	NEA	EAF (%)	n	β (s.e.)	P-value
ADMA	rs28489187	Intron of *DDAH1*	1	85797110	G	A	21.6	5105	0.37 (0.028)	1.39×10^{-40}
SDMA	rs37369	CDS of *AGXT2* (V140I)	5	35037115	T	C	8.8	5103	0.47 (0.035)	1.42×10^{-40}
SDMA	rs1884139	Intron of *DDAH1*	1	85845998	T	G	37.1	5103	-0.18 (0.020)	9.65×10^{-19}
SDMA	rs34400381	CDS of *SLC25A45* (R285C)	11	65143892	A	G	4.3	5102	0.36 (0.056)	2.48×10^{-10}
ADMA/SDMA	rs37369	CDS of *AGXT2* (V140I)	5	35037115	T	C	8.8	5102	-0.41 (0.035)	2.08×10^{-32}
ADMA/SDMA	chr1:85897175:D	Intron of *DDAH1*	1	85897175	A	ATCC	17.4	5102	0.27 (0.027)	3.85×10^{-24}

Chr., chromosome; position, variant position in NCBI human build 37; EA and NEA, effect allele and non-effect allele; EAF, effect allele frequency; CDS, coding sequence; β, effect size in standard deviations per copy of the effect allele.

Table 2. Associations between nonsynonomyous AGXT2 and SLC25A45 variants with SDMA, heart rate variability and resting heart rate.

				GWAS meta-analysis (n=5,110) SDMA		Young Finns (n=1,723) lnLF/HF		Mean HR (bpm)	
SNP	Gene	Effect allele	EAF (%)	β (SE)	P	β (SE)	P	β (SE)	P
rs37369 (V140I)	*AGXT2*	C	91.2	-0.47 (0.035)	1.4×10^{-40}	0.10 (0.052)	0.045	-0.54 (0.58)	0.36
rs16899974 (V498L)	*AGXT2*	C	77.2	-0.32 (0.024)	1.5×10^{-38}	0.13 (0.036)	0.00047	-0.73 (0.41)	0.073
rs34400381 (R285C)	*SLC25A45*	G	95.7	-0.36 (0.056)	2.5×10^{-10}	0.055 (0.064)	0.39	-2.02 (0.71)	0.0046

Per effect allele β (SE) from conditional linear regressions. Effect alleles are SDMA decreasing alleles from the GWAS meta-analysis analysis. Models for the ln transformed LF/HF component of short-term HRV are adjusted for age, sex and heart rate. Models for mean heart rate are adjusted for age, sex and lnLF/HF. Abbreviations: EAF, effect allele frequency; HR, heart rate; bpm, beats per minute; SE, standard error.

Table 3. Associations between nonsynonomyous *AGXT2* variants and SCD.

				All (n = 2748, 173 SCDs)		LVSP < 145 mmHg (n = 1220, 66 SCDs)		LVSP ≥ 145 mmHg (n = 1199, 83 SCDs)		
SNP	Gene	Effect allele	EAF (%)	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	P for interaction
rs37369	*AGXT2*	C	91.2	1.04 (0.70-1.55)	0.83	1.98 (0.88-4.45)	0.10	0.72 (0.44-1.19)	0.20	0.041
rs16899974	*AGXT2*	C	77.2	1.17 (0.87-1.55)	0.30	1.84 (1.10-3.09)	0.021	0.81 (0.55-1.21)	0.31	0.035

Per effect allele HRs (95% CI) from conditional Cox regressions. Effect alleles are SDMA decreasing alleles from the GWAS meta-analysis analysis. All models are adjusted for age, sex, Cystatin C, HbA1c, beta blocker use, left ventricular hypertrophy (ECG), and cardiomyopathy. Abbreviations: CDS, coding sequence; EAF, effect allele frequency; HR, hazard ratio per effect allele increase; CI, confidence interval; LVSP, left ventricular systolic pressure in coronary angiography.

Figure 1

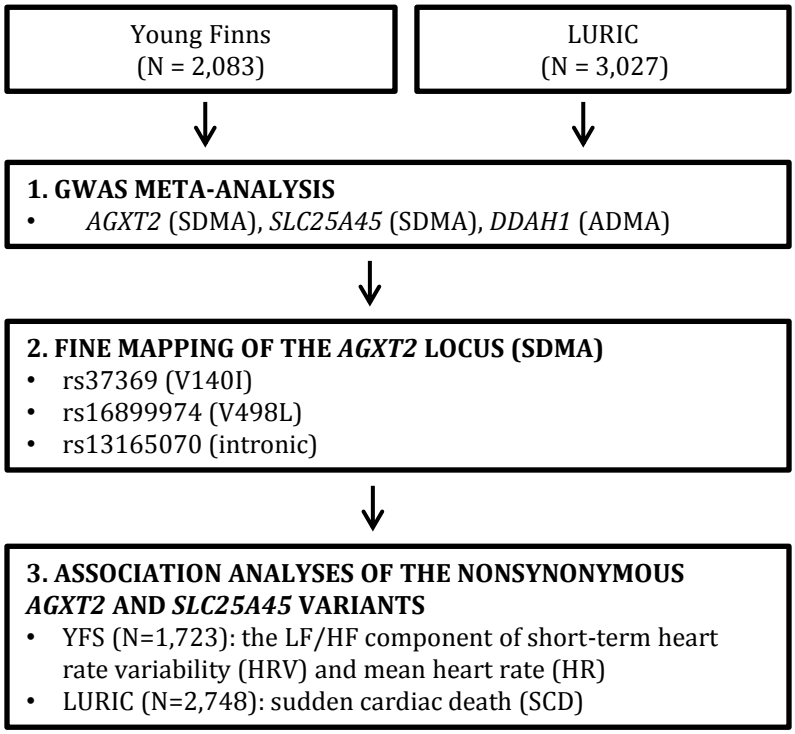
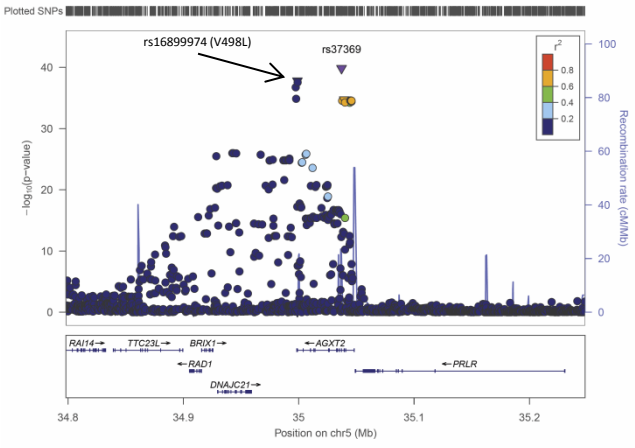
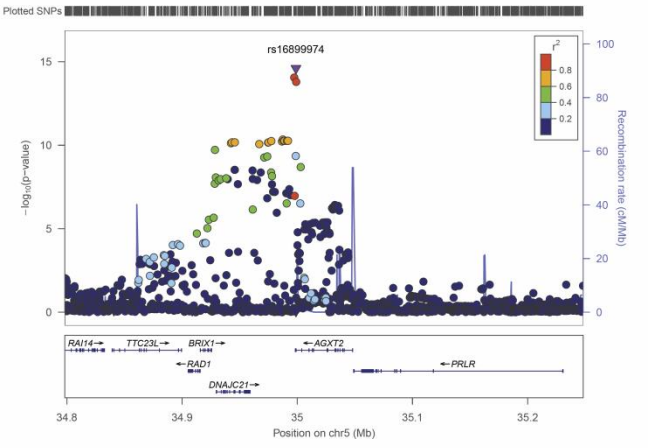

Young Finns
(N = 2,083)
LURIC
(N = 3,027)
1. GWAS META-ANALYSIS
• AGXT2 (SDMA), SLC25A45 (SDMA), DDAH1 (ADMA)
2. FINE MAPPING OF THE AGXT2 LOCUS (SDMA)
• rs37369 (V140I)
• rs16899974 (V498L)
• rs13165070 (intronic)
3. ASSOCIATION ANALYSES OF THE NONSYNONYMOUS AGXT2 AND SLC25A45 VARIANTS
• YFS (N=1,723): the LF/HF component of short-term heart rate variability (HRV) and mean heart rate (HR)
• LURIC (N=2,748): sudden cardiac death (SCD)

Figure 2

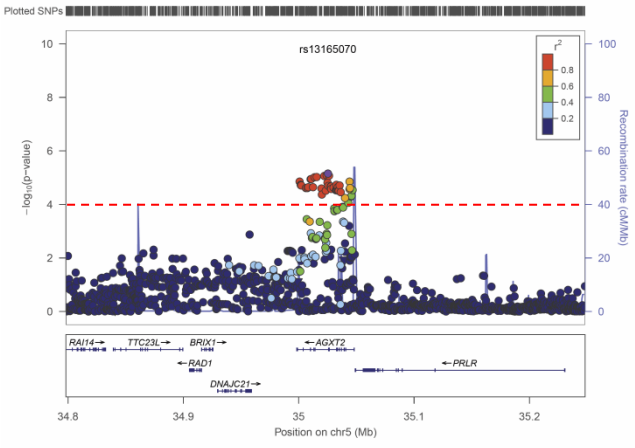
A



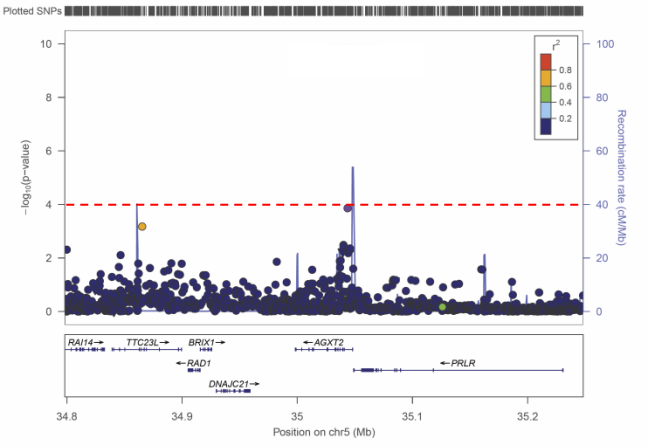
B



C



D



Genome-Wide Association Study Identifies 3 Genomic Loci Significantly Associated With Serum Levels of Homoarginine

The AtheroRemo Consortium

Marcus E. Kleber, PhD*; Ilkka Seppälä, MSc*; Stefan Pilz, MD, PhD; Michael M. Hoffmann, PhD; Andreas Tomaschitz, MD; Niku Oksala, MD, PhD, DSc; Emma Raitoharju, MSc; Leo-Pekka Lyytikäinen, MD; Kari-Matti Mäkelä, MSc, BM; Reijo Laaksonen, MD, PhD; Mika Kähönen, MD, PhD; Olli T. Raitakari, MD, PhD; Jie Huang, MD, MPH; Katharina Kienreich, MD; Astrid Fahrleitner-Pammer, MD; Christiane Drechsler, MD, PhD; Vera Krane, MD; Bernhard O. Boehm, MD; Wolfgang Koenig, MD; Christoph Wanner, MD; Terho Lehtimäki, MD, PhD; Winfried März, MD; Andreas Meinitzer, PhD

Background—Low serum levels of the amino acid derivative, homoarginine, have been associated with increased risk of total and cardiovascular mortality. Homoarginine deficiency may be related to renal and heart diseases, but the pathophysiologic role of homoarginine and the genetic regulation of its serum levels are largely unknown.

Methods and Results—In 3041 patients of the Ludwigshafen Risk and Cardiovascular Health (LURIC) study referred for coronary angiography and 2102 participants of the Young Finns Study (YFS), we performed a genome-wide association study to identify genomic loci associated with homoarginine serum levels and tested for associations of identified single-nucleotide polymorphisms with mortality in LURIC. We found genome-wide significant associations with homoarginine serum levels on chromosome 2 at the carbamoyl phosphate synthetase I locus, on chromosome 5 at the alanine-glyoxylate aminotransferase 2 locus, and on chromosome 15 at the glycine amidinotransferase locus, as well as a suggestive association on chromosome 6 at the *Homo sapiens* mediator complex subunit 23 gene/arginase I locus. All loci harbor enzymes located in the mitochondrium are involved in arginine metabolism. The strongest association was observed for rs1153858 at the glycine amidinotransferase locus with a *P* value of 1.25E-45 in the combined analysis and has been replicated in both the Die Deutsche Diabetes Dialyse Studie (4D study) and the Graz Endocrine Causes of Hypertension (GECOH) study.

Conclusions—In our genome-wide association study, we identified 3 chromosomal regions significantly associated with serum homoarginine and another region with suggestive association, providing novel insights into the genetic regulation of homoarginine. **(*Circ Cardiovasc Genet*. 2013;6:505-513.)**

Key Words: amino acids ■ arteriosclerosis ■ cardiovascular diseases ■ genome-wide association study

Homoarginine, a cationic amino acid, has been suggested previously to exert protective cardiovascular effects, putatively mediated by regulating the synthesis of the endothelial-derived relaxing factor, nitric oxide.[1,2] Data from the Ludwigshafen Risk and Cardiovascular Health (LURIC) study have shown that low homoarginine is a significant and independent risk factor for all-cause and cardiovascular mortality.[3] These findings among patients referred for coronary angiography were replicated in a cohort of dialysis patients with type 2 diabetes mellitus participating in the Die Deutsche Diabetes Dialyse Studie (4D study).[3] More detailed analyses on specific causes of death revealed an association of low homoarginine levels with fatal stroke[4] and sudden cardiac death,[5] as well as a positive correlation of homoarginine with angiographic left ventricular ejection fraction and an inverse correlation with N-terminal pro-B-type natriuretic peptide,[6] which might point to previously unknown pathophysiologic processes that could be relevant for the diagnosis and treatment of life-threatening heart and kidney diseases.[1–6]

Clinical Perspective on p 513

The current understanding of homoarginine metabolism is, however, in its infancy. Previous studies suggest that homoarginine is mainly derived from the kidney and may thus be associated with renal function.[7,8] Beyond this, we recently found an association between homoarginine and bone metabolism,[9,10] and a recent report found a key role of the enzyme glycine amidinotransferase (GATM) in homoarginine synthesis.[11]

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*Dr Kleber and I. Seppälä contributed equally to this work.

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Correspondence to Marcus Kleber, PhD, Mannheim Institute of Public Health, Social and Preventive Medicine, Ludolf-Krehl-Strasse 7–11, 68167 Mannheim, Germany. E-mail Marcus.kleber@medma.uni-heidelberg.de

l-Lysine can serve as a precursor for homoarginine synthesis, but detailed enzymatic key reactions determining homoarginine serum levels are largely unknown.[7,8]

We performed a genome-wide association study (GWAS) to identify genes that are involved in the regulation of homoarginine serum levels. Our study was performed on 3041 patients from the LURIC study, who were followed up for ≈10 years with respect to fatal events and in 2102 participants of the Young Finns Study (YFS). We further aimed to replicate identified genetic polymorphisms in 1244 hemodialysis patients with type 2 diabetes mellitus recruited from the 4D study[12] and 230 patients of the Graz Endocrine Causes of Hypertension (GECOH) study.[13]

Methods

Study Populations

LURIC Study

The LURIC study consists of 3316 white patients hospitalized for coronary angiography between 1997 and 2000 at a tertiary care center in Southwestern Germany.[14] Clinical indications for angiography were chest pain or a positive noninvasive stress test suggestive of myocardial ischemia. To limit clinical heterogeneity, individuals experiencing acute illnesses other than acute coronary syndrome, chronic noncardiac diseases, and a history of malignancy within the 5 past years were excluded. The study was approved by the ethics committee at the Ärztekammer Rheinland-Pfalz and was conducted in accordance with the Declaration of Helsinki. Informed written consent was obtained from all participants. In 3041 LURIC participants, genotypes, homoarginine levels, and all necessary covariates were available.

Cardiovascular Risk in YFS

In brief, the YFS cohort is a Finnish longitudinal population study sample on the evolution of cardiovascular risk factors from childhood to adulthood.[15] In the present study, we used the variables measured in 2001. For these subjects, genotyping was performed in 2009 using a custom-built Illumina Human 670k BeadChip at the Welcome Trust Sanger Institute. In 2102 subjects, genotype, risks factors, and phenotype data were complete, and they formed the present study population. This study was conducted according to the guidelines of the Declaration of Helsinki, and the study protocol was approved by local ethics committees. All participants gave their informed consent.

Tampere Vascular Study

Vascular sample series from femoral arteries, carotid arteries, and abdominal aortas were obtained during open vascular procedures between 2005 and 2008. The study has been approved by the Ethics Committee of Tampere University Hospital. All clinical investigations were conducted according to the Declaration of Helsinki. Tampere Vascular Study (TVS) whole blood and monocyte collections were performed in 2008, the angiographically verified patient samples to new TVS study were preselected from a larger population-based study,[16] and 55 individuals with angiographically verified coronary artery disease and 45 without were selected. The participant pool comprises patients who have undergone an exercise-stress test at Tampere University Hospital. A description of the studies used for replication is supplied in the online-only Data Supplement.

Clinical Definitions

In LURIC, coronary artery disease (CAD) has been defined angiographically using the maximum luminal narrowing estimated by visual analysis. CAD was defined as the presence of a visible luminal narrowing (>20% stenosis) in ≥1 of 15 coronary segments according to a classification of the American Heart Association. Diabetes mellitus has been defined according to American Diabetes Association (ADA) 2010 guidelines as increased fasting (>126 mg/dL), postchallenge (2 hours after the 75 g glucose load >200 mg/dL) glucose, elevated glycated hemoglobin (>6.5%), and history of diabetes mellitus. Hypertension was defined as a systolic and diastolic blood pressures >140 and >90 mm Hg, respectively, or a significant history of hypertension.

Laboratory Procedures

For LURIC patients, fasting blood samples were obtained by venipuncture in the early morning. Blood glucose, cholesterol, and triglycerides were measured by standard laboratory procedures as described previously.[14] High-density lipoprotein cholesterol was measured after separating lipoproteins with a combined ultracentrifugation-precipitation method.[14] Genomic DNA was prepared from EDTA-anticoagulated peripheral blood using a common salting-out procedure. Total homocysteine concentration in EDTA plasma was determined by high-performance liquid chromatography (Waters millennium chromatography with fluorescence detector 470). Arginine was measured in serum samples with a conventional amino acid analysis technique, involving separation of amino acids by ion exchange chromatography followed by postcolumn continuous reaction with ninhydrin.[17] Creatine was determined in serum by liquid chromatography tandem mass spectrometry.[18]

For YFS subjects, venous blood samples were drawn after an overnight fast. Standard enzymatic methods were used for serum total cholesterol, triglycerides, and high-density lipoprotein cholesterol.

Homoarginine was measured in all studies in serum stored at −80°C by a reversed phase high-performance liquid chromatography at the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz.[19,20] Intraday coefficients of variation at different concentrations (mean levels) were 4.7% (1.21 μmol/L) and 2.2% (3.53 μmol/L), and between-day coefficients of variation were 7.9% (1.25 μmol/L) and 6.8% (3.66 μmol/L).

Genotyping and Quality Control

In LURIC, genotyping was done using the Affymetrix Human SNP Array 6.0 at the LURIC study facility, whereas in YFS, genotyping

Table 1. Description of the Study Populations

	LURIC	YFS
No. of participants	3041	2102
Age, y (mean±SD)	62.7 (10.6)	31.7 (5.0)
Male sex, %	70.0	45.1
BMI, kg/m^2 (mean±SD)	27.5 (4.0)	25.1 (4.4)
Systolic blood pressure, mm Hg (mean±SD)	141.2 (23.6)	117.7 (13.1)
Diastolic blood pressure, mm Hg (mean±SD)	80.9 (11.4)	70.8 (10.8)
Hypertension, %	58.7	2.0
History of CAD, %	79.1	…
History of diabetes mellitus, %	40.5	0.6
Active smokers, %	23.1	23.9
Total cholesterol, mg/dL (mean±SD)	192.4 (10.8)	200.7 (38.0)
HDL-C, mg/dL (mean±SD)	38.7 (10.8)	50.3 (12.3)
Triglycerides, mg/dL		
Median (25th and 75th percentile)	146.0 (109.0–201.0)	97.9 (71.2–142.4)
Homoarginine, μmol/L		
Median (25th and 75th percentile)	2.4 (1.8–3.1)	1.8 (1.4–2.2)

BMI indicates body mass index; CAD; coronary heart disease; HDL-C, high-density lipoprotein cholesterol; LURIC, Ludwigshafen Risk and Cardiovascular Health; and YFS, Young Finns Study.

was done using a custom-built Illumina Human 670k BeadChip at the Welcome Trust Sanger Institute (Table I in the online-only Data Supplement). Genotype imputation in both studies was performed using MACH 1.0[21,22] and HapMap II CEU (release 22, National Center for Biotechnology Information build 36, dbSNP 126) samples as a reference. After imputation, 2 543 887 Single-nucleotide polymorphisms (SNPs) were available. SNPs with a squared correlation of ≥0.3 between imputed and true genotypes were considered well imputed. For TVS samples, genotyping was done using the Illumina HumanHap660W-Quad BeadChip (Illumina, Inc, San Diego, CA) according to the manufacturer's recommendation.

Expression Analyses

The expression levels of cells from whole blood, monocytes, and artery cells were analyzed with an Illumina HumanHT-12 v3 Expression BeadChip (Illumina).The BeadChips were scanned with the Illumina iScan system. Raw intensity data were exported using the Illumina GenomeStudio software. Further data processing was conducted by means of R language and appropriate Bioconductor modules. A more detailed description is provided in the online-only Data Supplement.

Statistical Analyses

For the main GWAS analysis, we did linear regression on homoarginine serum levels with adjustment for principle components to control for population stratification. As the distribution of homoarginine levels was a bit skewed to the left, we additionally performed an analysis based on residuals that were obtained using linear regression analysis in which homoarginine levels were adjusted for sex, age, and body mass index (relevant covariates that were identified using stepAIC function in R software), as well as principal components to control for population stratification, and inverse normal transformation was used to obtain a normal distribution.

SNPs were evaluated for association with homoarginine using linear regression analyses using the software QUICKTEST (http://toby.freeshell.org/software/quicktest.shtml) in LURIC and ProbABEL[23] in YFS. QQ and Manhattan plots were drawn for the analysis of the results. Regional plots were drawn using Locuszoom.[24] The P value for genome-wide significance was set at $P<5\times10^{-8}$, which corresponds to an α of 0.05 with a Bonferroni correction for 1 million tests. Meta-analysis of both studies was done using a fixed-effect, effective sample-weighted Z-score meta-analysis method, as implemented in the software METAL.[25]

Further statistical analyses were performed using the R statistical software version 2.15.0 (http://www.r-project.org) and IBM SPSS Statistics version 20.0 (IBM Corportion).

Expression quantitative trait locus analysis with lead SNPs was performed with the Genevar software using a window of ±1 Mb centered on the SNP.[26] The strength of the relationship between alleles and gene expression intensities was estimated using Spearman rank correlation and reported as nominal P values.

Results

Study Description

For the current analysis, 3041 samples from LURIC and 2102 samples from YFS have been included, making a total of 5143 samples. Basic characteristics for both studies are shown in Table 1. LURIC participants were on average significantly older than YFS participants with a much higher percentage of men, higher body mass index, higher blood pressure, lower high-density lipoprotein cholesterol, and higher triglycerides. Although in LURIC ≈80% of patients had CAD and ≈40% had diabetes mellitus, there were no known patients with CAD in the YFS, and only 0.6% had diabetes mellitus. Homoarginine levels were higher in LURIC than in YFS.

Association Analyses

A Manhattan plot of the combined meta-analysis is shown in Figure 1, and separate Manhattan plots for LURIC and YFS are shown in the online-only Data Supplement (Figures I and II in the online-only Data Supplement). The analysis revealed a total of 169 SNPs that passed the threshold of 5×10^{-8} for genome-wide significance (Table II in the online-only Data Supplement).

These SNPs are located on chromosome 2 at the *CPS1* locus (carbamoyl phosphate synthetase I: EC 6.3.4.16), on chromosome 5 at the *AGXT2* locus (alanine-glyoxylate aminotransferase: EC 2.6.1.44), and on chromosome 15 at the GATM locus (*GATM*: EC 2.1.4.1). We also identified another region on chromosome 6 at the *MED23/ARG1* locus (*Homo sapiens* mediator complex subunit 23 gene/arginase I), which we considered as a suggestive association (P value of 1.80×10^{-7} for the lead SNP rs17060430). Regional plots of the 3 genome-wide significant regions and the putative region on chromosome 6 are shown in Figures 2 to 5.

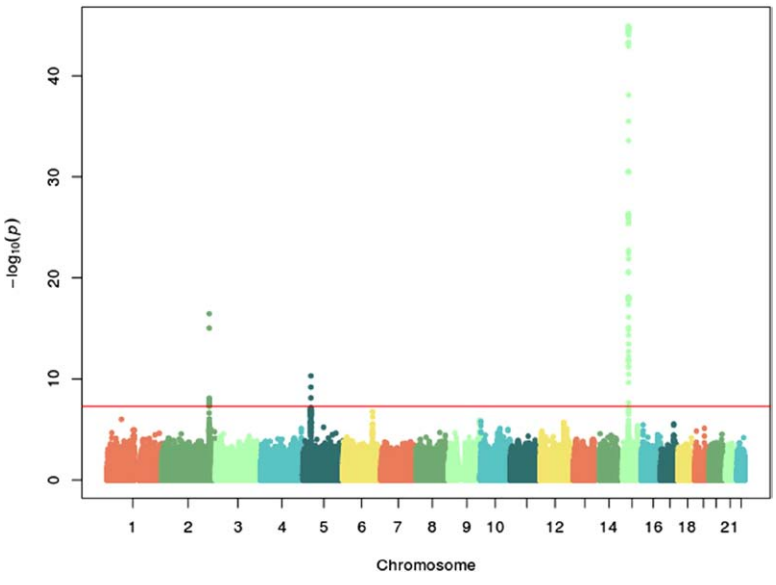

Figure 1. Manhattan plot of genome-wide association study meta-analysis of homoarginine. Each dot represents 1 single-nucleotide polymorphism present in the meta-analysis dataset, and its y axis coordinate indicates significance level in association with homoarginine.

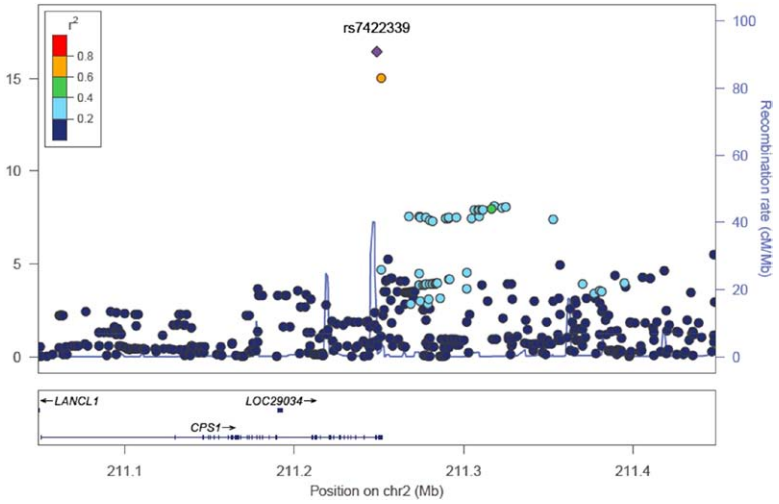

Figure 2. Regional plot of the carbamoyl phosphate synthetase 1 (*CPS1*) region on chromosome 2. Each dot represents 1 single-nucleotide polymorphism (SNP) present in the meta-analysis dataset, and its *y* axis coordinate indicates significance level in association with homoarginine level. For each SNP, linkage disequilibrium measured as r^2 with the lead SNP is indicated by the color scheme. Genome recombination rates are shown by blue lines, and genetic annotation is shown at the bottom.

QQ-plots for the individual studies are supplied in Figure III in the online-only Data Supplement.

The strongest association with homoarginine in the joined meta-analysis was observed for rs1153858 with a *P* value of 1.25×10^{-45} and a decrease in homoarginine serum concentration of 0.271 and 0.253 µmol/L per C allele in LURIC and YFS, respectively (Table 2). rs1153858 explained 3.3%, 5.3%, 2.0%, and 4.8% of the variance of homoarginine in LURIC, YFS, 4D, and GECOH, respectively.

We further sought to replicate the associations in 2 different cohorts, the 4D study and the GECOH study. In both studies, the association of rs1153858 with homoarginine could be replicated ($P<0.001$ in 4D; $P=0.001$ in GECOH; Table 3), whereas there was no association of rs37370 at the *AGXT2* locus. In the 4D study, we also were able to show the association of rs1047891 at the *CPS1* locus with homoarginine ($P=0.001$; Table 3).

Analysis of inverse normal transformed residuals of a regression model adjusted for sex, age, and body mass, as well as principal components, gave similar results like the analysis of the untransformed phenotype with *P* values of most SNPs even being a bit lower (Table V in the online-only Data Supplement).

Expression Quantitative Trait Locus Expression Analysis

We examined the association of the lead SNPs in the 3 identified loci with gene expression in transcriptomic profiles of 3 different tissues of the TVS. The major allele of rs1346268 at the *GATM* locus ($r^2=0.998$ with rs1153858) was significantly associated with increased mRNA levels of *GATM* and nearby genes in cells from the arterial plaque and whole blood, as well as in monocytes (Figure 6). For the *CPS1* locus and the *AGXT2* locus, no association of the lead SNPs with gene expression could be detected (data not shown).

Discussion

In our GWAS performed in 2 large cohorts of white patients, we identified sequence variants at 3 genomic loci as significant predictors of serum homoarginine levels. The strongest signal was observed on chromosome 15 at the *GATM* locus. GATM, also called l-arginine:GATM (AGAT; EC 2.1.4.1), plays a central role in energy metabolism by catalyzing the conversion of arginine and glycine to ornithine and guanidinoacetate.[27–29] Guanidinoacetate is subsequently converted to creatine, which serves as a buffer and an energy

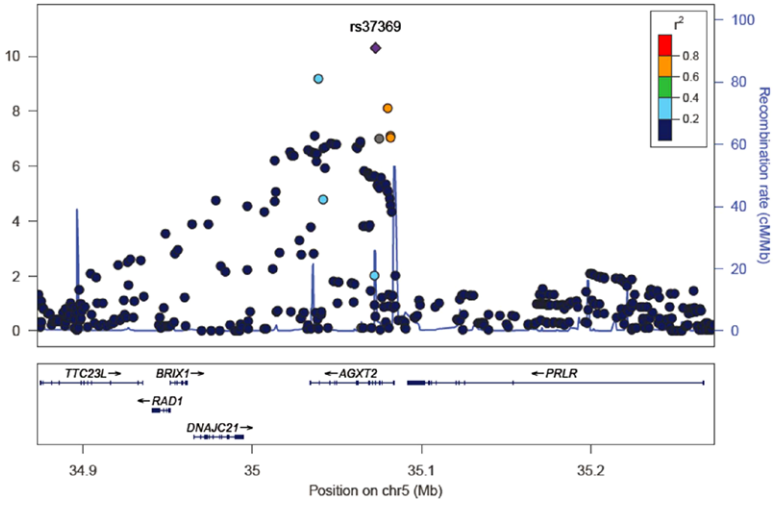

Figure 3. Regional plot of the alanine-glyoxylate aminotransferase 2 (*AGXT2*) region on chromosome 5. Each dot represents 1 single-nucleotide polymorphism (SNP) present in the meta-analysis dataset and its *y* axis coordinate indicates significance level in association with homoarginine level. For each SNP, linkage disequilibrium measured as r^2 with the lead SNP is indicated by the color scheme. Genome recombination rates are shown by blue lines, and genetic annotation is shown at the bottom.

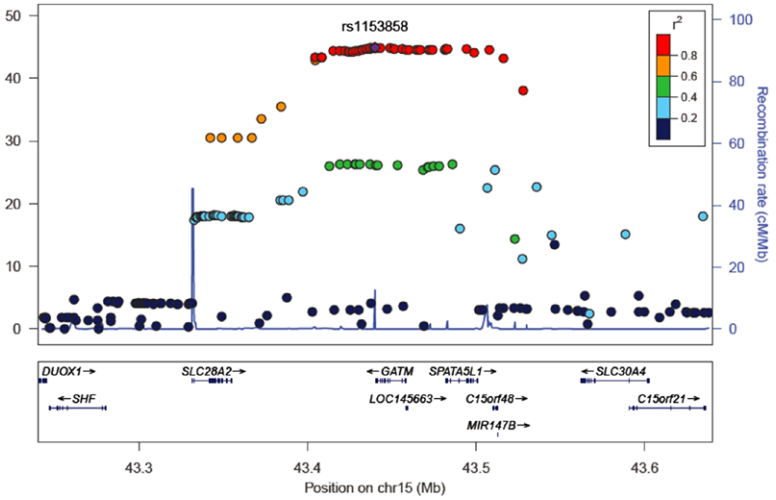

Figure 4. Regional plot of the glycine amidinotransferase (*GATM*) region on chromosome 15. Each dot represents 1 single-nucleotide polymorphism (SNP) present in the meta-analysis dataset, and its *y* axis coordinate indicates significance level in association with homoarginine level. For each SNP, linkage disequilibrium measured as r^2 with the lead SNP is indicated by the color scheme. Genome recombination rates are shown by blue lines, and genetic annotation is shown at the bottom.

shuttle for phosphate-bound energy, particularly for ATP. Our results suggest that GATM is significantly involved in the metabolism of homoarginine, which confirms a recent report by Davids et al,[11] who found that homoarginine synthesis was undetectable in GATM-deficient lymphoblasts. When arginine is replaced by homoarginine as a substrate, the GATM reaction yields guanidinoacetate and lysine instead of ornithine. Lysine, in turn, can be converted to homoarginine again via a reverse reaction by GATM or alternatively via homocitrulline and homoargininosuccinate similar to the reactions taking place in the urea circle.

Allelic variants in the *GATM* gene have been implicated with arginine:glycine amidinotransferase deficiency (online Mendelian inheritance in man no. 612718), an autosomal recessive disorder characterized by developmental delay, mental retardation, and severe depletion of creatine/phosphocreatine in the brain.[30] *GATM* mRNA expression is highest in the kidney, which is thought to be the major site of homoarginine synthesis.[31] In addition, *GATM* is also expressed in other tissues, such as liver and brain, skeletal muscle, heart, lung, and salivary glands.[31] The lead SNP identified in our GWAS, rs1153858, is located ≈600 bp downstream of the *GATM* gene. It is in high linkage disequilibrium (r^2=1) with a nonsynonymous coding variant, rs1288775 (P=8.52×10^{-56} in meta-analysis), which codes the exchange of glutamine against histidine at position 110. However, both Poly-Phen2[32] and Sorting Intolerant From Tolerant (SIFT)[33] predict this exchange to be benign. eQTL expression analysis identified another SNP in linkage to rs1153858 (rs1346268, P=1.3×10^{-55} in meta-analysis) that is associated with *GATM* mRNA levels in cells taken from artery and whole blood and in monocytes. The minor allele of rs1346268 was associated with lower levels of mRNA, whereas the minor allele of rs1153858 was associated with higher serum homoarginine.

A lookup in the ENCODE data using HaploReg[34] showed that rs1153858 changes a potential binding motif for the transcription factor paired box 1 (PAX-1), whereas rs1346268 changes an AIRE_2 motif. Several other potential binding motifs are reported in the same haploblock.

In line with the role of GATM in the synthesis of creatine, we observed a nominally significant association of rs1153858 with log-transformed creatine (P=0.031) in LURIC (Table VI in the online-only Data Supplement), which lost significance after Bonferroni correction for multiple testing.

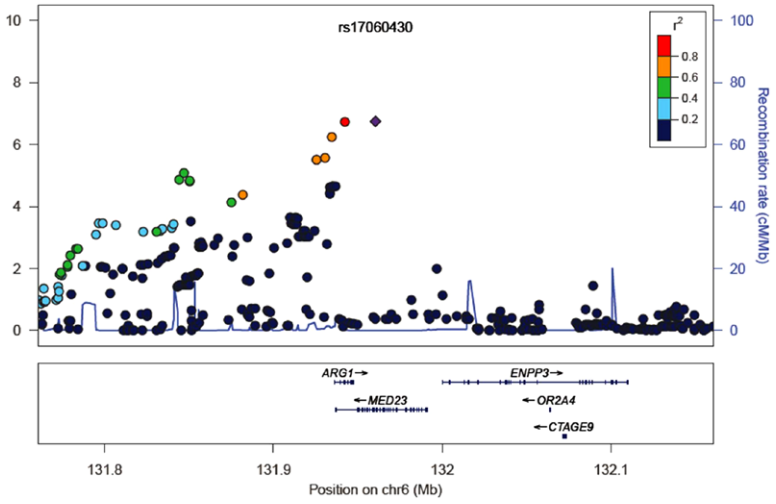

Figure 5. Regional plot of the arginase (*ARG*) 1 region on chromosome 6. Each dot represents 1 single-nucleotide polymorphism (SNP) present in the meta-analysis dataset, and its *y* axis coordinate indicates significance level in association with homoarginine level. For each SNP, linkage disequilibrium measured as r^2 with the lead SNP is indicated by the color scheme. Genome recombination rates are shown by blue lines, and genetic annotation is shown at the bottom.

Table 2. Lead SNPs in LURIC, YFS, and Combined Meta-Analysis

SNP	Chr	Position	Alleles	EAF		β		*P* Value			Candidate Gene
				LURIC	YFS	LURIC	YFS	LURIC	YFS	Combined	
rs7422339	2	211248752	C/A	0.69	0.64	0.189	0.148	1.22E–9	3.24E–14	3.55E–17	*CPS1*
rs37369	5	35072872	C/T	0.92	0.90	–0.220	–0.222	4.40E–06	1.69E–09	4.96E–11	*AGXT2*
rs17060430	6	131960430	G/A	0.97	0.94	–0.308	–0.154	3.98E–05	1.14E–03	1.80E–07	*MED23/ARG1*
rs1153858	15	43439995	C/T	0.72	0.70	–0.271	–0.253	1.02E–21	6.83E–27	1.25E–45	*GATM*

Alleles indicate effect allele/reference allele; Chr, chromosome; EAF, effect allele frequency; LURIC, Ludwigshafen Risk and Cardiovascular Health; and YFS, Young Finns Study.

The second locus associated with homoarginine levels was *CPS1* on chromosome 2. CPS1 (EC 6.3.4.16) is the rate-limiting enzyme of the hepatic urea cycle catalyzing the production of carbamoyl phosphate from ammonia, bicarbonate, consuming 2 molecules of ATP.[35] Deficiency of this enzyme results in hyperammonemia, which may be neonatally lethal or become manifest through environmental factors in adulthood. More than 200 mutations in the *CPS1* gene have been reported to date.[35] The top SNP in our GWAS, rs7422339, encodes the substitution of asparagine for threonine (T1406N) in a region that is critical for N-acetyl-glutamate binding and results in 20% to 30% higher enzymatic activity.[36] The same variation has been shown to influence nitric oxide metabolite concentrations and vasodilation,[37] the creatinine serum concentration,[38] homocysteine,[39] and it has also been associated with the risk of pulmonary hypertension in newborns.[40] In line with previous publications, the A allele of rs7422339 was associated with lower arginine levels in LURIC (although not significantly), beyond the association with lower homoarginine (Table VI in the online-only Data Supplement). Association with homocysteine was of borderline significance in LURIC with heterozygous individuals having higher levels, whereas there was no association with creatinine (data not shown).

The third locus influencing homoarginine was at 5p13.2 with the nearest gene being *AGXT2*. The *AGXT2* gene encodes the mitochondrial alanine-glyoxylate aminotransferase, which catalyzes the conversion of glyoxylate to glycine using l-alanine as the amino donor,[41] but it also catalyzes the transamination of asymmetrical dimethylarginine to α-keto-δ-(N,N-dimethylguanidino) valeric acid,[42] and overexpression of *AGXT2* has been shown to protect from asymmetrical dimethylarginine-induced impairment in nitric oxide production in endothelial cells.[43] Furthermore, a recent GWAS of metabolic traits in human urine identified rs37369 in the coding sequence of *AGXT2* to be strongly associated with β-aminoisobutyrate.[44] This polymorphism encodes a nonsynonymous valine-to-isoleucine (V140I) substitution and is a likely candidate to be the causative SNP of hyper-β-aminoisobutyric aciduria.[44] Another recent GWAS confirmed AGXT2 to be an important regulator of methylarginines and suggested that this gene represents a novel mechanism for the regulation of blood pressure through the kidney, with the T allele at rs37369 causing a modest increase in diastolic blood pressure.[45] The same SNP was identified in our GWAS to be associated with serum homoarginine. We did not observe a significant association with blood pressure, although there was a tendency toward higher systolic and diastolic blood pressures in carriers of the T allele (data not shown).

Furthermore, we identified another chromosomal region with a suggestive association to homoarginine serum levels at the *MED23/ARG1* locus on chromosome 6. Arginase (ARG1, EC 3.5.3.1) catalyzes the last step of the urea cycle. The isoform encoded by *ARG1* contributes 98% of the arginase activity in liver but is also present in red cells. The polymorphisms with suggestive association to homoarginine identified in our study reside in intronic regions of *Homo sapiens* mediator complex subunit 23 gene (*MED23*) or *ARG1* gene with both transcripts located on different DNA strands and showing a high degree of overlap. There was no linkage to several pathogenic SNPs in the *ARG1* gene causing arginase deficiency that have been described previously.

To attempt to replicate the association of SNPs identified in our GWAS with serum homoarginine, we genotyped the lead SNPs in 2 additional cohorts, the 4D study and the GECOH study. The association of rs1153858 at the *GATM* locus with homoarginine could be replicated in both studies, whereas there was no significant association for rs37370 at the *AGXT2*

Table 3. Replication of Lead SNPs in 4D and GECOH

Study	SNPID	Proxy	Gene	n	Effect Allele	Reference Allele	EAF	β	SE	*P* Value
4D	rs1047891	rs7422339	*CPS1*	1234	C	A	0.666	0.061	0.018	0.001
	rs37370	rs37369	*AGXT2*	1237	C	T	0.895	–0.027	0.027	0.318
	rs1153858	…	*GATM*	1235	C	T	0.745	–0.111	0.019	7.33E–09
GECOH	rs37370	rs37369	*AGXT2*	211	C	T	0.893	–0.144	0.158	0.364
	rs1153858	…	*GATM*	211	C	T	0.709	–0.364	0.112	0.001

4D indicates Die Deutsche Diabetes Dialyse Studie; EAF, effect allele frequency; and GECOH, Graz Endocrine Causes of Hypertension.

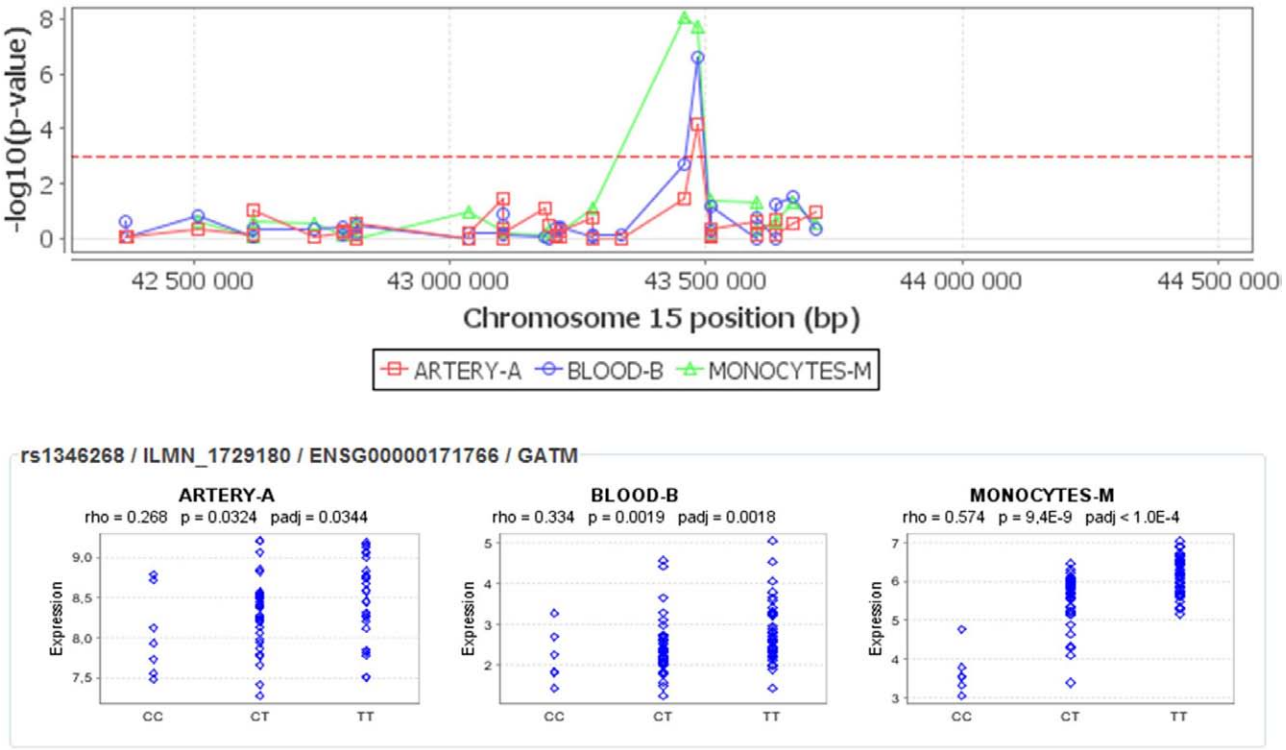

Figure 6. eQTL expression analysis of the association of rs1346268 (proxy of rs1153858) with the expression of glycine amidinotransferase and nearby genes in artery tissue, cells from whole blood, and monocytes in Tampere Vascular Study (TVS).

locus. In the 4D study, there was also a significant association of rs1047891 at the *CPS1* locus.

Conclusions

In our GWAS, we identified 3 chromosomal regions significantly associated with serum homoarginine and another region with suggestive association. All of these regions harbor genes that are located in the mitochondrium and have been linked to arginine metabolism. The strongest association, which was replicated in both the 4D and GECOH studies, was found for GATM, which synthesizes and catabolizes arginine/homoarginine, whereas CPS1 and ARG1 are involved in the urea cycle and the synthesis of arginine/homoarginine.

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Disclosures

None.

Appendix

From the Department of Internal Medicine II–Cardiology, University of Ulm Medical Center, Ulm, Germany (M.E.K., W.K.); Institute of Public Health, Social and Preventive Medicine, Mannheim Medical Faculty, Heidelberg University, Mannheim, Germany (M.E.K., W.M.); Department of Clinical Chemistry, Fimlab Laboratories, Tampere University School of Medicine, Tampere, Finland (I.S., N.O., E.R., L.-P.L., K.-M.M., R.L., T.L.); Department of Epidemiology and Biostatistics, EMGO Institute for Health and Care Research, VU University Medical Centre, Amsterdam, The Netherlands (S.P.); Department of Internal Medicine, Division of

Endocrinology and Nuclear Medicine (S.P.), Department of Cardiology (A.T.), Department of Internal Medicine, Division of Endocrinology and Metabolism (K.K., A.F.-P.), and Clinical Institute of Medical and Chemical Laboratory Diagnostics (W.M., A.M.), Medical University of Graz, Graz, Austria; Institute of Clinical Chemistry and Laboratory Medicine, University Medical Center Freiburg, Freiburg, Germany (M.M.H.); Division of Vascular Surgery, Departments of Surgery (N.O.) and Clinical Physiology (M.K.), Tampere University Hospital, Tampere, Finland; University of Tampere, Tampere, Finland (M.K.); Research Centre of Applied and Preventive Cardiovascular Medicine, University of Turku, Turku, Finland (O.T.R.); Department of Clinical Physiology and Nuclear Medicine, Turku University Hospital, Turku, Finland (O.T.R.); Department of Human Genetics, Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom (J.H.); Division of Nephrology, Department of Medicine, University of Würzburg, Würzburg, Germany (C.D., V.K., C.W.); Division of Endocrinology and Diabetes, Department of Internal Medicine I, Ulm University, Ulm, Germany (B.O.B.); LKC School of Medicine, Imperial College London and Nanyang Technological University, Singapore (B.O.B.); and Synlab Academy, Synlab Services GmbH, Mannheim, Germany (W.M.).

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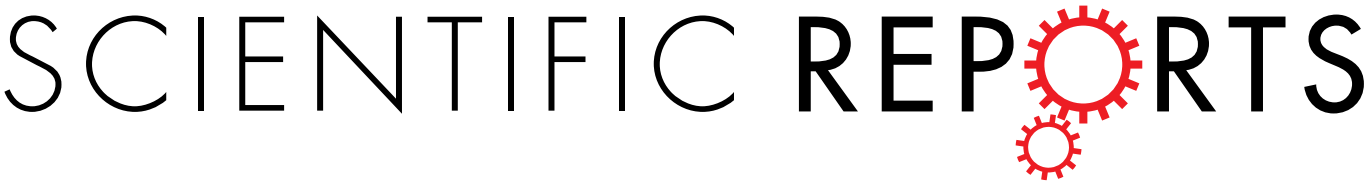
CLINICAL PERSPECTIVE

Deficiency of the cationic amino acid homoarginine has been shown previously to be an independent risk factor for all-cause and cardiovascular mortality. More detailed analyses on specific causes of death revealed an association of low homoarginine levels with fatal stroke and sudden cardiac death, a positive correlation of homoarginine with angiographic left ventricular ejection fraction and an inverse correlation with N-terminal pro-B-type natriuretic peptide. To date, 2 main hypotheses have emerged explaining how homoarginine exerts its protective cardiovascular effects, first by regulating the synthesis of the endothelial-derived relaxing factor nitric oxide and second by its possible involvement in energy metabolism. The present genome-wide association study identifies genes involved in the regulation of homoarginine serum concentration. The strongest association by far was observed for variants in the glycine amidinotransferase gene, which plays a central role in mitochondrial energy metabolism by catalyzing the conversion of arginine and glycine to ornithine and guanidinoacetate. This lends credit to the hypothesis of homoarginine and its protective effects through modulation of energy metabolism.



OPEN

Associations of functional alanine-glyoxylate aminotransferase 2 gene variants with atrial fibrillation and ischemic stroke

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Ilkka Seppälä[1], Marcus E. Kleber[2], Steve Bevan[3], Leo-Pekka Lyytikäinen[1], Niku Oksala[1,4], Jussi A. Hernesniemi[1,5], Kari-Matti Mäkelä[1], Peter M. Rothwell[6], Cathie Sudlow[7], Martin Dichgans[8], Nina Mononen[1], Efthymia Vlachopoulou[9], Juha Sinisalo[10], Graciela E. Delgado[2], Reijo Laaksonen[1], Tuomas Koskinen[11,12], Hubert Scharnagl[13], Mika Kähönen[14], Hugh S. Markus[15], Winfried März[2,13,16] & Terho Lehtimäki[1]

Asymmetric and symmetric dimethylarginines (ADMA and SDMA) impair nitric oxide bioavailability and have been implicated in the pathogenesis of atrial fibrillation (AF). Alanine–glyoxylate aminotransferase 2 (AGXT2) is the only enzyme capable of metabolizing both of the dimethylarginines. We hypothesized that two functional *AGXT2* missense variants (rs37369, V140I; rs16899974, V498L) are associated with AF and its cardioembolic complications. Association analyses were conducted using 1,834 individulas with AF and 7,159 unaffected individuals from two coronary angiography cohorts and a cohort comprising patients undergoing clinical exercise testing. In coronary angiography patients without structural heart disease, the minor A allele of rs16899974 was associated with any AF (OR = 2.07, 95% CI 1.59-2.68), and with paroxysmal AF (OR = 1.98, 95% CI 1.44–2.74) and chronic AF (OR = 2.03, 95% CI 1.35–3.06) separately. We could not replicate the association with AF in the other two cohorts. However, the A allele of rs16899974 was nominally associated with ischemic stroke risk in the meta-analysis of WTCCC2 ischemic stroke cohorts (3,548 cases, 5,972 controls) and with earlier onset of first-ever ischemic stroke (360 cases) in the cohort of clinical exercise test patients. In conclusion, *AGXT2* variations may be involved in the pathogenesis of AF and its age-related thromboembolic complications.

Circulating asymmetric and symmetric dimethylarginine (ADMA and SDMA) predict mortality in cardiovascular diseases[1,2]. Moreover, accumulating epidemiological and experimental evidence have implicated ADMA in the pathogenesis of atrial fibrillation (AF)[3–7], whereas little attention has been paid to the role of SDMA[2]. In patients

[1]Department of Clinical Chemistry, Fimlab Laboratories and School of Medicine, University of Tampere, Tampere, Finland. [2]Vth Department of Medicine (Nephrology, Hypertensiology, Endocrinology, Diabetology, Rheumatology), Medical Faculty Mannheim, University of Heidelberg, Heidelberg, Germany. [3]School of Life Science, University of Lincoln, Lincoln, UK. [4]Division of Vascular Surgery, Department of Surgery, Tampere University Hospital, Tampere, Finland. [5]Heart Hospital, Tampere University Hospital, Tampere, Finland. [6]Stroke Prevention Research Unit, Nuffield Department of Clinical Neuroscience, University of Oxford, Oxford, UK. [7]Division of Clinical Neurosciences and Insititute of Genetics and Molecular Medicine, University of Edinburgh, UK. [8]Institute for Stroke and Dementia Research, Klinikum der Universität München, Ludwig-Maximilians-Universität, Munich, Germany & Munich Cluster for Systems Neurology (SyNergy), Munich, Germany. [9]Transplantation Laboratory, Haartman Institute, University of Helsinki, Helsinki, Finland. [10]Heart and Lung Center, Helsinki University Hospital and Helsinki University, Helsinki, Finland. [11]Research Centre of Applied and Preventive Cardiovascular Medicine, University of Turku, Turku, Finland. [12]Satakunta Central Hospital, Department of Surgery, Pori, Finland. [13]Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Graz, Austria. [14]Department of Clinical Physiology, Tampere University Hospital and University of Tampere, Tampere, Finland. [15]Clinical Neurosciences, University of Cambridge, Cambridge, UK. [16]Synlab Academy, Synlab Services GmbH, Mannheim, Germany. Correspondence and requests for materials should be addressed to I.S. (email: ilkka.seppala@uta.fi)

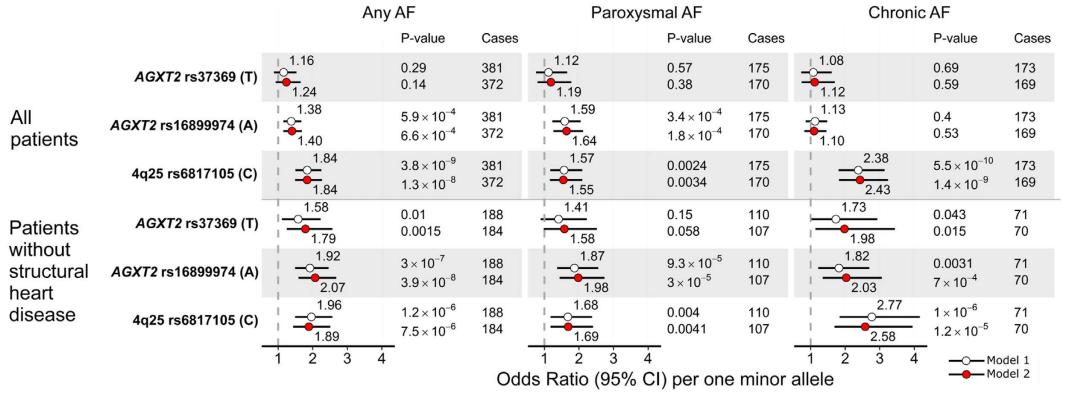

Figure 1. Associations of the AGXT2 and 4q25 variants with prevalent AF and its subtypes in LURIC. ORs per one minor allele increase from logistic regression models assuming additive genetic effect are shown. Model 1: adjusted for age, sex and body mass index. Model 2: Model 1 further adjusted for arterial hypertension, diabetes, coronary artery disease (>50% stenosis), serum NT-proBNP and eGFR. Structural heart disease refers to cardiomyopathy or valvular heart disease.

with a history of AF, the induction of AF during catheter ablation resulted in significantly elevated ADMA levels in the atria and systemic circulation[7], whereas in another study electrical cardioversion of AF decreased ADMA levels to normal values within 24 hours after AF was terminated[6]. Similarly, higher ADMA levels were associated with the recurrence of AF after electrical cardioversion or catheter ablation in patients with persistent AF[3,5]. These data suggests that elevated ADMA levels are not only a consequence of AF but may also be involved in the development of AF by modifying the atrial substrates for AF. Indeed, although only ADMA directly inhibits nitric oxide synthase (NOS), both dimethylarginines can reduce the bioavailability of nitric oxide (NO) by sifting the NOS enzyme from the production of NO to superoxide anion[8–10], and thus promote oxidative stress, endothelial dysfunction, and inflammation, all of which are associated with atrial remodelling and increased vulnerability to AF[11–13].

The primary pathway of ADMA deactivation is catalysed by the dimethylarginine dimethylaminohydrolase (DDAH) enzymes, whereas the renal clearance plays a major role in the elimination of SDMA from the systemic circulation. In contrast to DDAH, alanine–glyoxylate aminotransferase 2 (AGXT2), a nuclear-encoded mitochondrial protein, can metabolise not only ADMA but also SDMA in humans[14,15]. In addition to a strong expression of AGXT2 in the kidney and liver, *AGXT2* mRNA expression was recently detected in several other human organs including the heart[16]. As the activity of DDAH is reduced and the generation of ADMA increased via up-regulation of protein arginine methyltransferase 1 (PRMT1) in animal fibrillating atria[4] also explaining the increased ADMA levels in human AF, it can be hypothesized that the alternative AGXT2-mediated elimination pathway of dimethylarginines could play an important role in the pathogenesis of AF.

We recently fine-mapped the *AGXT2* gene region on chromosome 5p13 for associations between single-nucleotide polymorphisms (SNP) and serum SDMA levels and identified two missense mutations in *AGXT2* (rs37369, p.V140I; rs16899974, p.V498L) to be strongly and independently associated with SDMA levels in a meta-analysis of two large cohorts of European descent[15]. Experimental data indicates that rs37369 modifies the enzyme activity[16,17] whereas an *in silico* analysis showed that the rs16899974 variant may modify the enzyme stability[17]. Therefore, we considered that the two functional *AGXT2* variants may serve as naturally occurring genetic models to study the role of AGXT2 in human AF and its thromboembolic complications, i.e. stroke and its subtypes. More specifically, we hypothesized that these functional variants are (1) associated with paroxysmal and/or chronic AF in patients referred for coronary angiography, (2) associated more strongly with circulating levels of ADMA and SDMA in patients with AF compared to patients in sinus rhythm, and (3) associated with ischemic stroke and its subtypes. As a genetic control, we repeated some of the analyses with a known variant at 4q25 previously associated with AF.

Results

Associations of *AGXT2* and 4q25 control variants with atrial fibrillation and its subtypes in patients referred for coronary angiography (LURIC and Corogene). The clinical characteristics of the study participants for all studies are shown in Supplementary Table 1. In LURIC, 381 (13.0%) patients had prevalent AF at baseline of which 175 (50.3%) and 173 (49.7%) were further classified as paroxysmal or chronic AF, respectively. Those with AF were older (mean age 66.4 versus 62.2 years, $P < 0.0001$) and had less likely coronary artery disease (56.8% versus 71.6%, $P < 0.0001$) than patients in sinus rhythm. The associations of *AGXT2* and 4q25 variants with AF and its subtypes are shown in Fig. 1. In the whole study population, rs16899974 showed a significant association with any AF ($P = 6.6 \times 10^{-4}$) in a fully adjusted model. Moreover, when analysing paroxysmal and chronic AF cases separately, the association was especially marked with the paroxysmal subtype ($P = 1.8 \times 10^{-4}$), whereas there was no association with chronic AF ($P = 0.53$). We found strong evidence that a history of valvular heart disease attenuated the effect of rs16899974 and rs37369 on AF (p for interaction of 4.3×10^{-4} and 0.015, respectively, Table 1). Nominally significant interaction was also observed between rs16899974 and prevalent cardiomyopathy on AF ($P = 0.043$). When excluding the patients with structural heart

AF risk factor	Risk factor cases/controls	AF cases/controls	AGXT2 rs37369 (T)			AGXT2 rs16899974 (A)			4q25 rs6817105 (C)		
			OR_{int}	(95% CI)	p	OR_{int}	(95% CI)	p	OR_{int}	(95% CI)	p
Arterial hypertension	1,721/1,202	381/2,542	0.99	(0.56–1.74)	0.98	0.88	(0.60–1.28)	0.49	0.68	(0.46–1.02)	0.065
Diabetes	1,181/1,742	381/2,542	0.81	(0.46–1.40)	0.45	0.82	(0.56–1.19)	0.30	0.91	(0.61–1.36)	0.65
Valvular heart disease	518/2,405	381/2,542	0.45	(0.23–0.86)	0.015	0.46	(0.30–0.71)	0.00043	1.00	(0.63–1.59)	0.99
Aortic stenosis	146/2,405	272/2,279	0.45	(0.09–2.19)	0.32	0.46	(0.18–1.15)	0.096	1.46	(0.57–3.73)	0.43
Aortic insufficiency	80/2,403	269/2,214	0.18	(0.02–1.58)	0.12	0.38	(0.14–1.05)	0.063	1.37	(0.47–4.03)	0.57
Mitral stenosis	16/2,405	260/2,161	0.10	(0.01–1.18)	0.068	0.19	(0.02–1.59)	0.13	0.44	(0.08–2.35)	0.34
Mitral insufficiency	225/2,394	315/2,304	0.36	(0.15–0.88)	0.025	0.48	(0.27–0.85)	0.012	0.88	(0.47–1.63)	0.68
Other	34/2,403	262/2,175	2.66	(0.42–16.9)	0.30	0.83	(0.25–2.76)	0.76	0.39	(0.07–2.01)	0.26
Cardiomyopathy	307/2,616	381/2,542	0.79	(0.37–1.68)	0.54	0.61	(0.38–0.99)	0.043	1.31	(0.75–2.27)	0.34
Ischemic	134/2,612	326/2,420	0.62	(0.21–1.81)	0.38	0.58	(0.29–1.16)	0.12	1.10	(0.52–2.34)	0.79
Dilated	147/2,614	335/2,426	0.76	(0.25–2.26)	0.62	0.46	(0.24–0.90)	0.024	1.34	(0.62–2.91)	0.46
Restricted	24/2,616	296/2,344	1.76	(0.10–32.5)	0.70	2.52	(0.46–13.8)	0.29	0.67	(0.08–5.73)	0.71
Myocardial infarction	1,217/1,706	381/2,542	1.43	(0.81–2.51)	0.21	1.22	(0.82–1.80)	0.33	0.85	(0.55–1.30)	0.46
CVD event (stroke/TIA)	264/2,659	381/2,542	1.27	(0.58–2.78)	0.55	1.53	(0.86–2.74)	0.15	1.18	(0.64–2.18)	0.59

Table 1. Effect modification of *AGXT2* and 4q25 variants on atrial fibrillation in LURIC. Statistics: Results are from logistic regression analyses adjusted for age, sex and BMI. Interaction effects on a multiplicative scale (OR_{int}) per one minor allele increase assuming additive genetic model are shown. $OR_{int} = 1$ means no interaction on a multiplicative scale. Notes: In the subgroup analyses of valve disease and cardiomyopathy, controls did not have any valvular disease or cardiomyopathy, respectively. Abbreviations: CVD, cerebrovascular disease; TIA, transient ischemic attack.

disease from the analysis, a highly significant association for *AGXT2* rs16899974 was observed with any AF ($P = 3.9 \times 10^{-8}$), and with paroxysmal AF ($P = 3.0 \times 10^{-5}$) and chronic AF ($P = 7.0 \times 10^{-4}$) separately. In contrast to the *AGXT2* SNPs, the 4q25 variant showed a stronger association with chronic AF than paroxysmal AF in both the whole study population and when excluding the patients with structural heart disease (Fig. 1). When conducting a genome-wide scan using the fully adjusted model in patients without structural heart disease, no additional genome-wide significant associations were identified apart from the signal at the *AGXT2* locus (data not shown).

We sought to replicate the associations of the *AGXT2* variants with AF and its subtypes in an independent cohort. In contrast to LURIC, all subjects in the Corogene study with genotype data available had an acute coronary syndrome (Supplementary Table 1). Of the 2,208 patients included in the analysis, 265 (12.0%) had prevalent AF of which 141 (6.4%) and 107 (4.8%) had paroxysmal and chronic AF, respectively. No associations were observed between *AGXT2* variants and AF or its subtypes in Corogene (all $P > 0.05$, Supplementary Table 2). However, in accordance with the associations seen in LURIC, the 4q25 variant showed significant associations with any AF (OR = 1.50, 95% CI 1.18–1.91, P = 0.001) and chronic AF (OR = 1.84, 95% CI 1.29–2.61, P = 0.001) but not with paroxysmal AF (OR = 1.25, 95% CI 0.89–1.74, P = 0.20).

Associations with incident clinical AF and age at AF onset (FINCAVAS). Of the 3,862 FINCAVAS patients undergoing clinical exercise test between 2001 and 2008, 1,188 (30.8%) had their first AF event diagnosed between 1987 and 2015. The mean (SD) age at the diagnosis was 60.5 (13.3) and 64.6 (13.7) years for men and women, respectively (Supplementary Figure 1). The AF-free survival curves stratified by the rs16899974 genotypes for all patients and AF cases only are displayed in Fig. 2A,B, and for the *AGXT2* rs37369 and 4q25 variants in Supplementary Figures 2 and 3. No associations were seen between the rs16899974 genotypes and incident AF either in the whole study population or in the case-only-analysis (both $P > 0.05$). As seen in Fig. 2A,B, the survival curves of the three genotype groups start to separate only after around age 75, suggesting that other factors are responsible for clinical AF events in younger patients. In a multivariable analysis, rs16899974 was associated with incident AF and age at AF diagnosis in patients aged $\geq$75 years at the end of the follow-up, but not in patients aged $<$75 years at censoring of data (Table 2, Supplementary Table 3). The 4q25 variant showed a strong association in the whole study population; however, rs16899974 appears to be more strongly associated with incident AF in patients aged $\geq$75 years.

Associations with history of ischemic stroke and its subtypes (WTCCC2). We examined whether the two functional *AGXT2* variants are associated with ischemic stroke and its subtypes in the meta-analysis of WTCCC2 ischemic stroke cohorts. As shown in Supplementary Table 4, there was no significant association of rs37369 with all-cause ischemic stroke (IS) whereas rs16899974 showed statistically significant associations with all-cause IS and large-artery atherosclerosis (LAA) stroke subtype with per A allele ORs of 1.04 (95% CI 1.00–1.08, P = 0.032) and 1.10 (95% CI 1.01–1.19, P = 0.021), respectively. Moreover, rs16899974 showed a borderline significant association in the same direction for cardioembolic (CE) stroke with an OR of 1.08 (95% CI 1.00–1.16, P = 0.057). The SNPs were not associated with small-vessel disease (SVD) stroke subtype.

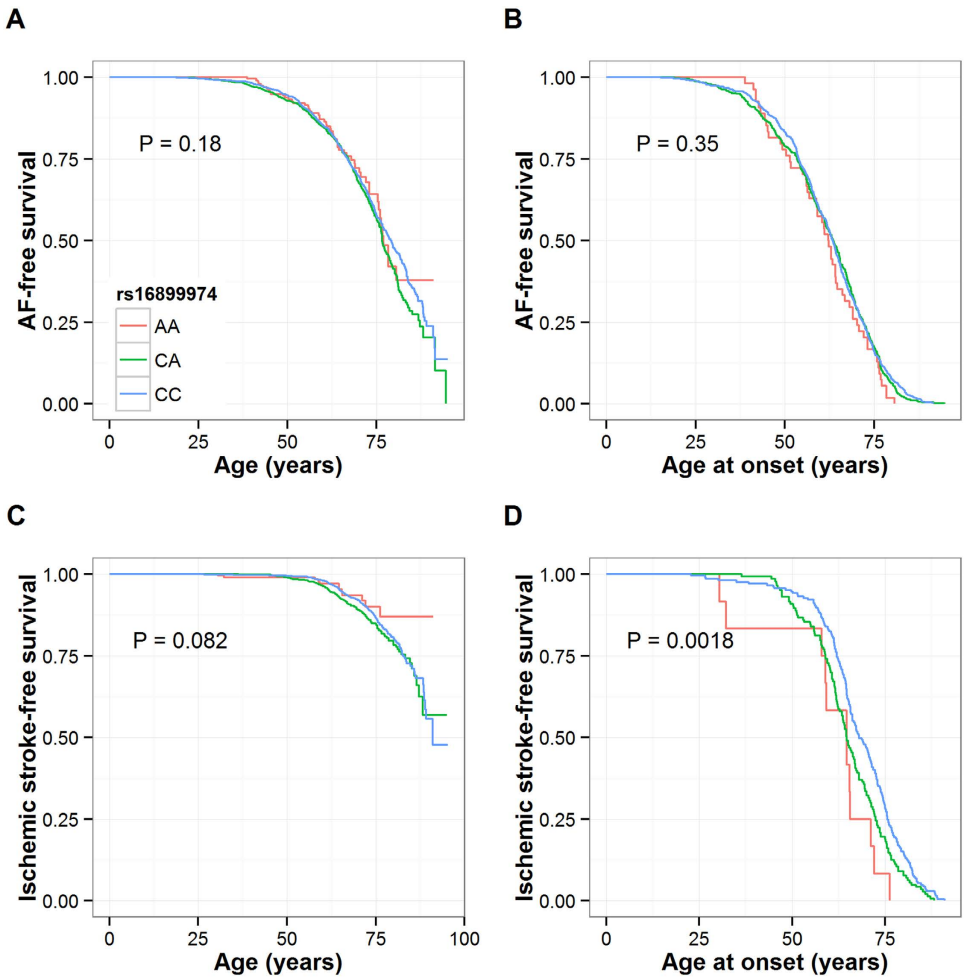

Figure 2. Kaplan-Meier event-free survival for patients participating the FINCAVAS study. Survival curves are shown as a function of the *AGXT2* rs16899974 genotype groups for incident AF (**A**) and ischemic stroke (**C**) and for cases of incident AF (**B**) and ischemic stroke (**D**) only (age of onset analysis).

Locus	SNP	cases/N	HR	(95% CI)	P
All					
AGXT2	rs37369	972/3,122	1.02	(0.87, 1.19)	0.83
AGXT2	rs16899974	1188/3,862	1.05	(0.96, 1.16)	0.28
4q25	rs6817105	972/3,122	1.51	(1.36, 1.69)	9.7×10^{-14}
Age < 75 years					
AGXT2	rs37369	802/2,562	0.95	(0.80, 1.12)	0.54
AGXT2	rs16899974	991/3,210	0.95	(0.85, 1.05)	0.32
4q25	rs6817105	808/2,587	1.55	(1.37, 1.74)	6.1×10^{-13}
Age ≥ 75 years					
AGXT2	rs37369	170/560	1.07	(0.71, 1.61)	0.75
AGXT2	rs16899974	197/662	1.38	(1.10, 1.74)	0.0063
4q25	rs6817105	170/560	1.26	(0.95, 1.67)	0.11

Table 2. Associations of *AGXT2* and 4q25 variants with incident clinical AF in FINCAVAS. Statistics: HRs per one minor allele increase are from Cox regression models, assuming an additive genetic effect. The baseline hazard function of Cox models are stratified by sex. Notes: Age is used as the time scale in Cox models. Analyses are stratified based on the age at the end of the follow-up.

Associations with incident ischemic stroke and age at first stroke diagnosis (FINCAVAS). Of the 3,862 FINCAVAS patients undergoing clinical exercise test between 2001 and 2008, 360 (9.3%) had their first ischemic stroke diagnosed between 1987 and 2015. The mean (SD) age at the diagnosis was 65.9 (10.7) and

Locus	SNP	Effect allele	EAF	cases/N	HR	(95% CI)	P
a) incident first ever ischemic stroke							
AGXT2	rs37369	T	0.094	295/3,122	0.97	(0.73, 1.30)	0.86
AGXT2	rs16899974	A	0.240	360/3,862	1.05	(0.88, 1.24)	0.61
4q25	rs6817105	C	0.156	295/3,122	1.22	(0.90, 1.40)	0.96
b) age at the first ischemic stroke diagnosis							
Linear regression							
Locus	SNP	Effect allele	EAF	N	β	(95% CI)	P
AGXT2	rs37369	T	0.086	295	−2.60	(−5.75, 0.55)	0.11
AGXT2	rs16899974	A	0.232	360	−3.24	(−5.24, −1.25)	0.0015
4q25	rs6817105	C	0.151	295	−2.60	(−5.14, −0.074)	0.044
Cox model							
Locus	SNP	Effect allele	EAF	N	HR	(95% CI)	P
AGXT2	rs37369	T	0.086	295	1.34	(0.99, 1,81)	0.062
AGXT2	rs16899974	A	0.232	360	1.44	(1.19, 1.75)	0.00018
4q25	rs6817105	C	0.151	295	1.18	(0.93, 1.51)	0.18

Table 3. Associations of the *AGXT2* and 4q25 variants with (a) incident ischemic stroke and (b) age at ischemic stroke onset in FINCAVAS. Statistics: HRs and βs (in years) per one minor allele increase are from Cox and linear regression models, respectively, assuming an additive genetic effect. Models are controlled for sex and a history of clinical atrial fibrillation.

68.5 (12.4) years for men and women, respectively. Of the 360 incident ischemic stroke cases, 83 (23%) had a previous AF diagnosis. The ischemic stroke-free survival curves stratified by the rs16899974 genotypes for all patients and ischemic stroke cases only are displayed in Fig. 2C,D, and for the *AGXT2* rs37369 and 4q25 variants in Supplementary Figures 2 and 3. None of the three SNPs showed a statistically significant association with incident ischemic stroke. However, rs16899974 was associated with age at onset of a first ischemic stroke both in the univariate analysis (Fig. 2D) and after controlling for sex and previously diagnosed clinical AF (Table 3). Interestingly, this finding was seen only in patients with cryptogenic ischemic stroke (n = 233) with a per minor allele HR of 1.57 (95% CI 1.21–2.04, P = 0.0007) and approximately 3.8 (95% CI 1.5–6.0, P = 0.0009) years earlier age at the diagnosis with each additional copy of the minor allele but not in those with another diagnosis of ischemic stroke (n = 127, both $P > 0.05$).

Associations with circulating dimethylarginines in patients with sinus rhythm and AF (LURIC). As displayed in Fig. 3A, there were statistically significant stepwise increases of ADMA and SDMA levels in patients with paroxysmal AF and chronic AF compared to patients in sinus rhythm. The trend across the AF status groups for ADMA remained highly significant after adjusting for age, sex and estimated glomerular filtration rate (eGFR) (p for linear trend $= 1.9 \times 10^{-9}$) whereas the association with SDMA was attenuated, but still significant (p for linear trend = 0.0023). The unadjusted associations of the *AGXT2* variants with ADMA, SDMA and eGFR by AF status are shown in Fig. 3B. As we expected, both SNPs showed strong associations with circulating SDMA levels in patients with sinus rhythm. Neither of the *AGXT2* variants was associated with ADMA or eGFR in any of the AF status groups; however, rs16899974 showed a borderline significant association with ADMA in patients with paroxysmal AF (P = 0.057). Moreover, it seems that the effect estimates for SDMA in the paroxysmal AF group are larger than in those with no AF or chronic AF (Fig. 3B). To test this hypothesis the interaction between the AF status groups and the *AGXT2* genotypes on SDMA levels were tested using a two-way ANOVA model with interaction. Statistically significant interaction was observed for rs16899974 in unadjusted model (F = 3.8, P = 0.0044), and when age, sex and eGFR were used as covariates (F = 2.6, P = 0.036). No such statistically significant interactions were observed for rs37369 or when using ADMA as a dependent variable.

Discussion

In the present study, we analysed the presence of associations of two functional missense *AGXT2* variants with AF and its thromboembolic complications in four independent study cohorts. The p.V498L variant (rs16899974) was associated with an increased risk of both paroxysmal and chronic AF in patients referred for coronary angiography but with no structural heart disease. Moreover, the same A allele of rs16899974 was nominally associated with an increased risk of any ischemic stroke in the meta-analysis of WTCCC2 ischemic stroke cohorts. Finally, rs16899974 was associated with an earlier age at the first ischemic stroke diagnosis in patients undergoing exercise stress testing and with incident clinical AF in patients aged ≥75 years at the time of AF diagnosis in the same cohort.

The two studied *AGXT2* SNPs have not been identified to be associated at a genome-wide significant level with the risk of prevalent or incident AF[18] or ischemic stroke subtypes[19] in previous large scale meta-analyses of genome-wide association studies. This is in accordance with the data from the FINCAVAS study showing lack of association with incident AF and ischemic stroke in the whole study population. Furthermore, no associations were observed with prevalent AF or its subtypes in the Corogene study including coronary angiography patients with acute coronary syndrome. In striking contrast with these findings we found a genome-wide significant association of rs16899974 with any AF in patients without structural heart disease in the LURIC study. This

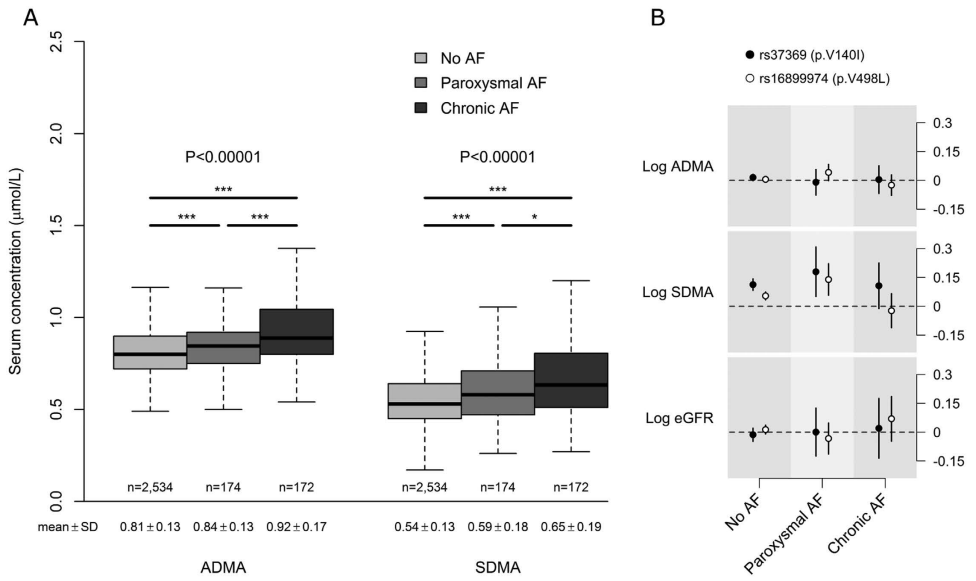

Figure 3. Box plots of untransformed concentrations of ADMA, and SDMA (µmol/l) according to the atrial fibrillation (AF) status in LURIC (**A**). Data are shown as the 25th, 50th, and 75th percentiles (represented by gray boxes), range (shown as whiskers; outliers have been removed), and the median (black horizontal line). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Unadjusted βs for ADMA, SDMA and eGFR by *AGXT2* SNPs and AF status assuming additive genetic effect on the log scale (**B**). Points represent point estimate of the β; vertical bars; the 95% CI.

association is supported with the observation in FINCAVAS showing that rs16899974 is associated with hospital discharge data based incident clinical AF in patients aged 75 and over at the time of AF diagnosis. Although, there is a separate meta-analysis for lone AF[20], defined as AF with an onset before 66 years of age in individuals without overt heart disease, no large-scale studies have sought to identify common genetic variants underlying AF risk in the elderly or for paroxysmal and more persistent forms of AF separately.

Interestingly, rs6817105 at 4q25, previously associated with AF in genome-wide association studies, was mainly associated with prevalent chronic AF in both LURIC and Corogene. These associations suggest that the risk allele carriers have a relatively high rate of progression from paroxysmal AF to chronic AF, at least in patients referred for coronary angiography. In contrast, the associations of rs16899974 with both paroxysmal and chronic AF in LURIC, with late onset clinical AF and with earlier age of onset of cryptogenic ischemic stroke suggest a role for AGXT2 in more subclinical forms of AF characterized by a relatively low rate of progression to chronic AF; however, this hypothesis needs to be validated in a prospective setting.

In line with the AF associations in LURIC and FINCAVAS, the AF risk allele of rs16899974 was associated with an earlier onset of cryptogenic ischemic stroke although no association was observed with the ischemic stroke risk in the whole FINCAVAS study population. The association with age at ischemic stroke diagnosis was strengthened after adjusting the analysis for gender and prior AF diagnosis. Moreover, the majority of ischemic stroke cases did not have a prior AF diagnosis and were not having an oral anticoagulation therapy against cardioembolic stroke. The association with the age at ischemic stroke diagnosis could be explained with the observed associations with paroxysmal AF in LURIC and AF in patients aged 75 and over in FINCAVAS. Paroxysmal AF is more likely to be subclinical and asymptomatic than more persistent forms of AF and therefore acute ischemic stroke may be the first clinical manifestation of the underlying subclinical atrial fibrillation. The possible bias due to the underdiagnosed paroxysmal AF cases in FINCAVAS may explain the observation that the association with incident AF is seen only in the elderly with probably more symptoms and more frequent screening for AF than younger patients.

In the LURIC study, circulating ADMA and SDMA levels showed step-wise increases as the AF status was changed from sinus rhythm through paroxysmal and chronic AF independent of age, sex and renal function. In line with these results, a similar step-wise increase in ADMA levels were seen in patients with paroxysmal AF and non-paroxysmal AF compared to patients with no AF in a previous study of coronary angiography patients whereas the association of SDMA with AF was not investigated[21]. More recently, ADMA, but not SDMA, was independently associated with the development of symptomatic AF in patients with acute myocardial infarction[22]. In contrast, SDMA, but not ADMA, was significantly associated with atrial fibrillation in patients with acute ischemic stroke[2]. The lack of association between SDMA and AF in most of the previous studies may reflect the relatively small number of AF cases studied. Moreover, the method of ADMA and SDMA quantification is not standardized complicating comparisons between different studies.

We observed a statistically significant interaction between rs16899974 and AF status groups on circulating SDMA levels independent of age, sex and renal function. Together with the observation that AGXT2 is expressed in the human heart[16] this interaction suggests a possible role for AGXT2 in the local dimethylarginine metabolism

in cardiac tissue; however, due to the observational nature of our study, this interesting hypothesis clearly needs further investigation. Furthermore, the lack of association of rs16899974 with the systemic ADMA levels does not exclude the possibility that AGXT2 could contribute to the ADMA metabolism locally in cardiac tissues. This can be explained by the fact that, unlike SDMA, ADMA is primarily metabolised by the DDAH enzymes which may be able to compensate impaired metabolism by AGXT2 both locally and at the systemic level[23].

To further support the role of dimethylarginines behind the associations with AF, in a recent large population-based study[24], SDMA was correlated with markers of atrial remodelling such as left atrial size and atrial conductance time although no independent association was seen between SDMA and AF. These associations are in line with the concept that dimethylarginines could promote the electrical and structural atrial remodeling during AF secondary to oxidative stress and NO synthase uncoupling. It is also possible that the genetic variants of AGXT2 within cardiac tissues alter the NO-related signalling pathways that regulate almost all cardiac ionic channels and modulate the susceptibility to both atrial and ventricular arrhythmias[25]. Interestingly, a recent report showed that ADMA was independently associated with left atrial appendage thrombus in patients with non-valvular atrial fibrillation[26], further supporting the concept that ADMA-induced endocardial dysfunction could contribute to the increased risk of cardioembolic stroke in AF. Finally, because the AGXT2 enzyme have several substrates other than dimethylarginines[27] and the systemic levels of dimethylarginines can merely reflect the systemic and/or local activity of AGXT2, our results support further mechanistic studies on the role of dimethylarginines behind the observed associations.

Whether future genome-wide association studies on age at onset of any ischemic or cryptogenic stroke could identify novel genetic variants associated additionally with paroxysmal or subclinical forms of AF is worth investigating because diagnosing of silent AF is challenging. Moreover, as the risk of AF increases substantially with age, an age-at-onset informed[28] or age stratification-based[29] approaches could detect novel AF loci whose magnitude of genetic effects differ with age. Whether the *AGXT2* variants could predict the presence of a silent or paroxysmal AF in patients with acute cryptogenic ischemic stroke warrants further investigation. Finally, experimental data for the functional role of rs16899974 is currently lacking.

Some degree of misclassification of AF cases is expected, although this would most likely attenuate the true genetic effect rather than create false positive findings. Because atrial fibrillation is often intermittent and asymptomatic, it is not possible to exclude the possibility that some of those how are classified to have no AF have undetected silent AF. However, this limitation is likely to be present in all clinical data sets in which AF is defined based on hospital discharge diagnostic codes rather than prolonged ambulatory ECG monitoring. In contrast, acute ischemic stroke with neurologic deficits is less likely to be left undetected and without a diagnosis. Moreover, the associations with AF and age at ischemic stroke onset cannot be directly generalized to the general population, other patient groups or individuals of other ancestral backgrounds. Finally, rs16899974 may be in linkage disequilibrium with the causal variant(s) underlying the associations with AF and ischemic stroke phenotypes.

Conclusions

We found strong evidence that the *AGXT2* p.V498L polymorphism is associated with both paroxysmal and chronic forms of AF in coronary angiographic patients without structural heart disease in ultrasound, and earlier age at onset of ischemic stroke in patients undergoing exercise stress testing. Hence, our study suggests that *AGXT2* variations are involved in the genesis of AF and its age-related thromboembolic complications. Future mechanistic studies should investigate whether these associations are mediated through local metabolism of dimethylarginines by AGXT2 in cardiac tissues.

Methods

Study populations. The LURIC study consists of 3,316 Caucasian patients hospitalized for coronary angiography between 1997 and 2000 at a tertiary care center in Southwestern Germany. Clinical indications for angiography were chest pain or a positive non-invasive stress test suggestive of myocardial ischemia. To limit clinical heterogeneity, individuals suffering from acute illnesses other than acute coronary syndrome (ACS), chronic non-cardiac diseases and a history of malignancy within the five past years were excluded.

The Corogene study included 5295 consecutive Finnish patients assigned to coronary angiogram in 4 hospitals servicing 1.5 million people in the Hospital District of Helsinki and Uusimaa. Of the Corogene study, 2500 patients with acute coronary syndrome (ICD-10: I20–I25) were included in a genome-wide association study.

The FINCAVAS study included all consecutive patients referred for a clinically indicated exercise test using a bicycle ergometer at Tampere University Hospital between October 2001 and the end of 2008 and willing to participate. A total of 4,068 participants had a technically successful exercise test. The main indications for the exercise test were suspicion of coronary heart disease (CHD, frequency 46%), evaluation of work capacity (26%), testing vulnerability to arrhythmia during exercise (25%), and adequacy of the CHD treatment (13%); some patients had more than one indication.

Discovery stroke cohorts in WTCCC2 ischemic stroke GWAS included samples from the UK and Germany, with a total of 3,548 cases and 5,972 controls. See further description of the study cohorts and laboratory analyses in Supplementary Methods 1–2 and Supplementary Tables 1, 5 and 6.

This study was conducted according to the Declaration of Helsinki principles and written informed consent was obtained from all participants. The study was performed in accordance with approved guidelines and regulations. The LURIC study was approved by the ethics committee at the Ärztekammer Rheinland-Pfalz. The Corogene study was approved by appropriate Ethics Committees of the Helsinki and Uusimaa Hospital region. The FINCAVAS study was approved by the Ethical Committee of the Hospital District of Pirkanmaa, Finland. For the WTCCC2 ischemic stroke cohorts, the recruitment of patients was approved by the relevant local ethics committees from all the participating centers.

Genotyping and quality control. In LURIC, genotyping was done by using the Affymetrix Human SNP Array 6.0 at the LURIC Study facility. Genotype imputation was performed using the IMPUTE2 software and the 1000 Genomes March 2012 haplotypes as a reference. Genotyped data was used for rs37369 and imputed data for rs16899974 with an excellent imputation quality (info~0.935).

The Corogene cohort was genotyped with Illumina 660 K BeadChip array at the Sanger Institute (Hixton, Cambridge, UK) and imputed using the 1000 Genomes April 2012 reference panel as a reference.

In FINCAVAS, we genotyped rs16899974 successfully for 3,889 participants using Taqman@SNP Genotyping Assay C__25742181_10 and ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). No evidence for deviation from the Hardy-Weinberg equilibrium was observed ($\chi_1^2 = 0.21$, $p = 0.65$). The data for rs37369 and rs6817105 were obtained for 3,195 individuals by genotyping using the Illumina HumanCardio-Metabo BeadChip or HumanCoreExome chip arrays and imputation using the IMPUTE2 software and 1000 Genomes March 2012 haplotypes as a reference.

For the WTCCC2 samples, Illumina BeadChips were used for genome-wide genotyping and genotype imputation was performed using MACH based on HapMap Phase 2 European (CEU) reference data.

Classification of atrial fibrillation cases. In LURIC, 2,923 patients had both genotype and atrial fibrillation (AF) status data available. Of the 2,923 participants, 360 had a history of AF at baseline. In addition, 161 individuals were in AF rhythm during the index coronary angiography, of which 21 were not included in the 360 cases diagnosed previously. Therefore, a total of 381 individuals were classified as any AF. Of the 381 AF cases, 348 were further classified as having either paroxysmal AF (n = 175) or chronic AF (n=173).

For the Corogene study, potential prevalent AF cases were screened from the baseline database. For these patients, we ascertained the AF status and its subtype from their medical records. Of the 2,208 patients included in this study, 265, 141 and 107 had any AF, paroxysmal AF and chronic AF, respectively.

For the FINCAVAS participants, clinical AF was ascertained from the central university hospital discharge diagnostic codes (ICD-9-CM 427.3, 427.31, or 427.32; or ICD-10 I48) from 1987 to 2015 (1179 incident AF cases), and study population area-wide electrical ECG recordings from 2005 onward (16 additional incident AF cases). In addition, to further ascertain the AF status at the index exercise stress test, we utilized the data from the baseline examination. According to the FINCAVAS baseline database, 13 patients had a history of AF/flutter or developed one during the exercise stress test but did not have any previous discharge diagnosis of AF with the date of diagnosis and were therefore excluded from all analyses. Both the genotype and phenotype data were available for 1,188 incident AF cases and 2,674 censored controls.

Classification of ischemic stroke cases. For the WTCCC2 ischemic stroke cohorts, Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification[30] was performed by an in-house neurologist and all stroke cases were classified into mutually exclusive aetiologic subtypes: large-artery atherosclerosis (LAA), small-vessel disease (SVD), cardioembolic stroke (CE), other aetiology, or unknown aetiology.

For the FINCAVAS cohort, age at the first ischemic stroke was ascertained from the central university hospital discharge diagnostic codes (ICD-9 433.x1, 434 (excluding 434.x0), or 436; or ICD-10 I63.0–I63.9). Of the 3,862 individuals with both genotype and phenotype data available, there were 360 incident ischemic stroke cases diagnosed between 1987 and 2015. In the majority of the cases 233 (65%), the etiology (LAA, SVD or CE) was uncertain at the time of the diagnosis and were therefore diagnosed as a cryptogenic ischemic stroke (I63.9).

Statistical methods for cross-sectional association and interaction analyses. Statistical analyses were performed under the R statistical environment. The associations of the *AGXT2* and 4q25 variants with AF and its subtypes were tested using multivariable logistic regression analyses assuming an additive genetic model. In addition, we tested the interactions of the studied polymorphisms with established AF risk factors on any AF in LURIC. For the WTCCC2 cohorts, associations of the *AGXT2* variants were tested with unadjusted logistic regression using PLINK[31] under an additive genetic model and results were meta-analyzed with inverse-variance-weighted method implemented in the METAL software[32].

Statistical methods for survival and age of onset analyses. Survival analyses were used to assess associations with incident AF/stroke and differences in age of AF/stroke onset as a function of the studied variants. In addition, for the age of onset analyses, we applied linear regression considering the age of onset as a quantitative trait. Survival curves of time to AF/stroke onset were estimated using the Kaplan–Meier method. We used a Cox regression analysis to examine the effect of the SNPs together with covariates on the survival functions of incident AF and ischemic stroke. We used chronological age as the fundamental time scale in all analyses. All patients were followed to a fixed date (April 2015). Those with no incident AF or ischemic stroke during the observation period were right censored at their last visit at the central hospital or at their last electrical ECG recording in the community, whichever came later. Significance was accepted at $P < 0.05$ in all analyses.

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Author Contributions

I.S. wrote the main manuscript text. I.S., S.B. and E.V. analysed the data. M.E.K., S.B., L.-P.L., N.O., J.A.H., K.-M.M., G.E.D., T.K. and H.S. provided expertise for data analysis and interpretation, commented the paper. P.M.R., C.S., M.D., J.S., R.L., M.K., H.S.M., W.M. and T.L. contributed to data collection. N.M. performed the genotyping in FINCAVAS. All authors contributed to and have approved the final manuscript.

Additional Information

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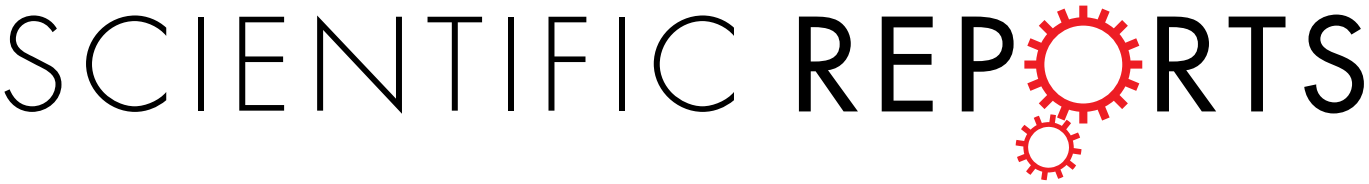

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The biomarker and causal roles of homoarginine in the development of cardiometabolic diseases: an observational and Mendelian randomization analysis

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Ilkka Seppälä[1], Niku Oksala[1,2], Antti Jula[3], Antti J. Kangas[4,5], Pasi Soininen[4,5], Nina Hutri-Kähönen[6], Winfried März[7,8,9], Andreas Meinitzer[9], Markus Juonala[10,11], Mika Kähönen[12], Olli T. Raitakari[10,13] & Terho Lehtimäki[1]

High L-homoarginine (hArg) levels are directly associated with several risk factors for cardiometabolic diseases whereas low levels predict increased mortality in prospective studies. The biomarker role of hArg in young adults remains unknown. To study the predictive value of hArg in the development of cardiometabolic risk factors and diseases, we utilized data on high-pressure liquid chromatography-measured hArg, cardiovascular risk factors, ultrasound markers of preclinical atherosclerosis and type 2 diabetes from the population-based Young Finns Study involving 2,106 young adults (54.6% females, aged 24–39). We used a Mendelian randomization approach involving tens to hundreds of thousands of individuals to test causal associations. In our 10-year follow-up analysis, hArg served as an independent predictor for future hyperglycaemia (OR 1.31, 95% CI 1.06–1.63) and abdominal obesity (OR 1.60, 95% 1.14–2.30) in men and type 2 diabetes in women (OR 1.55, 95% CI 1.02–2.41). The MR analysis revealed no evidence of causal associations between serum hArg and any of the studied cardiometabolic outcomes. In conclusion, lifetime exposure to higher levels of circulating hArg does not seem to alter cardiometabolic disease risk. Whether hArg could be used as a biomarker for identification of individuals at risk developing cardiometabolic abnormalities merits further investigation.

Accumulating evidence indicates that low levels of serum non-proteinogenic amino acid L-homoarginine (hArg) are associated with an increased risk of death from cardiovascular diseases, including heart failure, sudden cardiac death and fatal strokes, in various patient populations[1–6]. The association of hArg with adverse cardiovascular outcomes also appears to hold true in a population-based cohort of older adults[7] as well as a multi-ethnic United States population[8]. On the other hand, in cross-sectional analyses of these population samples, hArg was positively associated with several cardiovascular risk factors including hypertension, obesity, dysglycaemia, insulin resistance and dyslipidaemia, referring to the possibility that hArg could also play a causal role in the

[1]Department of Clinical Chemistry, Fimlab Laboratories, Faculty of Medicine and Life Sciences, University of Tampere, Tampere, Finland. [2]Division of Vascular Surgery, Department of Surgery, Tampere University Hospital, Tampere, Finland. [3]Health and Functional Capacity, National Institute for Health and Welfare, Turku, Finland. [4]Computational Medicine, Faculty of Medicine, University of Oulu, Oulu, Finland. [5]NMR Metabolomics Laboratory, School of Pharmacy, University of Eastern Finland, Kuopio, Finland. [6]Department of Paediatrics, University of Tampere and Tampere University Hospital, Tampere, Finland. [7]Institute of Public Health, Social and Preventive Medicine, Mannheim Medical Faculty, University of Heidelberg, Mannheim, Germany. [8]Synlab Services GmbH, Mannheim, Germany. [9]Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Graz, Austria. [10]Research Centre of Applied and Preventive Cardiovascular Medicine, University of Turku, Turku, Finland. [11]Department of Medicine, Turku University Hospital and University of Turku, Turku, Finland. [12]Department of Clinical Physiology, Tampere University Hospital, and Faculty of Medicine and Life Sciences, University of Tampere, Tampere, Finland. [13]Department of Clinical Physiology and Nuclear Medicine, Turku University Hospital, Turku, Finland. Correspondence and requests for materials should be addressed to I.S. (email: ilkka.seppala@uta.fi)

development of cardiometabolic diseases. Interestingly, recent experimental data indicates that hArg promotes the calcification of vascular smooth muscle cells[9] and may thus indeed have a causal role in the pathogenesis of atherosclerosis.

Supplementation with L-arginine (Arg), the primary precursor of nitric oxide (NO), unexpectedly increased cardiovascular events and mortality in a small randomized controlled trial (RCT) in patients with acute coronary syndrome[10]. Whether hArg administration in the secondary prevention setting would have the same detrimental effects as Arg, or whether increased levels of hArg would reduce the rate of cardiovascular events and mortality as suggested by most of the prospective studies, is currently not known. However, before any clinical trials using hArg supplementation or a pharmacological intervention to modify circulating hArg levels, it is rational to conduct an epidemiological investigation of its causality and potential pleiotropic effects, i.e. both adverse and/or beneficial effects, by using genetically determined hArg to avoid inaccurate conclusions due to the reverse causation and residual confounding related to all observational studies. Moreover, because common cardiometabolic diseases such as type 2 diabetes mellitus (T2DM) and coronary artery disease (CAD) develop over several decades during one's lifespan before any symptoms or clinical manifestations, any clinical trials on the effects of the lifetime exposure to high hArg in primary prevention settings are not feasible in practice.

So far, no prospective studies have been conducted to study the predictive value of circulating hArg levels on the development of metabolic abnormalities in early adulthood. As metabolic syndrome (Mets) or a high body mass index (BMI) are strong predictors of future T2DM and cardiovascular disease manifestations[11,12], we investigated whether hArg levels could predict the incidence of Mets components or obesity (BMI $>30\,kg/m^2$) and high insulin as well as incident T2DM and preclinical atherosclerosis in a population-based prospective cohort of young adults without clinical cardiovascular diseases. Moreover, we applied quantitative serum nuclear magnetic resonance (NMR) metabolomics to study the detailed molecular associations with hArg in young men and women. Furthermore, although we have individual-level genome-wide genetic data available for the Cardiovascular Risk in Young Finns Study (YFS) participants, we used summary-level data from several large-scale meta-analyses of genome-wide association studies involving tens to hundreds of thousands of individuals available in the public domain to increase the statistical power to detect potentially causal associations between hArg and metabolites, cardiometabolic risk factors, T2DM and CAD.

Results

An overview of the study flow, data sources and statistical analyses is illustrated in Fig. 1.

Clinical characteristics and cross-sectional determinants of serum hArg levels in YFS. Baseline characteristics of the YFS study subjects are presented in Table 1. To examine the determinants of hArg levels at baseline (2001), we performed a multivariate linear regression analysis using a bi-directional step-wise procedure. When all cardiometabolic risk factor variables shown in Table 1, along with serum SHBG, were included in the same stepwise multivariable linear regression model, hArg was independently and positively associated with male sex, BMI, SHBG, triglycerides and LDL cholesterol (Table 2). Inverse associations of hArg were observed with daily smoking and age. The overall R^2 for hArg was 0.114, suggesting that 11.4% of the variation in hArg concentrations was explained by the selected variables. When the three hArg-related SNPs were further added to the model selection procedure, the overall R^2 statistic increased to a value of 22.4% ($n = 1895$) while the significance of the other explanatory variables remained roughly the same. Prompted by the association with SHBG and a detailed metabolic profiling, the association of hArg with different hormonal contraceptive methods in women is shown in Figure S1. In women, HArg was positively associated with the use of combined oral contraceptives (containing oestrogen), but not with the use of progestin-only contraceptives (Figure S1).

Longitudinal and cross-sectional associations of hArg with 228 serum metabolites in YFS. The cross-sectional and longitudinal (6- to 10-year follow-up) associations of baseline hArg with 228 serum metabolites are shown in Figures S2–S3. For both sexes, the strong negative association of glycine with hArg persisted over time during 10 years of follow-up. For all subjects, glycine, histidine and tyrosine showed consistent associations with hArg at each time point (Figure S3). The cross-sectional associations of hArg with 73 selected metabolomics measures adjusted for age, BMI and daily smoking are illustrated separately for men and women in Figure S4. In women, more metabolic measures were associated with hArg compared to men, i.e. 33 measures for women and 9 for men ($P < 0.002$). In women, hArg was positively associated with the smallest very-low-density lipoprotein (VLDL), small and medium HDL, the triglyceride content of different lipoprotein classes, as well as fatty acids, several amino acids and glycoprotein acetyls (GlycA), which is a novel marker of inflammation (Figure S4). Indeed, when further adjusting for oral contraceptive use (in women) and serum SHBG (in both sexes), these associations were largely reduced to non-significant, except for the positive associations of amino acids and docosahexaenoic acid with hArg in women (Fig. 2). For both sexes, glycine showed a strong negative association with hArg, whereas the negative association of HDL subclasses and hArg was seen only in men (Fig. 2). Significant men-specific interactions were observed between hArg and BMI on glucose, valine and saturated fatty acids (%) (Fig. 2).

Longitudinal relations of hArg with incident obesity, Mets components, preclinical atherosclerosis and T2DM in YFS. In our 6- to 10-year follow-up (depending on the endpoints used) in unadjusted analyses, hArg was significantly and directly associated with incident obesity (BMI $\geq 30\,kg/m^2$), abdominal obesity (high waist circumference), hyperglycaemia, high insulin, high-risk carotid intima-media thickness and distensibility in men, and with incident T2DM in women (Fig. 3). In our 10-year follow-up analysis after further adjustments with other cardiovascular risk factors, hArg served as a significant biomarker for future hyperglycaemia (OR 1.31, 95% CI 1.06–1.63) and abdominal obesity (OR 1.60, 95% 1.14–2.30) in men and predicted

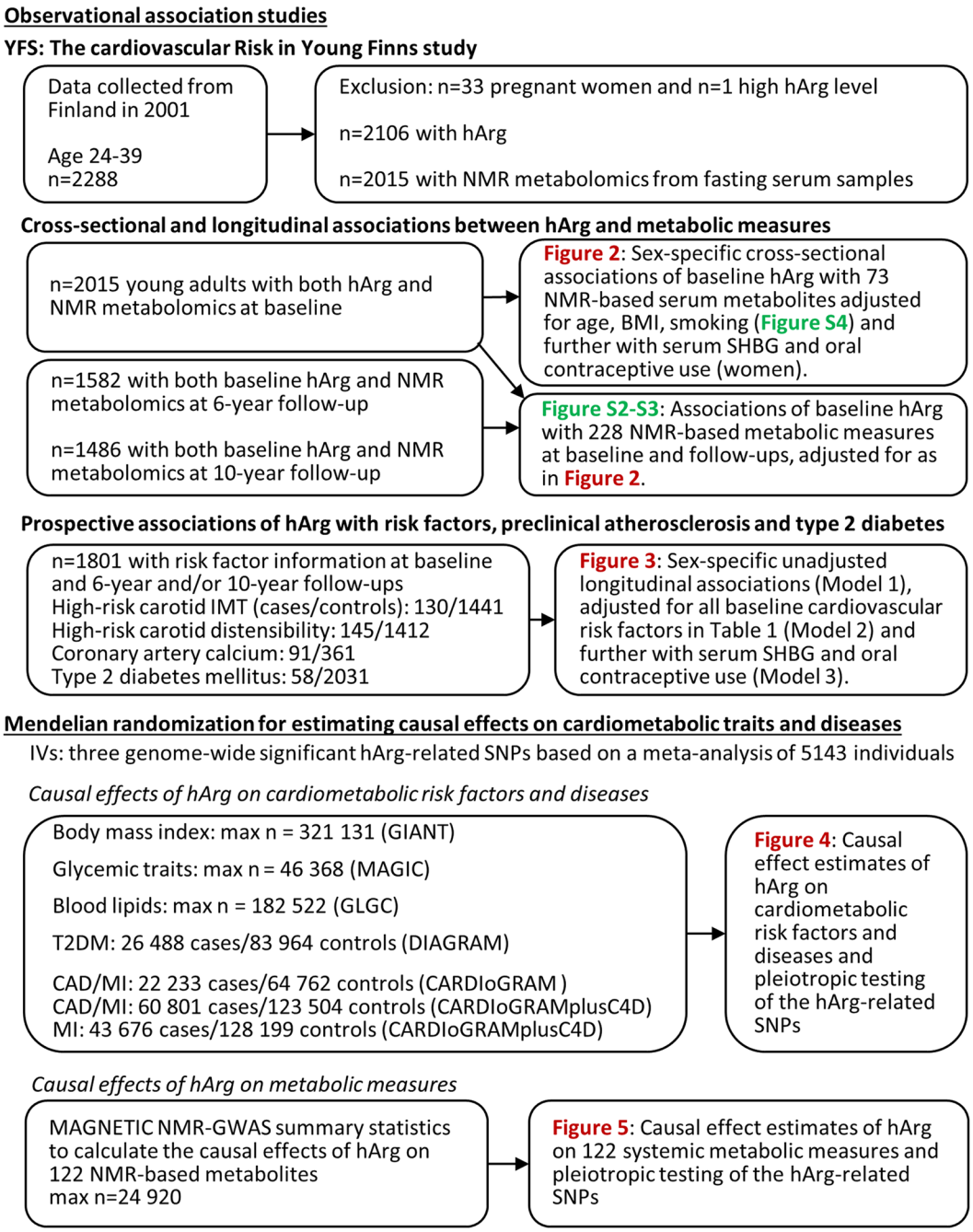

Figure 1. Overview of the study design, data sources and statistical analyses.

T2DM in women (OR 1.55, 95% CI 1.02–2.41) (Fig. 3). Furthermore, in the cross-sectional study (in 2001), for both men and women separately, hArg was strongly associated with BMI (for men $P = 5.7 \times 10^{-23}$, for women $P = 8.9 \times 10^{-8}$ and for all subjects combined $P = 1.4 \times 10^{-26}$), but not with BMI-adjusted waist circumference, in the observational analyses using the YFS data (Fig. 4).

Estimating causal effects of hArg on obesity, lipids and glycaemic traits as well as T2DM and CAD. In this part of the study, utilizing summary-level data from different GWAS meta-analyses available in the public domain (Fig. 1), no evidence was found that serum hArg was causally associated with any of the studied obesity traits (BMI or BMI-adjusted waist circumference), glycaemic or lipid traits, or T2DM or CAD (Fig. 4).

Estimating causal effects of hArg on 122 metabolites. We tested the causality of serum hArg on 122 NMR-based metabolic measures, and the results of these analyses are illustrated in Fig. 5. No evidence was found of a causal relationship between hArg and any of the tested 122 metabolites (for all traits, P-value for combined causal estimates >0.05). For three metabolites (creatinine, histidine and glycine), heterogeneity in the causal

	All	Men	Women
Number of subjects (%)	2106	957 (45.4)	1149 (54.6)
Age (years)	31.7 (5.0)	31.6 (5.0)	31.7 (5.0)
Homoarginine (hArg) (μmol/L)	1.85 (0.65)	1.93 (0.61)	1.79 (0.68)
*ln*hArg (μmol/L)	0.56 (0.34)	0.61 (0.30)	0.52 (0.36)
LDL cholesterol (mmol/L)	3.27 (0.84)	3.42 (0.90)	3.14 (0.77)
HDL cholesterol (mmol/L)	1.29 (0.31)	1.17 (0.27)	1.39 (0.30)
Triglycerides (mmol/L)	1.26 (0.64)	1.41 (0.70)	1.13 (0.55)
Systolic blood pressure (mmHg)	117 (13)	121 (12)	113 (12)
Diastolic blood pressure (mmHg)	72 (11)	73 (11)	69 (10)
C-reactive protein (mg/L)	1.9 (4.0)	1.5 (3.4)	2.1 (4.4)
Glucose (mmol/L)	5.1 (0.85)	5.2 (0.93)	4.9 (0.75)
Insulin (IU/L)	7.7 (5.7)	7.6 (5.8)	7.8 (5.7)
Body mass index (kg/m^2)	25.0 (4.4)	25.6 (4.1)	24.4 (4.5)
Waist circumference (cm)	84 (12)	90 (11)	79 (11)
Daily smokers (%)	520 (24.7)	288 (30.1)	232 (20.2)
Family history of CAD (%)	281 (13.3)	123 (12.9)	158 (13.8)

Table 1. Baseline descriptive data for the YFS cohort in 2001. Statistics are mean (SD) or n (%); *ln*hArg is natural log-transformed. CAD, coronary artery disease; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Explanatory variable	β	95% CI	P-value
Male sex	0.14	[0.10, 0.17]	4.1×10^{-13}
Body mass index (kg/m^2)	0.014	[0.0093, 0.018]	1.2×10^{-9}
Daily smoking	−0.089	[−0.12, −0.056]	1.3×10^{-7}
Age (years)	−0.0067	[−0.0097, −0.0038]	7.8×10^{-6}
*ln*SHBG (nmol/L)	0.061	[0.032, 0.089]	3.2×10^{-5}
*ln*Triglycerides (mmol/L)	0.045	[0.019, 0.072]	8.2×10^{-4}
LDL cholesterol (mmol/L)	0.022	[0.0045, 0.040]	0.014
*ln*CRP (mg/L)	0.016	[0.0023, 0.029]	0.021

Table 2. Cross-sectional stepwise multivariable linear regression modelling for homoarginine (hArg) (n = 2057). **Statistics:** In the bi-directional stepwise regression modelling applied, serum hArg was used as a dependent variable and all the variables shown in Table 1 and *ln*SHBG as explanatory variables. Those variables that were selected by Akaike's information criterion (AIC) using the stepAIC R function with the default settings and had a p-value < 0.05 are shown above. HArg, SHBG, triglycerides and CRP were natural log-transformed. For the continuous variables, β (95% CI) are shown for each 1-unit change in the variable. **Abbreviations:** SHBG, sex hormone-binding globulin; LDL, low-density lipoprotein; CRP, C-reactive protein.

estimates was detected due to strong associations with the *CPS1* variant and a lack of association or inconsistent association with the two other genetic variants (see Table S1).

Discussion

Consistent with the positive cross-sectional association of hArg with BMI in the present and several previous studies[1, 6, 8, 13], we now also showed that, in our 10-year follow-up analysis after adjustments with other cardiovascular risk factors, hArg served as a significant biomarker (predictor) for future hyperglycaemia and for abdominal obesity in men and incident T2DM in women. On the other hand, our MR analyses using large-scale GWAS meta-analysis summary data did not indicate causal roles for hArg in the development of high BMI or BMI-adjusted waist circumference, or any other studied clinical or metabolic trait.

In line with several previous population and patient studies[1, 6, 8, 13], we observed a strong positive association between hArg and BMI in young adults, whereas the causal effects of hArg on BMI and waist circumference were absent. The lack of a causal role of circulating hArg in body weight is supported by an experimental study on mice and a small clinical trial showing that several-fold elevations of plasma hArg by oral hArg supplementation for several weeks did not have any effect on body weight[14, 15]. Moreover, L-arginine:glycine amidinotransferase (AGAT) -deficient mice exhibiting creatine and hArg[5] deficiencies were completely protected from diet-induced

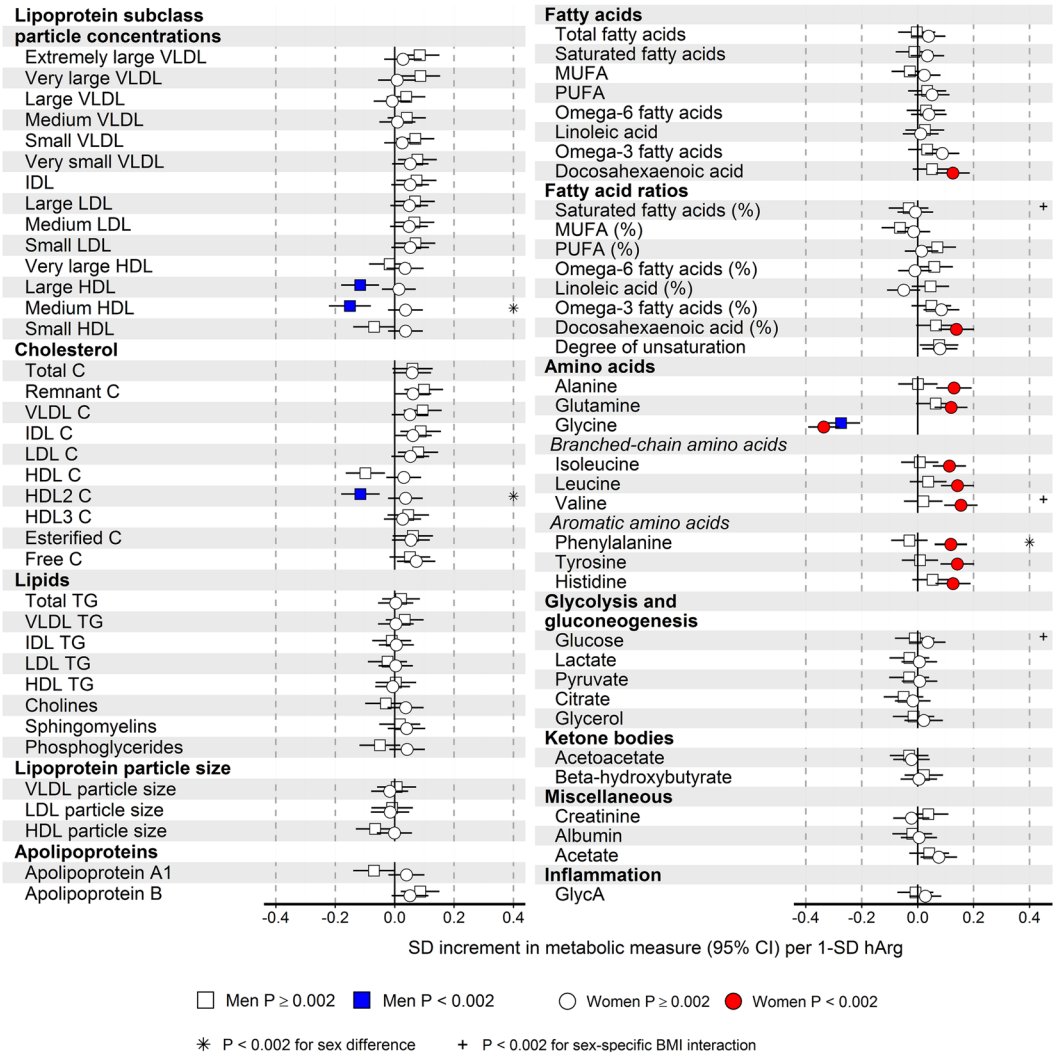

Figure 2. Sex-specific cross-sectional associations of baseline hArg with 73 NMR-based serum metabolites, adjusted for age, body mass index (BMI), daily smoking, serum SHBG and oral contraceptive use (in women). The analyses were conducted for 867 men and 1097 women. Squares indicate men, and circles represent women. Open and closed symbols indicate $P \geq 0.002$ and $P < 0.002$, respectively. Sex differences with $P < 0.002$ are marked by asterisks. BMI interactions with $P < 0.002$ are marked by the plus or minus sign, depending on the direction of the estimated interaction effect.

obesity and metabolic syndrome, whereas oral creatine supplementation resulted in a complete normalisation of the body weight and composition of these animals[16], suggesting that the effect of AGAT deficiency on BMI is hArg-independent. Therefore, it is possible that hArg is a biomarker of AGAT activity and creatine synthesis and that higher creatine levels predispose to weight gain, as suggested by the mouse model. This hypothesis is supported by our result showing that hArg has some predictive value for the development of abdominal obesity during a 10-year follow-up in young men despite careful adjustment for various baseline risk factors, including BMI. Concordantly, no change in plasma or tissue hArg concentrations was observed in a small study of patients undergoing bariatric surgery despite dramatic weight loss, suggesting that there is no causal effect of BMI on hArg levels[17]. Furthermore, the strong inverse association of baseline hArg and 10-year glycine suggests that hArg has some predictive value for creatine synthesis via AGAT (Figure S5) at least 10 years ahead of time. Taken together, hArg endogenously synthesized by AGAT might be an excellent marker of systemic creatine synthesis, explaining the positive cross-sectional association of hArg with BMI in large population and patient cohorts.

We observed a longitudinal association between baseline hArg levels and 10-year incident hyperglycaemia and a positive interaction between baseline hArg and BMI on NMR-based fasting serum glucose in young men. However, cross-sectional associations between hArg and fasting glucose were absent in both men and women, in line with the lack of causal effects on glycaemic traits in individuals without T2DM. In contrast, hArg was independently associated with fasting glucose in older adults[13]. Moreover, in an RCT on oral hArg supplementation lasting 4 weeks in 20 young volunteers, a moderate increase in plasma glucose in comparison to placebo was observed[15]. Compared to a 7-fold increase in hArg plasma levels after supplementation with 125 mg hArg once

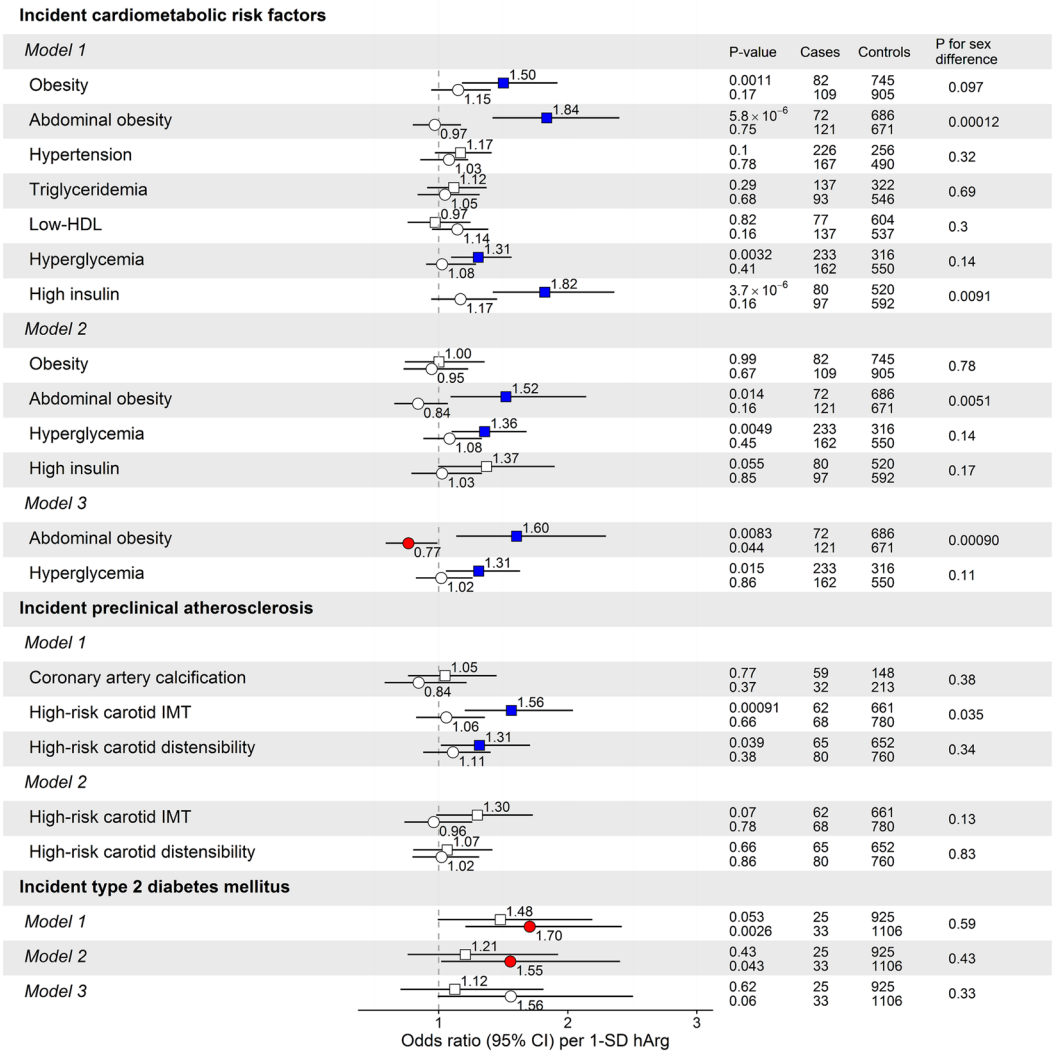

Figure 3. Sex-specific prospective (observational) associations of baseline hArg (in 2001) and cardiometabolic risk factors, preclinical atherosclerosis and type 2 diabetes mellitus (T2DM) during a 10-year follow-up in YFS. The prospective associations are shown as unadjusted (Model 1), adjusted for all baseline cardiometabolic risk factors shown in Table 1 (Model 2), and further adjusted for baseline serum steroid hormone binding globulin (SHBG) and oral contraceptive use in women. Open and closed squares and circles indicate $P \geq 0.05$ and $P < 0.05$, respectively.

daily, the hArg-associated lead *GATM* variant used in the MR analyses showed a relatively small per allele effect of ~0.25 µmol/L (or ~14% increase from the mean value of 1.85 µmol/L). Therefore, the possible effect of hArg on blood glucose is likely seen at much higher concentrations of hArg than what is normally present in the blood, in line with the tightly regulated glucose homeostasis in humans without overt T2DM. A 16-week oral hArg supplementation resulted in a 6-fold increase in plasma hArg, as well as increased insulin secretion and reduced blood glucose in mice on a high-fat diet but not in mice on a normal diet[14]. This contrasts with a moderate increase in blood glucose in the RCT, probably due to the differences in glucose metabolism between mice and humans.

In concordance with several previous studies[8,13], we observed an independent negative association between daily smoking and circulating hArg levels, while the molecular underpinnings of this association remain elusive. In contrast to some studies on older adults from general and patient populations[6,13], we observed no associations between baseline hArg and incident arterial hypertension during a 10-year follow-up, or baseline diastolic or systolic blood pressure in young adults. We were unable to study the causality of lifelong exposure to hArg on blood pressure due to the lack of publicly available complete summary statistics on the hArg-associated SNPs, and further MR studies are thus warranted to address whether there is any causal effect of circulating hArg on blood pressure in humans. However, the RCT on hArg supplementation in young volunteers did not detect any differences in diastolic or systolic blood pressure between hArg and placebo after a 4-week supplementation[15].

Baseline hArg was positively associated with incident T2DM especially in women, although the association was attenuated after adjusting for cardiometabolic risk factors and the use of oral contraceptives. A causal effect of hArg on T2DM was absent, suggesting that lifelong exposure to higher circulating hArg levels does not increase

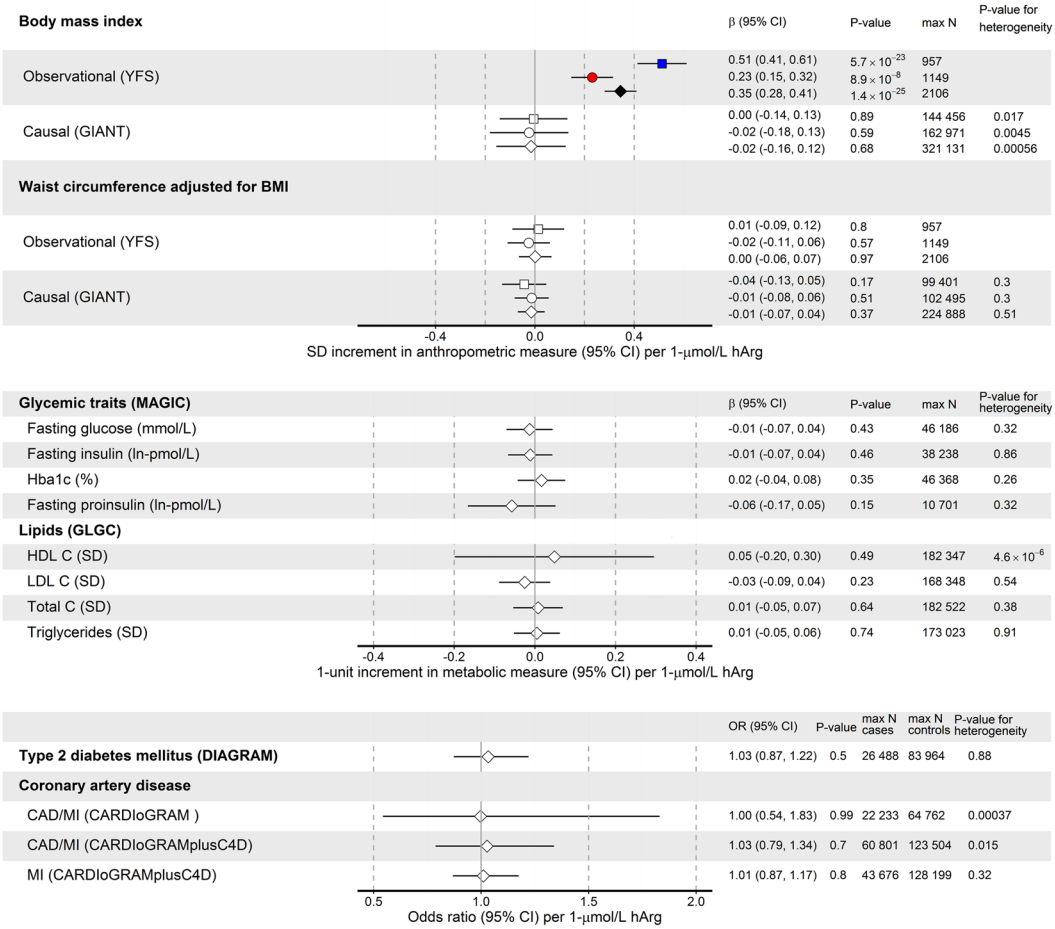

Figure 4. Combined causal effect estimates (β = beta, odds ratio and 95% CI confidence intervals) of hArg with cardiometabolic risk factors, type 2 diabetes mellitus (T2DM) and coronary artery disease (CAD). For each metabolite, the summary-level data across the three hArg-associated SNPs (*GATM* rs1153858, *CPS1* rs1047891 and *AGXT2* rs37369) was combined using weighted linear regression, and the heterogeneity in the causal effects from different individual variants was tested by Cochran's Q statistic. All p-values for combined causal effects >0.05. A p-value of >0.05 from Cochran's Q statistic indicates that there is no more heterogeneity between causal effects estimated using the variants individually than would be expected by chance.

the risk of developing T2DM. In some population and patient cohorts, observational cross-sectional associations between hArg and prevalent T2DM were absent[6,7,18], whereas in some studies, T2DM was associated with lower hArg levels compared to non-diabetic controls[8], and, in patients with T2DM undergoing maintenance haemodialysis, low hArg levels correlated with longer duration of T2DM[1].

No independent associations were observed between hArg and 6-year incident high-risk carotid intima-media thickness or carotid distensibility. Neither was the baseline hArg associated with the presence of coronary artery calcification assessed seven years after the hArg quantification. These results are consistent with the lack of a cross-sectional association of hArg with coronary calcium in the population-based Dallas Heart Study[8]. In addition, there were no associations between genetically determined hArg and CAD or myocardial infarction, suggesting that serum hArg does not have a causal effect on the development of coronary atherosclerosis or its clinical manifestations in humans, at least at concentrations normally observed in the circulation. This is in line with the lack of a cross-sectional association between hArg and prevalent CAD in over 3000 patients referred for coronary angiography[18] as well as with the absence of associations between baseline hArg and future cardiovascular events and myocardial infarction in haemodialysis patients[2]. The experimental evidence on the role of hArg in atherosclerosis is not conclusive, as one study showed that hArg augments the osteo-/chondrogenic transformation of human aortic vascular smooth muscle cells and aortic calcification[9], whereas another study reported a beneficial role of hArg in balloon-injured rat carotids trough the inhibition of neointimal formation[19].

There are no earlier systematic studies investigating the causal role of hArg in the regulation of serum metabolite profiles. In our analysis, we found no evidence for causal relationships between circulating hArg and 122 systemic metabolite measures. There are several potential explanations for this. Firstly, it is possible that hArg is an innocent AGAT metabolite and, at best, a biomarker with no direct causal effects on systemic metabolic pathways or diseases. This view is supported by experiments in rats and pigs, showing that >95% of orally administered

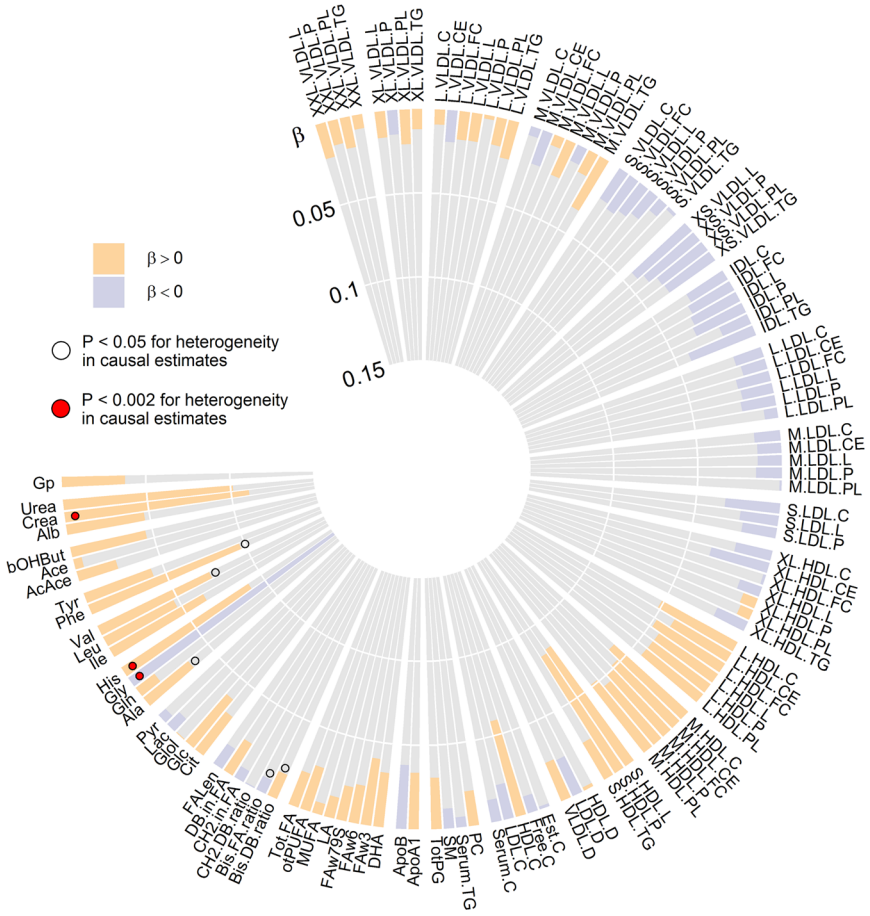

Figure 5. Combined causal effect estimates (β = beta; coloured bars indicate the direction and magnitude of the effect; yellow bars, $\beta > 0$, blue bars, $\beta < 0$) of hArg with 122 systemic metabolic measures using summary-level data from previous meta-analyses of genome-wide association studies. For each metabolite, the summary-level data across the three hArg associated SNPs (*GATM* rs1153858, *CPS1* rs1047891 and *AGXT2* rs37369) was combined using weighted linear regression, and the heterogeneity in the causal effects from different individual variants was tested by Cochran's Q statistic. All p-values for combined causal effects >0.05. A p-value of >0.05 from Cochran's Q statistic indicates that there is no more heterogeneity between causal effects estimated using the variants individually than would be expected by chance. The numbers of individuals used to estimate SNP–metabolite associations vary between 8 905 and 24 924, depending on the metabolite.

hArg was recovered in urine and not metabolized in tissues[20]. Moreover, the RCT on oral hArg supplementation in 20 young volunteers did not detect any systemic effects, with the exception of a moderate increase in blood glucose, despite a 7-fold increase in plasma hArg compared to placebo[15]. Secondly, hArg may have specific effects on metabolic pathways not covered by the metabolomics platform used or may play a more important role in certain pathologies rather than in healthy individuals.

The use of oral oestrogen contraceptives in young women was associated with a marked increase in hArg levels. A previous study of pregnant YFS participants reported that hArg levels were increased during the second and third trimesters of normal pregnancy when compared to non-pregnant women and that hArg was positively correlated with gestational age[21]. The gradual increase in hArg levels during normal pregnancy is likely due to a parallel increase in maternal oestrogen levels. This reasoning is supported by experiments showing that the expression of the *AGAT* gene is modulated by oestrogen in chick liver[22] and consistent with a marked increase in the renal *AGAT* mRNA and protein expression of pregnant spiny mice at term when compared to non-pregnant animals[23]. The fact that the hArg synthesis by AGAT is modulated by sex hormones might aid to explain the observed sex differences in the associations between hArg and incident cardiometabolic risk factors and T2DM. This hypothesis warrants further examination.

Our study has limitations that are general to all MR studies, and these should be considered when interpreting the results. The three MR assumptions in the context of the present study are as follows: 1) the genetic variants should be associated with the circulating hArg levels, 2) they should affect the outcome only through the circulating hArg levels (exclusion restriction assumption), and 3) they should be independent of hArg-outcome confounders (independence assumption). Regarding the first assumption, we acknowledge that the sample size used to quantify the strength of the SNP–hArg associations is limited (n = 5143). On the other hand, the lead

GATM variant is a strong instrument for MR analyses, explaining ~5.3% of the variability in serum hArg levels in YFS, and all three variants included in the MR analyses showed genome-wide significant ($P < 5 \times 10^{-8}$, F-statistic >120, Table S2) associations with serum hArg levels in the original GWAS[24]. In addition, the statistical power in two-sample MR analyses is mainly determined by the number of individuals used to assess SNP–outcome associations[25], for which we used publicly available summary statistics from large consortiums involving tens to hundreds of thousands of individuals. An adequate statistical power to detect causal effects is evident by the inspection of the 95% confidence intervals for, e.g., BMI-adjusted waist circumference in Fig. 4 showing that the confidence intervals for the combined causal estimates are comparable to those from the observational association analyses when there is no heterogeneity between the causal estimates. Moreover, some degree of sample overlap between individuals used to estimate SNP–hArg and SNP–outcome associations is present for some of the outcomes studied; however, the possible effect of this sample overlap on the causal estimates is likely to be small, because the individuals used in the hArg GWAS constitute a very small proportion of the total number of individuals used in the large meta-analyses of GWASs on outcomes. The risk that the independence assumption is violated due to a population stratification in GWASs is minimized by adjustments for principal components. Another potential concern in our MR analyses, as in all MR studies, is a horizontal pleiotropy (i.e. the genetic variants affect the outcome through pathways not mediated by hArg levels). We could avoid too precise causal estimates in the cases in which some of the genetic variants showed a pleiotropic effect on outcome by using weighted linear regression, and formally tested the presence of heterogeneity between the causal estimates using Cochran's Q statistic. Moreover, the three genome-wide significant hArg-related SNPs used to estimate causal effects have biologically plausible and functional roles in the endogenous hArg synthesis and/or catabolism as illustrated in Figures S6 and S7. Finally, despite single-variant pleiotropy in some SNP–outcome associations, the other two of the three independent variants showed not even a weak association with the outcome (Tables S1 and S3–S7). As discussed before[26], although both negative and positive results are potentially subject to a violation of the assumptions required for MR, in the case of negative results, the biases would have to balance out perfectly to result in an effect estimate of zero when there is in fact a true effect, making negative findings from MR more reliable than positive ones.

In the observational analyses, the 10-year follow-up was not long enough to investigate very long term predictive value of hArg on studied cardiometabolic outcomes. However, we observed a significant association between baseline hArg and new onset T2DM in young women indicating that the follow-up period was sufficiently long to detect such associations despite limited number of new-onset cases of T2DM. Furthermore, the follow-up period of 6 years used to study the association between hArg and high-risk carotid atherosclerosis is relatively short. However, traditional cardiometabolic risk factors were associated with these outcomes suggesting that the follow-up time was long enough to detect such association also with hArg.

In conclusion, our results show that circulating hArg is not causally associated with various cardiometabolic risk factors, the systemic metabolic profile, T2DM or CAD. Our results do not provide any consistent evidence that interventions aimed at modifying hArg levels will improve the metabolic profiles or risk of T2DM or CAD. Whether hArg could be used as a biomarker to predict future abdominal obesity in young men remains to be investigated in future studies.

Methods

Study population and data sources. YFS is a Finnish longitudinal population study on the evolution of cardiovascular risk factors from childhood to adulthood[27]. The study began in 1980, when 3,596 children and adolescents aged 3–18 years were randomly selected from five university hospital catchment areas in Finland. In 2001, 2,288 participants aged 24–39 years attended the 21-year follow-up. We excluded 33 pregnant women, as their hArg levels have been shown to be elevated during pregnancy in the YFS[21], as well as 11 subjects who had type 1 diabetes at baseline and one participant who had an exceptionally high hArg serum concentration (20.2 µmol/L) from all analyses. Therefore, 2,106 participants contributed to the cross-sectional association analysis of hArg and cardiometabolic risk factors at baseline (in 2001). We considered the participants of the longitudinal analyses who attended the 2001 follow-up and at least one later follow-up in 2007 or 2011. Of these participants, we included those for whom hArg and covariate data at the 2001 follow-up (used as baseline for the present study) and cardiometabolic risk factor data at the 2007 and/or 2011 follow-up were available and who were not pregnant in 2001 (n = 1801). The YFS was approved by the 1st ethical committee of the Hospital District of Southwest Finland (on September 21st, 2010) and by local ethical committees. The methods were carried out in accordance with the relevant guidelines and regulations. The participants gave written informed consent.

Genetic instrument selection for Mendelian randomization (MR) studies. For MR analyses, we used three single-nucleotide polymorphisms (SNPs) – *GATM* (glycine amidinotransferase) rs1153858, *CPS1* (carbamoyl-phosphate synthase 1) rs1047891 (formerly rs7422339), and *AGXT2* (alanine-glyoxylate aminotransferase 2) rs37369 – that were all associated with serum hArg levels on a genome-wide significance level ($P < 5 \times 10^{-8}$) in a recent genome-wide association study (GWAS) of 5,143 individuals[24]. The *GATM* rs1153858 variant alone, also associated with *GATM* mRNA expression in a tissue-specific manner (see Figure S6), explained ~5.3% of the variability in serum hArg levels in the YFS[24]. The summary statistics for the SNP-outcome associations were extracted from the summary-level data available in the public domain from large GWASs (see number of subjects in Fig. 1) investigating the associations of gene variants with conventional cardiometabolic risk factors, including BMI[28,29], waist circumference[30], fasting glucose[31], fasting insulin[31], HbA_1c[32], proinsulin[33], total cholesterol[34], high-density lipoprotein (HDL) cholesterol[34], triglycerides[34], low-density lipoprotein (LDL) cholesterol[34] as well as CAD[35,36] and T2DM[37]. The summary-level data for 122 NMR-method-based serum metabolites from the MAGNETIC NMR GWAS consortium[38] were downloaded from http://computationalmedicine.fi/data. All

associations extracted from the summary-level data on hArg-related variants and outcomes are presented in Tables S1–S7.

Serum hArg measurements and other biochemical assays. HArg was measured in serum stored at −80 °C by means of reversed-phase high-performance liquid chromatography at the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz[39]. Details of the biochemical measurements and clinical data are presented in Supplementary Methods.

Metabolic profiling. High-throughput NMR spectroscopy was used for the absolute quantification of serum metabolites. The metabolite set (includes 228 quantified metabolites) covers multiple metabolic pathways, including lipoprotein lipids and subclasses, fatty acids and fatty acid compositions, as well as amino acids and glycolysis precursors. All molecular measures are quantified in a single experimental setup, constituting both established and novel metabolic risk factors. This NMR-based metabolite profiling has previously been used in various epidemiological[40,41] and genetic studies[38] and has been reviewed recently[42]. Details of the experimentation have been described elsewhere[42,43].

Incident outcome definitions for the studied traits. In the YFS, the metabolic syndrome components were defined according to harmonized criteria[11]: waist circumference ≥102 cm in males and ≥88 cm in females (1), hypertriglyceridemia >1.7 mmol/l (2), HDL cholesterol <1.0 mmol/l in males and <1.3 mmol/l in females (3), blood pressure ≥130/85 mmHg or treatment for blood pressure (4), and fasting plasma glucose ≥5.6 mmol/l or previously diagnosed T2DM. High insulin was defined as a 10-year fasting insulin in the >90th percentile using sex-specific values corresponding with a fasting insulin of ≥18.8 IU/L for men and ≥15.2 IU/L for women. Obesity was defined as BMI ≥30 kg/m^2. In 2008, cardiac computed tomography was performed to measure coronary artery calcification (CAC) for a subsample of 589 participants then aged 40–46 years[44]. The absence of CAC was defined as an Agatston score of 0, and individuals with an Agatston score of 1 or greater were classified as having CAC. In 2001 and 2007, carotid artery intima-media thickness (IMT) and distensibility were measured as described in detail previously[45,46]. As previously[47], high-risk carotid IMT and distensibility were defined as falling within the age- and sex-specific ≥90 percentile and ≤10 percentile, respectively. Participants were classified as having T2DM if they had fasting a plasma glucose level of 7.0 mmol/l or greater, reported the use of oral glucose-lowering medication or insulin but had not reported having type 1 diabetes mellitus, or reported a diagnosis of T2DM by a physician. Participants were also classified as having T2DM if they had HbA1c ≥6.5% (48 mmol/mol).

Statistical Methods. All statistical analyses were conducted with R version >3.1.2 (https://www.r-project.org/).

Clinical determinants of hArg levels in YFS. We used linear regression analysis to explore the associations of hArg with common cardiovascular risk factors at baseline (age, sex, LDL cholesterol, HDL cholesterol, triglycerides, systolic and diastolic blood pressure, CRP, glucose, insulin, BMI, waist circumference, smoking and family history of CAD) as well as serum sex hormone-binding globulin (SHBG) and hormonal oral contraceptive use in women. Stepwise model selection by Akaike's information criterion (AIC) was performed to determine the most important determinants of hArg levels using the stepAIC R function.

Cross-sectional association analyses of hArg and metabolites in YFS. To facilitate the log-transformation, zero values in NMR-based metabolic measures were replaced by half of the minimum positive value found for each metabolite, assuming this to be the best estimation of detection limit. Prior to statistical analyses, NMR-based metabolic measures were log-transformed and scaled to standard deviations (SD) to facilitate comparisons across metabolites. Association magnitudes are reported in SD units of metabolite concentration per 1-SD increment in log-transformed hArg. For sex-stratified cross-sectional analysis, sex-specific scaling was applied for NMR metabolites. Due to the correlated nature of the systemic metabolite measures, over 95% of the variation in the metabolic data was explained by 25 principal components, and a P-value of 0.05/25 = 0.002 was thus required after multiple testing correction[40].

Cross-sectional and longitudinal associations of baseline hArg with circulating metabolites at baseline and at the follow-ups were analysed using linear regression models, stratified by sex, with each metabolic measure as the outcome and hArg as the explanatory variable. The regression models were adjusted for age, BMI, daily smoking, serum SHBG and oral contraceptive use (in women). For each metabolic measure, the sex-related differences in observational estimates were tested by using the Z-statistic. Interactions between BMI and each metabolite measure in relation to hArg levels were tested in men and women separately by adding the interaction term to the linear regression models.

Longitudinal association analyses of hArg with cardiometabolic risk factors, preclinical atherosclerosis and T2DM in YFS. The prospective associations of hArg with 6- or 10-year incident Mets components, high insulin (see incident outcome definitions above), and incident obesity (BMI >30 kg/m^2) were assessed using logistic regression models with scaled log-transformed baseline hArg as an explanatory variable. Incident T2DM during the 10-year follow-up was used as an outcome variable to assess the prospective association of hArg with T2DM. The follow-up period for carotid artery ultrasound parameters was six years. Coronary calcification was assessed once seven years after the baseline in 2008. Those with the condition at baseline were excluded from the analysis. We fitted unadjusted models (Model 1) and models that were adjusted for all cardiometabolic risk factors shown in Table 1 (Model 2) and, further, for serum SHBG and oral contraceptive use (in women, Model 3).

Instrumental variable analyses using publicly available summary-level data on hArg, metabolites, cardiometabolic traits, T2DM and CAD. The strengths of the genetic instruments used for hArg were assessed based on the F-statistic derived from the summary statistics of the hArg GWAS (Table S2). Despite the relatively small sample size (n = 5143) in the meta-analysis of GWASs on hArg[24], all F-statistics for the instruments were well over 10 (between 120 and 370) (Table S2), indicating that all three instruments separately are sufficiently strong to be used in MR studies. We then used these instruments in the Mendelian randomization (MR) analyses to quantify the strengths of the causal associations of hArg with NMR metabolites, cardiometabolic traits, T2DM and CAD. As previously[48], to evaluate combined causal estimates with 95% confidence intervals (95% CI) from summary statistics of the three independent SNPs, we performed a weighted linear regression of the genetic associations with each outcome variable on the genetic associations with hArg using first-order weights described in more detail with an R code implementation elsewhere[49]. This multiplicative random-effects model was used for combining causal estimates to obtain the same point estimate as from a fixed-effects model while avoiding too precise estimates in the case of heterogeneity in the causal effects obtained from different genetic variants. The combined MR estimates are in units of 1-unit increment of a continuous outcome (or odds ratio of disease risk) per 1-μmol/L increment in hArg. We performed heterogeneity tests based on Cochran's Q statistic to detect potential dissimilarity between the causal effects across the hArg SNPs (instrumental variables).

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Author Contributions

I.S. planned the study flow, conducted the analyses and wrote the first draft of the paper. N.O. contributed to the discussion, in addition to reviewing/editing the manuscript. A.J.K. and P.S. contributed to the NMR-based metabolite profiling and reviewed the manuscript. W.M. and A.M. organized the hArg quantification of participants in the YFS and reviewed the manuscript. A.J. participated in cohort collection and reviewed the manuscript. N.H.-K. participated in cohort collection and reviewed the manuscript. M.J. participated in cohort collection and reviewed the manuscript. M.K. participated in cohort collection and reviewed the manuscript. O.T.R. handled funding, participated in cohort collection, and reviewed the manuscript. T.L. handled funding and supervision, participated in cohort collection and contributed to the discussion, in addition to reviewing/editing the manuscript.

Additional Information

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Competing Interests: A.J.K. and P.S. are shareholders of Brainshake Ltd (www.brainshake.fi), a company offering NMR-based metabolite profiling. A.J.K. and P.S. report employment and consulting for Brainshake Ltd. Other authors did not report any competing interests.