ORIGINAL RESEARCH

Dimethylguanidino Valerate: A Lifestyle-Related Metabolite Associated With Future Coronary Artery Disease and Cardiovascular Mortality

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***Background*—**Identification of lifestyle modifiable metabolic pathways related to cardiometabolic disease risk is essential for improvement of primary prevention in susceptible individuals. It was recently shown that plasma dimethylguanidino valerate (DMGV) levels are associated with incident type 2 diabetes mellitus. Our aims were to investigate whether plasma DMGV is related to risk of future coronary artery disease and with cardiovascular mortality and to replicate the association with type 2 diabetes mellitus and pinpoint candidate lifestyle interventions susceptible to modulate DMGV levels.

***Methods and Results*—**Plasma DMGV levels were measured using liquid chromatography-mass spectrometry in a total of 5768 participants from the MDC (Malmö Diet and Cancer Study—Cardiovascular Cohort), MPP (Malmö Preventive Project), and MOS (Malmö Offspring Study). Dietary intake assessment was performed in the MOS. Baseline levels of DMGV associated with incident coronary artery disease in both the MDC (hazard ratio=1.29; CI=1.16–1.43; $P<0.001$) and MPP (odds ratio=1.25; CI=1.08–1.44; $P=2.4e\text{-}3$). In the MDC, DMGV was associated with cardiovascular mortality and incident coronary artery disease, independently of traditional risk factors. Furthermore, the association between DMGV and incident type 2 diabetes mellitus was replicated in both the MDC (hazard ratio=1.83; CI=1.63–2.05; $P<0.001$) and MPP (odds ratio=1.65; CI=1.38–1.98; $P<0.001$). Intake of sugar-sweetened beverages was associated with increased levels of DMGV, whereas intake of vegetables and level of physical activity was associated with lower DMGV.

***Conclusions*—**We discovered novel independent associations between plasma DMGV and incident coronary artery disease and cardiovascular mortality, while replicating the previously reported association with incident type 2 diabetes mellitus. Additionally, strong associations with sugar-sweetened beverages, vegetable intake, and physical activity suggest the potential to modify DMGV levels using lifestyle interventions. **(*J Am Heart Assoc*. 2019;8:e012846. DOI: 10.1161/JAHA.119.012846.)**

Key Words: coronary artery disease • diabetes mellitus • lifestyle • metabolome • metabolomics

Nontargeted metabolomics has emerged as a powerful tool to generate candidate biomarkers for metabolic disease. Once validated, these candidate biomarkers could eventually improve risk stratification or help identifying molecular disturbances that could be subjected to pharmacological or lifestyle interventions.[1] However, thus far no candidate marker generated by nontargeted metabolomics has made it to clinical application, indicating that more efforts should be put into validating already-known candidates.[2] Given that the number of patients with type 2 diabetes (T2DM) is increasing globally[3] and given that T2DM is a major risk factor for cardiovascular morbidity and mortality,[4] it is particularly important to identify metabolites associated with diabetes mellitus–related cardiovascular disease (cardiometabolic disease; CMD). Metabolites which not only cluster with metabolic risk factors and predict risk of incident CMD, but also are causally linked with lifestyle habits are good candidates for intense interventions directed to individuals with disturbed levels of these metabolites.

Circulating dimethylguanidino valerate (DMGV) levels were recently reported to associate with increased biopsy proven amount of liver fat and incidence of T2DM. The study also showed that DMGV is strongly associated with variants in the

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Accompanying Data S1, Tables S1 throgh S9, and Figures S1 through S4 are available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.119.012846

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Clinical Perspective

What Is New?

- Plasma levels of the diabetes mellitus–associated metabolite dimethylguanidino valerate associates with cardiovascular risk factors and future risk of coronary artery disease and cardiovascular mortality in 3 Swedish prospective cohorts, comprising over 5000 participants.
- Plasma dimethylguanidino valerate correlates with levels of leisure-time physical activity dietary intakes of vegetables and sugar-sweetened beverages.

What Are the Clinical Implications?

- Understanding the mechanisms between plasma levels of dimethylguanidino valerate, lifestyle, and coronary artery disease may facilitate development of future personalized preventive strategies for individuals with high coronary artery disease risk.

gene encoding alanine glyoxylate aminotransferase II (*AGXT2*), which catalyzes the conversion of asymmetric dimethylarginine (ADMA) to DMGV.[5] Previous studies have indicated that variants in *AGXT2*, apart from DMGV levels, also associate with levels of ADMA,[6] a known inhibitor of nitric oxide synthesis.[7] Earlier studies have established that ADMA levels predict incident coronary artery disease (CAD) and stroke in several population-based cohorts,[8] and a causal link between ADMA and atherosclerosis and hypertension has been suggested.[9] Given that *AGXT2*-related metabolites might be involved in the pathogenesis of atherosclerosis and CAD, the connection between plasma DMGV and CAD should be studied.

Here, we investigated the relation between plasma DMGV levels and incidence of CAD and T2DM and with cardiovascular mortality in 2 independent Swedish prospective cohorts of 4848 participants in total. We hypothesized that both T2DM and CAD are preceeded by increased levels of DMGV. Furthermore, we used a third cohort of 920 participants, to test whether dietary habits and level of physical activity are related to DMGV plasma concentration, in order to pave way for potential strategies to modulate DMGV levels.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Samples

Plasma levels of DMGV was measured in a total of 5768 participants from 3 different Swedish cohorts. The 2 prospective cohorts, the case-control study the MPP (Malmö Preventive Project) and the population-based MDC (Malmö Diet and Cancer—Cardiovascular Cohort), were used to investigate the association between plasma DMGV and incident CAD and T2DM. The population-based nature of the MDC enabled additional analysis of associations between DMGV and cardiovascular mortality. The cross-sectional MOS (Malmö Offspring Sudy) was used to investigate whether lifestyle factors correlated with plasma DMGV levels.

The MPP is a population-based prospective cohort of 33 346 individuals, enrolled between 1974 and 1992. Between 2002 and 2006, 18 240 individuals (aged 65–80 years) were re-examined for cardiometabolic risk factors, and overnight-fasting EDTA plasma was collected and stored at −80°C for later analyses. Among a random sample of 5386 individuals from the re-examination, 1406 were excluded because of history of T2DM, CAD, or stroke before re-examination, because of incomplete data on CAD risk factors or because of missing plasma samples. In the remaining 3980 individuals, 384 developed CAD before December 31, 2013 with a mean follow-up time of 7.2 years and 202 developed T2DM with a mean follow-up time of 6.3 years. Controls were defined as individuals that did not develop CAD or T2DM during the follow-up time. Among 3361 individuals, qualified as controls, a random sample of 500 individuals was included in the analyses. Plasma samples for 4 control participants were missing, resulting in a total of 496 controls. This resulted in a case-control cohort of 1049 individuals.

The MDC is a population-based cohort, designed to study the epidemiology of carotid artery disease with participants being enrolled between 1991 and 1996. Among the 5405 participants who came fasted, citrate plasma was obtained from 3799 participants for analysis. The 3799 subjects included are compared with participants with missing plasma samples in Table S1. During an average follow-up time of 18.2, 19.0, and 19.6 years, 402 participants developed T2DM, 423 developed CAD, and 340 died of cardiovascular causes, respectively. MDC participants included in a previous publication (N=220) establishing an association between DMGV and T2DM incidence[5] were excluded (Data S1) from the data analysis of T2DM incidence in sensitivity analyses (Table S2).

The MOS is a population-based cohort study where adult (aged >18 years) children and grandchildren from the MDC study[10] were recruited. Participants were invited by letter and visited the research clinic where overnight-fasting EDTA plasma was collected and anthropometric measurements were performed. The subcohort of 920 MOS participants, that was analyzed, has previously been described in more detail.[11] An overview of the 3 cohorts can be found in Figure S1. The ethics committee of Lund University approved the study protocols for the MOS (DNR 2012/594), MPP, and MDC (DNR

2009/633), and all participants provided written informed consent.

End Point Definitions and Biochemical Measurements

CAD was defined as coronary revascularization, fatal or nonfatal myocardial infarction, or death atributed to ischemic heart disease. The study subjects were followed for incident CAD through record linkage using the Swedish personal identification number with the previously validated Swedish Hospital Discharge Register, the Swedish Cause of Death Register, and the Swedish Coronary Angiography and Angioplasty Registry (SCAAR).[12] T2DM was defined as a fasting plasma glucose of >7.0 mmol/L or a history of physician diagnosis of T2DM or being on antidiabetic medication or having been registered in local or national Swedish diabetes mellitus registries.[13] *International Classification of Diseases, Ninth and Tenth Revision (ICD-9* and *ICD-10)* codes and details about biochemical measurements are found in Data S1.

Genome-Wide Associations Studies

Genome-wide genotyping of participants from the MOS and MPP was conducted using the Illumina GSA Bead Chip (Illumina Inc, San Diego, CA). Genome-wide association studies for DMGV plasma levels were performed using a linear additive genetic model adjusting for age and sex. PLINK v1.9 [14] was used to analyze the MPP whereas the MOS was analyzed using the EMMAX implementation in Efficient and Parallelizable Association Container Toolbox (EPACTS) to handle the nonindependence of family data.

Analytical Procedure

Profiling of plasma metabolites was performed using an ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry system (Agilent Technologies 1290 LC, 6550 MS; Agilent Technologies, Santa Clara, CA) and has previously been described in detail.[15] Briefly, plasma samples stored at −80°C were thawed and extracted by addition of 6 volumes of extraction solution. The extraction solution consisted of 80:20 methanol/water containing stable isotope-labeled ADMA (d6), purchased from Cambridge Isotope Laboratories (Andover, MA). Extracted samples were separated on an Acquity UPLC BEH Amide column (1.7 μm, 2.1×100 mm; Waters Corporation, Milford, MA). Samples were analysed in batches of 90 samples, where quality-control samples were run in the beginning of each batch and every sixth analytical sample, in order to condition the column and capture analytical drift, respectively. Tandem mass spectrometry spectra for DMGV and ADMA were collected by isolating m/z 202.1192 and 203.1503, respectively, and subjecting them to 10, 20, and 40 eV of collision energy. The isolation width was 1.3 m/z.

Metabolite Identification

DMGV was putatively identified by matching the measured mass-over charge ratio (m/z; Data S1) and fragmentation spectra (Figure S2) with the Human Metabolome Database [16] (HMDB0240212) and previously published data of DMGV.[5] Given that a synthetic standard was unavailable, the identity of DMGV was confirmed by conducting a genome-wide association study of DMGV (Table S3 and Figure S3) and matching the genetic associations with previously published data.[5] ADMA was identified by matching retention times and spectra to synthetic ADMA.

Data Processing

ADMA, ADMA-d6, and DMGV peak areas were integrated using Agilent Profinder B.06.00 (Agilent Technologies). Quality-control samples were injected every 6 analytical samples, in order to ensure high analytical repeatability. ADMA was normalized to the internal standard, ADMA-d6, and DMGV was normalized using DMGV measurements in the quality-control samples. First, a low-order nonlinear locally estimated smoothing function was fitted to the DMGV signals in the quality-control samples as a function of the injection order. The α-parameter, reflecting the proportion of samples to be used when constructing the correction curve, was set to 2/3. Using this function, a correction curve for the analytical samples was interpolated, to which the DMGV measurements in the analytical samples were normalized.[17] The normalization was performed in R software (version 3.4.3; R Foundation for Statistical Computing, Vienna, Austria). The variability of measured DMGV in the quality-control samples is presented in Figure S4).

Dietary Intake and Physical Activity Assessment

Dietary intake for participants in the MOS was assessed with a 4-day web-based food record, Riksmaten2010, developed by the Swedish National Food Agency.[18] Energy-adjusted intakes (g/MJ) of vegetables, fruits and berries, whole grain, red meat, fish, dairy, and sugar-sweetened beverages (SSBs) were calculated by dividing intakes with nonalcohol energy intake.

The relative validity of the Riksmaten2010 method was evaluated by comparing the reported energy intake to objectively measured total energy expenditure with the doubly labeled water technique (r=0.40)[19] and by comparing intake of fiber sources with objective plasma biomarkers.[19]

The Pearson correlation coefficients for fruit and vegetable intake were 0.46 and 0.20 in women and men, respectively, and for whole grain intake 0.30 and 0.29.

Physical activity was assessed in the MOS by a question regarding leisure time physical activity. Participants were allocated into 1 of 4 categories according to their level of physical activity (1, light exercise <2 hours per week; 2, light exercise >2 hours per week; 3, moderate exercise, 1–2 times per week; and 4, regular exercise >3 times per week).

Statistical Analyses

Because of skewed distributions, both DMGV and ADMA levels were log transformed before statistical analyses. Correlations between plasma DMGV and traditional CMD risk factors were analyzed using partial Spearman's rho correlations, adjusted for age and sex. Cox proportional hazards models were used to analyze the associations between DMGV and incident CAD and T2DM and cardiovascular mortality in the MDC cohort. The study design of the MPP, where all incident cases of T2DM and CAD were analyzed together with a random selection of healthy controls, implicates that Cox proportional hazards models are invalid, attributable to a higher rate of events compared with the background population. Additionally, the selection of cases does not enable analysis of cardiovascular mortality. Thus, cardiovascular mortality is not investigated in the MPP and logistic regression models are used to investigate incidence of T2DM and CAD. The analyses on metabolite concentrations in relation to incident CAD, T2DM, and cardiovascular mortality were, in the primary model 1, adjusted for age and sex. In subsequent analyses in model 2, the regression models were additionally adjusted for CMD risk factors that were significantly associated with plasma DMGV in the MDC, MPP, and MOS. Associations between quartiles of dietary intake and plasma DMGV were analyzed using linear regression models, where model 1 was adjusted for age and sex and model 2 additionally adjusted for body mass index (BMI), physical activity, and smoking status. The association between level of physical activity and plasma DMGV was analyzed in the same manner. In all regression models, associations were considered significant at $P<0.05$. All statistical analyses were performed using R software (version 3.3.0; R Foundation for Statistical Computing).

Results

The relationship between DMGV and CMD risk factors was investigated in the MPP, MDC, and MOS. Baseline characteristics of the 3 cohorts can be found in Table 1. Strong positive correlations were observed between DMGV levels and BMI and triglycerides, whereas inverse correlations were observed with high-density lipoprotein cholesterol, in all 3 cohorts (Figure 1). DMGV was also correleated with higher prevalence of hypertension and with fasting glucose levels, although these correlations were not equally strong in the 3 cohorts (Table S4). Overall, BMI, hypertension, fasting glucose, triglycerides, and high-density lipoprotein cholesterol were all associated with plasma DMGV and could therefore be considered as DMGV-related CMD risk factors. Moreover, baseline DMGV levels were higher in the 376 participants with baseline diabetes mellitus (odds ratio [OR]=2.45; CI=2.01–2.99; P=6.2e-19) and in the 83 participants with history of CAD (OR=2.29; CI=1.58–3.41; P=3.3e-5), using age- and sex-adjusted logistic regression models.

In the MPP, baseline levels of DMGV were significantly higher in incident cases of T2DM compared with controls (OR=1.65; CI=1.38–1.98; $P<0.001$). After multivariable adjustment, the association between DMGV and incidence of T2DM was attenuated, but remained borderline significant (OR=1.35; CI=0.98–1.53; P=0.07). Next, the association between plasma DMGV and risk of T2DM was tested in a population-based setting in the MDC. Plasma DMGV levels were strongly associated with an increased risk of future T2DM, an association that remained significant after adjustments for DMGV-related CMD risk factors (Table 2).

Given the connection between *AGXT2*-related metabolites and atherosclerosis, we further investigated the relationship between plasma DMGV levels and risk for CAD. Compared with controls, plasma DMGV was significantly higher in participants from the MPP that developed CAD during the follow-up time (OR=1.25; CI=1.08–1.44; P=2.4e-3). However, the association between plasma DMGV and incident CAD was attenuated after adjustments for DMGV-related traditional CMD risk factors (OR=1.09; CI=0.93–1.28; P=0.30). The attenuation observed in the MPP cohort was not driven by any single CMD trait, given that DMGV remained significantly associated with CAD when adding 1 CMD trait at a time on top of the age- and sex-adjusted model (Table S5). Furthermore, the association between plasma DMGV and incident CAD was tested in a population-based setting, in the MDC. Plasma DMGV was associated with an increased risk of future CAD (hazard ratio=1.29; $P<0.001$) in an age- and sex-adjusted Cox proportional hazards model. Adjustments for DMGV-related CMD risk factors clearly attenuated the association, but it remained borderline significant (Table 2). High plasma DMGV was also associated with cardiovascular mortality (Figure 2), an association that remained significant after adjustments for DMGV-related CMD risk factors (Table 2).

Given that ADMA, the substrate of AGXT2-mediated DMGV production, has been described as a CMD risk factor, it is a potential mediator in the connection between DMGV and

Table 1. Characteristics of the Participants in MDC, MPP, and MOS

	MDC			MPP			MOS
Trait	Whole Cohort (N=3799)	Incident T2DM (N=402)	Incident CAD (N=423)	Controls (N=496)	Incident T2DM (N=202)	Incident CAD (N=384)	(N=920)
Age, y	57.7 (±6.0)	57.8 (±5.8)	59.7 (±5.8)	68.7 (±5.9)	69.3 (±5.7)	70.6 (±6.3)	39.3 (±13.5)
Sex (% female)	58.8	55.0	41.8	37.2	31.4	21.3	52.6
BMI, kg/m^2	25.7 (±3.9)	27.4 (±4.6)	26.7 (±4.3)	26.5 (±4.2)	29.2 (±4.8)	27.1 (±4.1)	25.8 (±4.6)
Fasting glucose, mmol/L	5.2 (±1.4)	5.2 (±0.4)	5.8 (±2.2)	5.4 (±0.5)	6.0 (±0.6)	5.5 (±0.6)	5.5 (±0.9)
LDL cholesterol, mmol/L	4.2 (±1.0)	4.3 (±1.0)	4.4 (±1.0)	3.7 (±0.9)	3.7 (±1.0)	3.9 (±1.1)	3.1 (±1.0)
HDL cholesterol, mmol/L	1.4 (±0.4)	1.3 (±0.3)	1.3 (±0.3)	1.4 (±0.4)	1.3 (±0.4)	1.3 (±0.4)	1.6 (±0.5)
Triglycerides, mmol/L	1.3 (±0.6)	1.5 (±0.7)	1.5 (±0.7)	1.2 (±0.6)	1.3 (±0.6)	1.4 (±0.7)	1.1 (±0.6)
Hypertension, %	16.3	22.9	23.6	24.6	43.6	38.9	6.9
Current smokers, %	27.4	28.9	35.4	18.1	22.1	25.7	5.2
Diabetes mellitus, %	4.9	0	11.3	0	0	0	2.9

Plasma metabolomics in MPP was performed in 1049 individuals; 202 developed T2DM, 384 coronary artery disease, and 496 remained free from disease. In the MOS, plasma metabolomics was performed in 920 individuals. In the MDC, plasma metabolomics was performed in 3799 participants, where 402 developed T2DM and 423 developed CAD. Table displays the average of traditional risk factors for cardiometabolic disease in the 3 groups. Numbers in parenthesis indicate the SDs. BMI indicates body mass index; CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MDC, Malm€o Diet and Cancer Study; MMP, Malm€o Preventive Project; MOS, Malm€o Offspring Study; T2DM, type 2 diabetes mellitus.

CMD risk. Circulating levels of ADMA and DMGV were found to be correlated in all 3 cohorts (Table S6), but including ADMA as a covariate in the prediction model of incident T2DM or CAD (Table S7) did not influence the models, supporting a view that these associations were independent from ADMA.

To investigate whether DMGV levels could be modifiable by lifestyle factors, we assessed its relationship with energy-adjusted dietary intake and physical activity in the MOS. Circulating levels of DMGV were lower in participants with higher intake of vegetables (beta=−0.23 [per quartile]; $P<0.001$), fruits and berries (beta=−0.15 [per quartile]; $P<0.001$), whole grain (beta=−0.10 [per quartile]; $P=0.0061$), and fish (beta=−0.07 [per quartile]; $P=0.049$), using age- and sex-adjusted linear regression models. Conversely, higher intake of SSBs (beta=0.38 [per quartile]; $P<0.001$) was significantly associated with increased DMGV levels. There was also a strong negative association between DMGV and self-reported leisure-time physical activity (beta=−0.24 [per physical activity group]; $P<0.001$). Intake of vegetables (beta=−0.17 [per quartile]; $P<0.001$), SSBs (beta=0.14 [per quartile]; $P<0.001$), and fruit and berries (beta=−0.08 [per quartile]; $P=0.028$) were significantly associated with plasma DMGV after additional adjustments for BMI, physical activity, and smoking status (Figure 3). All data on connections between dietary variables and DMGV can be found in Table S8. Physical activity and dietary intake of vegetables and SSBs were all significantly associated with plasma DMGV when included in the same regression model, whereas the association with intake of fruit and berries was attenuated (Table S9). In contrast to DMGV, ADMA did not associate with any dietary intake nor with the level of physical activity (beta=−0.006; $P=0.076$; Table S7).

Discussion

This study shows, for the first time, that increased plasma DMGV levels are associated with incident CAD and cardiovascular mortality. Furthermore, the previously shown association between DMGV and incident T2DM was replicated in 2 independent Swedish cohorts. Last, we discovered novel and strong associations between DMGV levels and dietary intake, showing that individuals with higher DMGV levels had higher intake of SSBs, whereas individuals with lower DMGV had higher intake of vegetables.

Increased Plasma DMGV Is a Novel Risk Factor for CAD and Cardiovascular Mortality

This study provides novel findings of associations between plasma DMGV levels and incident CAD. Although it was clear that much of the association between DMGV and incident CAD was related to traditional CMD risk factors, given that multivariable adjustments clearly attenuated the association in the MPP, increased DMGV levels were independently associated with an increased risk of both future CAD and cardiovascular mortality in the largest investigated cohort, the MDC.

The association between plasma DMGV and incident T2DM has previously been described in a smaller case-control

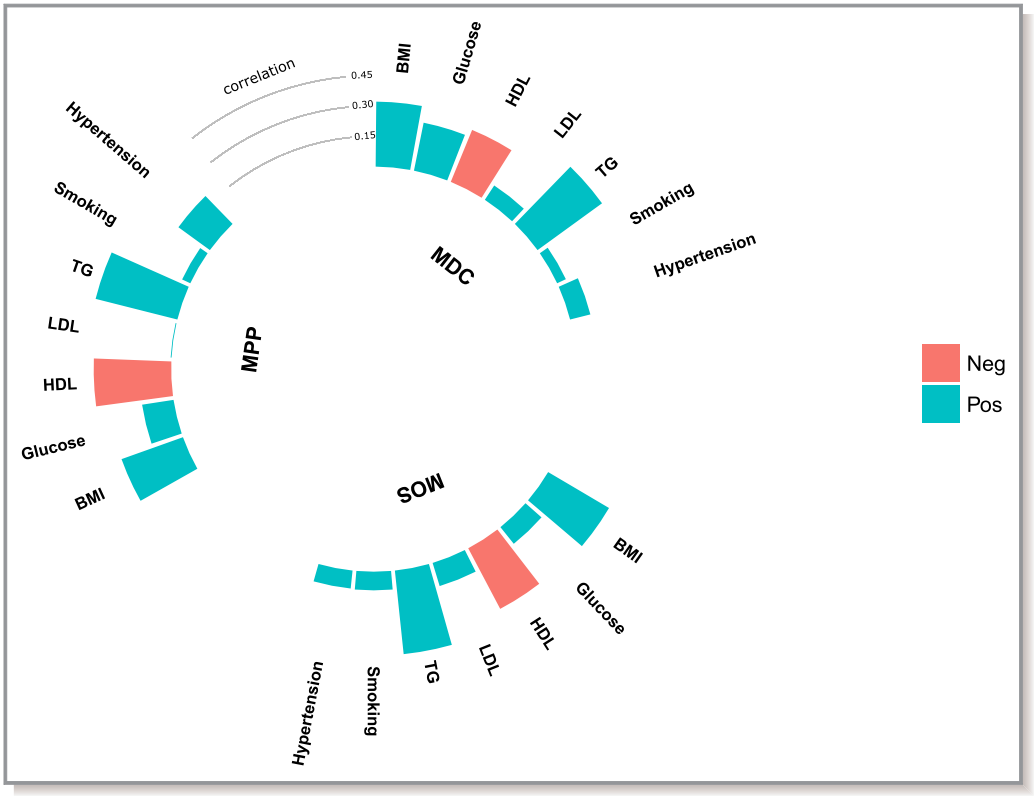

Figure 1. Correlations between plasma dimethylguanidino valerate (DMGV) levels and cardiometabolic risk factors in Malmö Diet and Cancer—Cardiovascular Cohort (MDC; N=3799), Malmö Preventive Project (MPP; N=1049), and Malmö Offspring Study (MOS; N=920). Correlations are Spearman's correlation coefficients. BMI indicates body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Neg, negative; Pos, positive; TG, triglyceride.

study.[5] This study provides an important replication of the previous findings in an independent case-control study and additionally shows that plasma DMGV can be utilized to identify individuals at high risk for future T2DM in a population-based setting. Particularly interesting was that the correlation between DMGV and fasting glucose, a core phenotype of T2DM, was weaker than to obesity and dyslipidemia, indicating that the potential pathophysiological connection with diabetes mellitus development could be more related to a dysmetabolic cardiovascular risk profile rather than with beta-cell dysfunction. This is in line with the established link between DMGV and hepatic steatosis.[5] Notably, DMGV could potentially be seen as a marker for metabolic syndrome, given that it correlates strongly with its different components. This is in agreement with the attenuated assocations between DMGV and incidence of CAD, T2DM, and cardiovascular mortality after adjustment for CMD risk factors. Importantly, these associations remained significant after adjustments, indicating that DMGV is also connected to cardiometabolic disease-related mechanisms

Table 2. Association Between Plasma Dimethylguanidino Valerate Levels and Incidence of T2DM and CAD and Cardiovascular Mortality

	T2DM (N=3423)		CAD (N=3716)		Cardiovascular Mortality (N=3799)	
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2
HR per SD	1.83 (1.63–2.05)	1.29 (1.14–1.46)	1.29 (1.16–1.43)	1.12 (1.00–1.25)	1.28 (1.14–1.45)	1.14 (1.01–1.30)
P Value	8.7e-25	4.9e-5	3.3e-6	0.053	3.2e-5	0.04

Hazard ratios (HR) are expressed per SD increment of plasma dimethylguanidino valerate. Model 1 is adjusted for age and sex. Model 2 is additionally adjusted for BMI, fasting glucose, HDL cholesterol, triglycerides, and hypertension. Analyses are performed in participants from the Malmö Diet and Cancer—Cardiovascular Cohort (N=3799), where participants prevalent T2DM at baseline (N=376) were excluded in the analysis on incident T2DM and participants with prevalent CAD (N=83) where excluded in the analyses on incident CAD. BMI indicates body mass index; CAD, coronary artery disease; HDL, high-density lipoprotein; T2DM, type 2 diabetes mellitus.

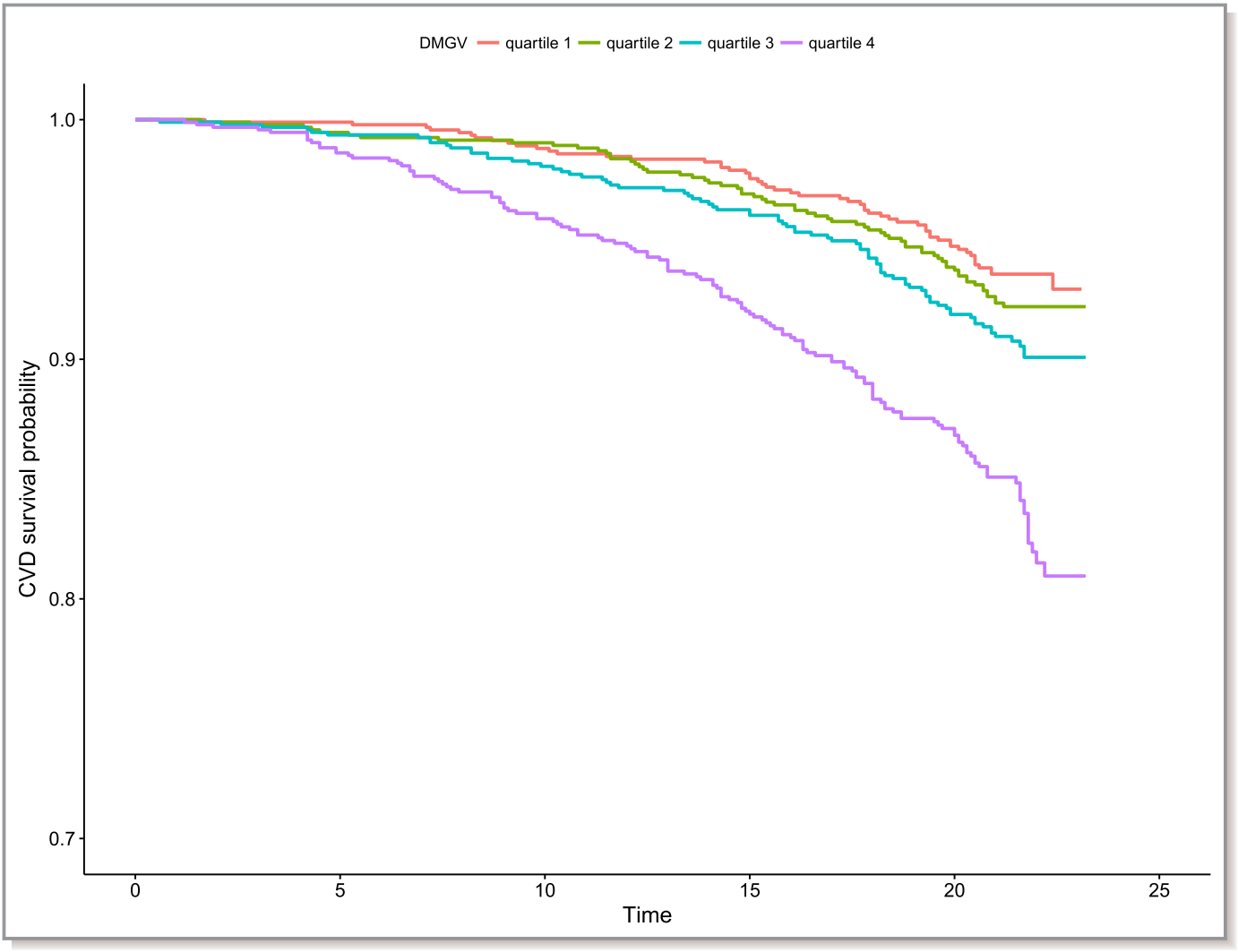

Figure 2. Kaplan-Meier estimates for cardiovascular mortality according to quartiles of plasma DMGV (dimethylguanidino valerate) levels. CVD indicates cardiovascular disease.

that are unrelated to the components of metabolic syndrome. Moreover, the strong connection to overweight and dyslipidemia separates DMGV from its AGXT2 precursor, ADMA, which CMD-related pathways are mainly through hypertension, by decreased nitric oxide production.[7]

Although DMGV is associated with incident T2DM and CAD independently of CMD risk factors, these associations might not be strong enough to result in better risk stratification. Thus, the most important prospect of DMGV in cardiometabolic research lies in understanding the mechanisms connecting it to disease. Regardless of its potential as a predictive biomarker, we have found that dysregulation of DMGV homeostasis reflects an overweight and dyslipidemic state that precedes CMD. This is particularly important given that altered plasma DMGV levels might represent molecular metabolic disturbances contributing to the clustering of T2DM and CAD.

Genetic Variants in AGXT2 Associate With Plasma DMGV Levels

Apart from synthesizing DMGV from ADMA, AGXT2 is known to catalyze the transamination of several substrates in humans, including alanine, beta-aminoisobutyrate (BAIB), and homoarginine.[20,21] Homoarginine,[22,23] BAIB,[23] and particularly ADMA[8] are metabolites that have been associated to CMD or its risk factors, suggesting an important role of AGXT2 in CMD development. *AGXT2* is expressed in liver and kidney,[24] and previous studies have shown that knockout of *AGXT2* in mice depleted DMGV in plasma and kidney, indicating that DMGV is produced exclusively from ADMA by the AGXT2 pathway.[25] This knockout study of *AGXT2* resulted in slightly increased blood pressure,[25] and overexpression of *AGXT2* in endothelial cells has been shown to protect against ADMA-related inhibition of nitric oxide production.[26] In the

Figure 3. Associations between plasma dimethylguanidino valerate (DMGV) levels and dietary intake of vegetables and sugar-sweetened beverages. Boxes represent the first and third quartile of plasma DMGV. Indiviudal measurements are presented as scatterplots, superimposed on the box plots.

present study, increased DMGV primarily correlates with dyslipidemia and obesity and is less strongly correlated with hypertension. This lower correlation could correspond to a countereffect of lower ADMA levels, leading to increased nitric oxide production. Given that it is hard to disentangle whether effects from AGXT2 knockouts arise from raised ADMA levels or lowered DMGV levels, future studies should investigate supplementation of DMGV in animal models in order to refine the effects of DMGV on markers for cardiometabolic health. It is tempting to utilize the relationship between genetic variants in *AGXT2* and plasma DMGV to investigate the possible causal links between DMGV and cardiometabolic outcomes, either by using *AGXT2* as an instrumental variable in a Mendelian randomization study or in *AGXT2* knockout experiments. Such studies are, however, very difficult to interpret because of pleiotropic associations of genetic variants in *AGXT2* and because of the number of different biochemical pathways that could be influenced by *AGXT2* knockout.

We showed here that the association between plasma DMGV and T2DM and CAD is independent of ADMA, but it cannot be excluded that the link between plasma DMGV and CMD could be mediated through other AGXT2-related metabolites. Increased BAIB has been connected with exercise, increased production of leptin, and browning of adipose tissue.[27] Low circulating BAIB, earlier shown to associate with CMD risk factors,[27] could decrease the substrate competition for AGXT2, resulting in increased conversion of ADMA to DMGV. Our data support this theory by establishing a link between physical activity and plasma DMGV levels, suggesting that BAIB production in skeletal muscle could be 1 factor influencing plasma DMGV levels and possibly contributing to the connection with CMD traits.

Lifestyle-Related Determinants of Plasma DMGV Levels

Metabolite levels could be influenced by lifestyle factors, such as dietary intake[28] and physical activity.[29] We found that plasma DMGV levels were related to lifestyle factors such as dietary intake and physical activity. In the MOS, we observed that intake of vegetables as well as self-reported leisure-time physical activity were inversely related to plasma DMGV levels, whereas intake of SSBs was associated with higher DMGV. This indicates the potential to modify plasma DMGV levels by dietary or other lifestyle interventions. Naturally, many factors could drive the associations between plasma DMGV, dietary intake, and physical activity, and our study cannot by any means prove a causal link. Intake of vegetables and SSBs could be considered to be related to overall dietary quality and other lifestyle factors.[30] However, the associations between DMGV and intake of vegetables and SSBs were not markedly attenuated after adjustment for age, sex, physical activity, BMI, and smoking status. Interestingly, ADMA did not significantly associate with either physical activity or with dietary intake, demonstrating that the association between these lifestyle factors and plasma DMGV levels is independent of plasma ADMA levels. The associations with physical activity are in line with a recent publication showing that DMGV levels are lowered in response to a 20-week exercise intervention. The change in DMGV levels tracked closely to improvements in several cardiometabolic traits. Participants with high baseline DMGV levels did, however, show attenuated improvement in these cardiometabolic markers, indicating that these individuals are partially resistant to exercise interventions.[31] Thus, DMGV levels could potentially pinpoint individuals who particularly could benefit from lifestyle modifications and be used as a marker of adherence to such interventions. The association between DMGV and lifestyle factors should motivate further studies that can lead to more-personalized strategies for cardiometabolic disease prevention in the future. Such studies also need to evaluate a potential causal effect of lifestyle factors on DMGV levels and whether DMGV-reducing interventions can be translated into decreased risk for CMD.

Limitations

The present study has several limitations. Levels of DMGV are presented as relative concentrations, given that no isotope-labeled internal standard was used in the mass spectrometry analysis. The different study designs in the MPP and MDC required different regression models to study incidence of T2DM and CAD. This ultimately results in that effect estimates cannot be compared between the studies. Although we have used a validated method for dietary assessement, there may be inaccuracies in the measurements. Moreover, many other lifestyle factors could be analyzed in connection to DMGV, but the present study has focused on diet and physical activity, given that they are cornerstones in prevention and lifestyle treatment of T2DM and CAD. The associations between DMGV and lifestyle factors were performed in the MOS, where participants were younger and more healthy than in the prospective cohorts, the MPP and MDC. This calls for a replication study to confirm that the associations between DMGV and lifestyle factors are valid in populations similar to those where it has been used to predict future CMD. Finally, additional replication is needed in nonwhite populations.

Conclusions

Plasma DMGV levels associate with higher incidence of CAD and T2DM and with cardiovascular mortality. Among the studied lifestyle factors, higher intake of vegetables, lower

intake of SSBs, and a higher level of physical activity were associated with lower plasma DMGV, suggesting the possibility that targeted lifestyle interventions could beneficially affect DMGV-related dysmetabolic traits.

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Author Contributions

Ottosson contributed to study design, acquisition and interpretation of data, statistical analyses, and drafted the manuscript. Ericson, Almgren, and Smith contributed to collection and interpretation of data and statistical analysis. Brunkwall and Hellstrand contributed to collection and interpretation of data. Nilsson and Orho-Melander assisted in data interpretation. Fernandez and Melander contributed to study concept and design, interpretation of data, and statistical analyses. All authors made intellectual contributions to drafting and/or revising the manuscript and approved the final version.

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Disclosures

None.

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Supplemental Material

Data S1.

Endpoint definitions and biochemical measurements

Myocardial infarction was defined on the basis of International Classification of Diseases, ninth revision (ICD-9) code 410 or ICD-10 code I21. Coronary artery bypass surgery was identified from national Swedish classification systems of surgical procedures and defined as procedure codes 3065, 3066, 3068, 3080, 3092, 3105, 3127, or 3158 in the Op6 system; or as procedure code FN in the KKÅ97 system. Percutaneous intervention was identified from SCAAR. Death from cardiovascular causes was defined based on the ICD-9 codes 390-459 and ICD-10 codes C00-D48.

Cigarette smoking was elicited by a self-administered questionnaire, with current cigarette smoking defined as any use within the past year. Measurements of fasting total cholesterol, HDL cholesterol, triglycerides, and glucose were made according to standard procedures at the Department of Clinical Chemistry at Malmö University Hospital. LDL cholesterol was estimated with the Friedewald equation. Hypertension was defined as being on anti-hypertensive medication.

Peak picking

Peak picking was performed by using the Find by Formula algorithm in Agilent MassHunter Profinder B.06.00. The algorithm searched for spectral peaks that match the molecular formulae and retention times for DMGV, ADMA and ADMA-d6. Allowed ion adducts included $(M+H)^+$ and $(M+NH_4)^+$.

Metabolite identifications

DMGV was identified by matching the measured mass-over charge ratio (m/z), fragmentation spectra and genetic associations with previously published data of DMGV [5]. The m/z of the parent ion (202.1194) and product ions (71.0601 and 70.0600) matched with a mass error of 3.9 ppm, 16.0 ppm, and 14.3 ppm respectively (Figure S1). The m/z 202.1194 also matched DMGV in the publically available metabolite database The Human Metabolome Database (HMDB0240212). To support the correctly matched m/z, two genome-wide associations studies (GWAS) were performed on plasma DMGV in MPP (N =1049) and a subset of MOS (N = 639). In MOS, four single nucleotide polymorphisms (SNP) in *AGTX2* showed

genome wide significant associations with DMGV levels (figure 2A). These findings were replicated in MPP, showing that all four SNPs and eight additional SNPs in *AGXT2* were associated with plasma DMGV at a genome-wide significant level (figure 2B). These findings replicate the previous study by O'Sullivan et al., and thus confirm the identity of DMGV.

ADMA was identified by matching the mass-to-charge ratio (m/z) 203.1503 to METLIN and HMDB and by matching the retention time and fragmentation spectra of m/z 203.1503 with synthetic ADMA (Sigma Aldrich, St Louis, MI, USA).

Participants in MDC overlapping with a previous publication

In O'Sullivan et al [5], some participants from the Malmö Diet and Cancer – Cardiovascular Cohort were used to create a case-control study, where the association between baseline DMGV levels and future T2DM was investigated. Those individuals (i.e., 226 participants) were now excluded from the data analysis of T2DM incidence in sensitivity analyses presented in Table S2.

Table S1. Baseline characteristics of participants with analyzed plasma samples in MDC and participants where plasma samples were missing.

Trait	Analyzed participants (N=3799)	Excluded participants (N=1606)
Age (years)	57.7 (±6.0)	57.3 (±5.8)
Sex (% female)	58.8	58.6
BMI (kg/m^2)	25.7 (±3.9)	26.0 (±4.0)
Fasting glucose (mmol/l)	5.2 (±1.4)	5.1 (±1.3)
LDL cholesterol (mmol/l)	4.2 (±1.0)	4.2 (±1.0)
HDL Cholesterol (mM)	1.4 (±0.4)	1.3 (±0.4)
Triglycerides (mM)	1.3 (±0.6)	1.5(±1.1)
Hypertension (%)	16.3	18.3
Current Smokers (%)	27.4	26.8

In MDC, plasma metabolomics was performed in 3799 participants, where citrate plasma samples were available, while the remaining 1606 participants' plasma could not be analyzed. Traditional risk factors are given for both groups above, where standard deviations are given in parentheses.

Table S2. Sensitivity analysis – Association between DMGV and incident T2DM when excluding participants examined in previous publication.

	HR	P
Model 1: Incident T2DM all participants (N=3423)	1.29 (1.15-1.46)	4.9e-5
Model 2: Incident T2DM excluding previously examined participants (N=3203)	1.22 (1.06-1.41)	6.7e-3

HR = Hazard ratio of incident T2DM per standard deviation increment of DMGV. Both models are adjusted for age, sex, BMI, fasting glucose, HDL cholesterol and hypertension. MDC participants included in a prior publication (122 incident T2DM and 98 controls) are excluded in model 2.

Table S3. SNPs significantly associated with plasma dimethylguanidino valerate (DMGV) levels.

SNP	Gene	MPP (N = 1049)		MOS (N = 639)		
		Beta	**P**	**Beta**	**P**	**Beta**
rs37369	AGXT2	-0.46	3.1e-28	-0.55	1.4e-16	-0.55
rs40200	AGXT2	-0.42	3.9e-25	-0.46	1.2e-10	-0.43
rs28305	AGXT2	-0.42	9.3e-25	-0.47	3.4e-11	-0.43
rs468327	AGXT2	-0.26	3.5e-21	-0.28	3.6e-10	-0.27
rs114286107	AGXT2	-0.55	6.4e-11	-0.63	1.6e-6	-0.57
rs3846633	AGXT2	-0.15	5.1e-10	-0.12	1.7e-3	-0.14
rs163841	AGXT2	0.14	2.0e-09	0.20	3.9e-7	0.16
rs185186	AGXT2	-0.16	2.0e-09	-0.14	1.1e-3	-0.15
rs72730595	AGXT2	-0.61	4.0e-09	-0.10	0.54	-0.47
rs16899972	AGXT2	-0.14	1.3e-08	0.13	6.5e-4	-0.13
rs163588	AGXT2	-0.14	4.5e-08	-0.14	3.5e-4	-0.14

SNPs that are genome-wide significantly associated with plasma DMGV levels in Malmö Preventive Project (MPP) and Malmö Offspring Study (MOS).

Table S4. Correlations between dimethylguanidino valerate (DMGV) and cardiometabolic risk factors in the three cohorts studied.

	MDC (N=3799)		MPP (N=1049)		MOS (N=920)	
Risk factor	**Correlation**	**P**	**Correlation**	**P**	**Correlation**	**P**
BMI	0.321	1.25e-84	0.322	1.2e-26	0.349	1.1e-27
Fasting glucose	0.241	1.42e-47	0.157	3.4e-7	0.105	1.4e-3
Fasting HDL cholesterol	-0.277	1.1e-62	-0.383	8.2e-38	-0.338	5.4e-26
Fasting LDL cholesterol	0.084	5.3e-7	0.004	0.89	0.12	2.9e-4
Fasting Triglycerides	0.393	9.2e-130	0.416	4.2e-45	0.415	1.7e-39
Hypertension	0.103	9.0e-10	0.192	3.9e-10	0.089	6.9e-3
Smoking	0.048	0.0037	0.045	0.15	0.092	5.2e-3
Age	0.22	8.8e-44	0.15	8.9e-9	0.18	6.5e-8
Sex	-0.13	4.1e-15	-0.095	2.3e-4	-0.16	8.6e-7

The connection between DMGV and cardiometabolic risk factors are investigated using partial Spearman's correlations, adjusted for age and sex.

Table S5. Attenuation of association between dimethylguanidino valerate (DMGV) and cardiometabolic disease (CMD) end-points in Malmö Preventive Project (N=1049) by adjusting for CMD traits.

	T2DM		CAD	
Trait adjustment	**OR**	**P**	**OR**	**P**
Model 1 + BMI	1.41 (1.17-1.71)	4.7e-4	1.21(1.04-1.41)	0.014
Model 1 + Glucose	1.48 (1.22-1.81)	8.1e-5	1.24(1.07-1.44)	0.0036
Model 1 + TG	1.49 (1.23-1.82)	6.8e-5	1.18(1.01-1.37)	0.040
Model 1 + HDL	1.52 (1.26-1.85)	2.0e-5	1.19(1.02-1.39)	0.026
Model 1 + Hypertension	1.54 (1.29-1.86)	4.3e-6	1.19(1.03-1.38)	0.021

Odds ratios (OR) are expressed as odds ratio of developing T2DM or CAD per standard deviation unit increase of metabolite. 95 % confidence intervals of the OR are reported in parenthesis. Model 1 is a logistic regression, adjusted for age and sex.

Table S6. Correlations between dimethylguanidino valerate and asymmetric dimethylarginine.

Cohort	Correlation	P
MDC	0.09	1.2e-8
MPP	0.14	1.2e-7
MOS	0.19	5.0e-9

Correlation coefficients are spearman's correlations. MDC: Malmö Diet and Cancer – Cardiovascular Cohort (N=3799), MPP: Malmö Preventive Project (N=1049) and MOS: Malmö Offspring Study (N=920).

Table S7. Associations between DMGV and incidence of T2DM and CAD are independent of ADMA.

	MPP		MDC	
	OR	**P**	**HR**	**P**
T2DM	1.64 (1.36-1.98)	1.63 e-7	1.83 (1.63-2.05)	8.7e-25
CAD	1.25 (1.08-1.45)	2.6e-3	1.29 (1.16-1.43)	3.3e-6

Odds ratios (OR) and hazard ratios (HR) are expressed per standard deviation increment of plasma DMGV. Regression models are adjusted for age, sex and plasma ADMA.

Table S8. Associations between dietary intake variables and circulating levels of metabolites (ADMA and DMGV) in MOS (n=920).

Diet group	DMGV model 1		DMGV model 2		ADMA model 1		ADMA model 2	
	Beta	**P**	**Beta**	**P**	**Beta**	**P**	**Beta**	**P**
Vegetables	-0.23	9.8e-11	-0.17	3.7e-7	-0.02	0.58	-0.01	0.78
Sugar-sweetened beverages	0.18	1.8e-7	0.14	7.6e-6	0.04	0.38	0.03	0.45
Fruit and berries	-0.15	4.5e-5	-0.08	0.028	-0.02	0.62	0.00	0.92
Whole grain	-0.1	6.1e-3	-0.01	0.66	0.01	0.70	0.03	0.40
Fish	-0.07	0.049	-0.06	0.16	0.01	0.78	0.02	0.66
Red Meat	-0.01	0.73	-0.06	0.09	0.01	0.86	0.00	0.94
Dairy	0.01	0.87	0.02	0.69	-0.04	0.30	-0.03	0.34

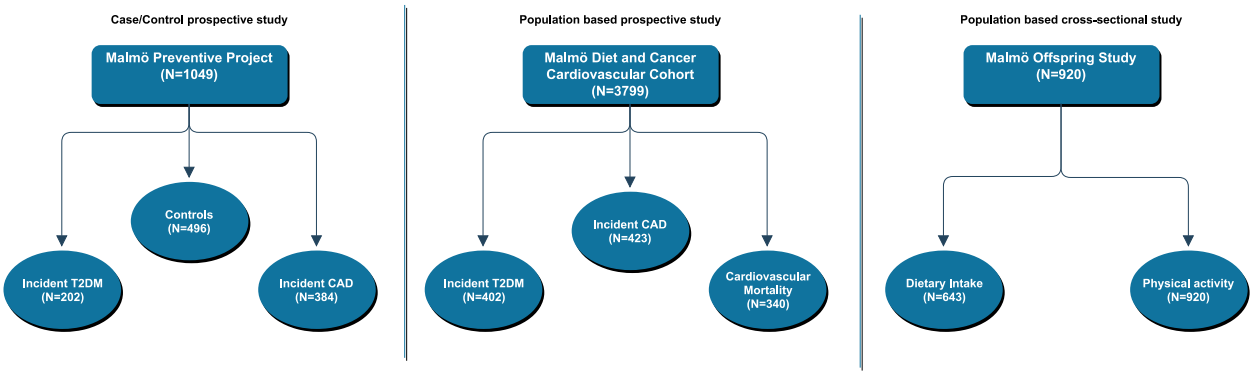
Betas are expressed as standard deviation change of metabolite per quartile increment of dietary intake. The linear regression model 1 is adjusted for age and sex and model 2 for age, sex, BMI, level of physical activity and current smoking status.

Table S9. Life-style factors associating with plasma dimethylguanidino valerate (DMGV) levels in MOS (N=920).

Life style factor	Beta	P
Physical activity	-0.09 (-0.20- -0.05)	9.8e-4
Vegetable intake	-0.14 (-0.20- -0.07)	5.2e-5
SSB intake	0.12 (0.05-0.18)	1.1e-3
Fruit and berries intake	-0.04 (-0.11-0.02)	0.22

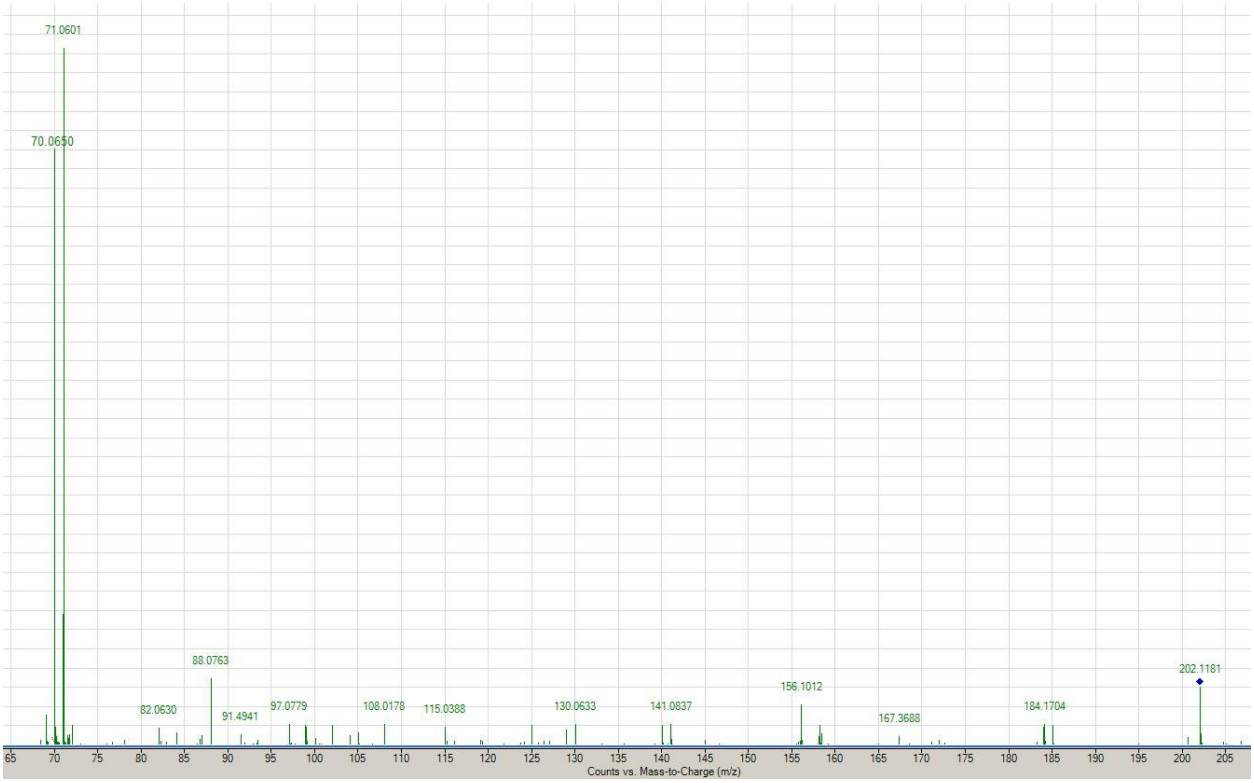
Betas are expressed as standard deviation change of metabolite per quartile increment of dietary intake or physical activity. The linear regression model is adjusted for age, sex, BMI, level of physical activity, current smoking status and dietary intake of vegetables, sugar-sweetened beverages and fruits and berries. 95 % confidence intervals are presented in parentheses.

Figure S1. Overview of the three investigated cohorts.



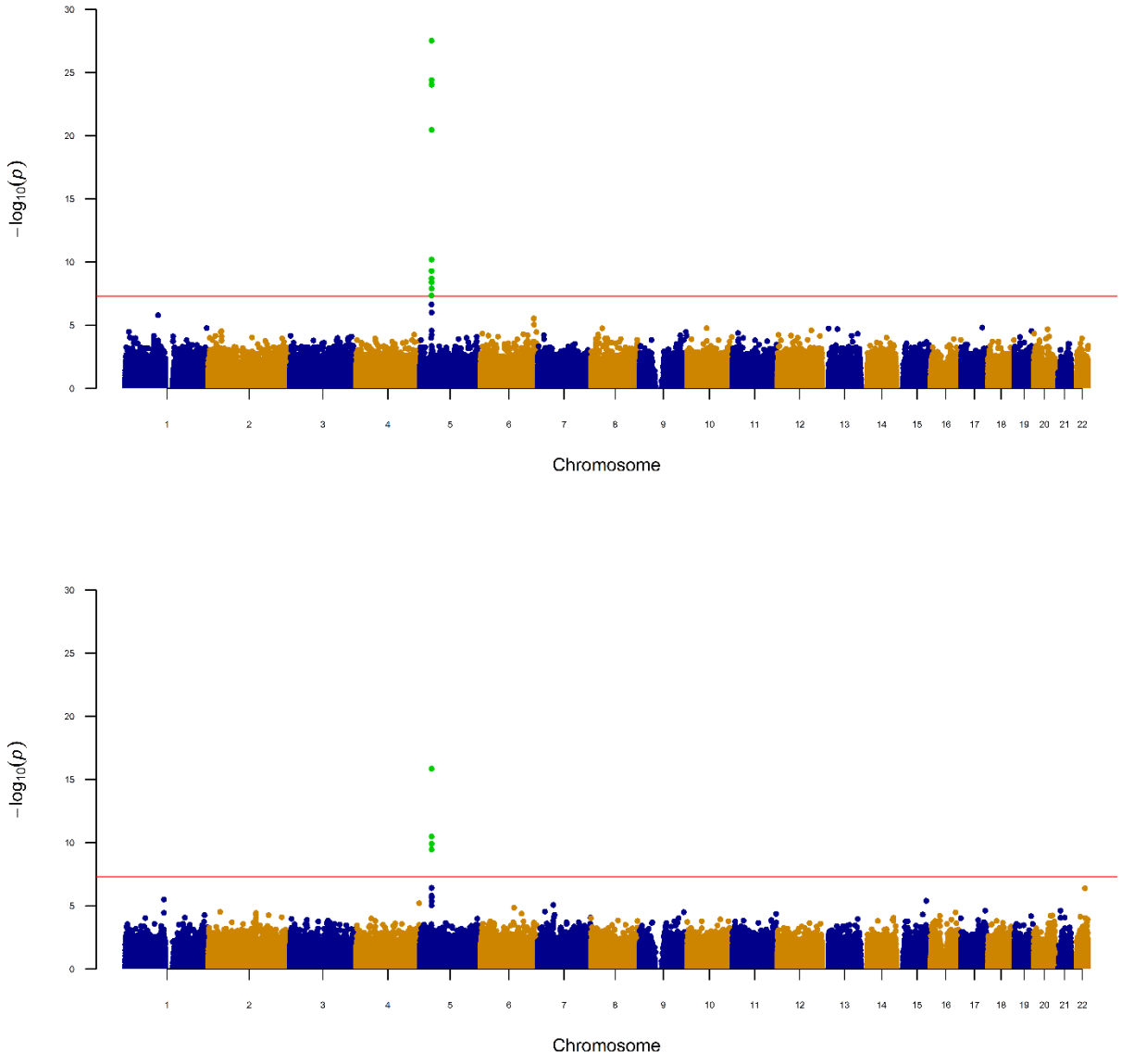
The subcohort in Malmö Preventive Project (MPP) is a prospective case-control study designed to study the baseline levels of metabolites in participants that later develop type 2 diabetes (T2DM) or coronary artery disease (CAD) in comparison to healthy controls. Malmö Diet and Cancer – Cardiovascular Cohort (MDC) is a population based study where all available plasma samples were analyzed in order to study the relation between baseline levels of metabolites and future T2DM, CAD and cardiovascular mortality. In the population based cross-sectional Malmö Offspring Study (MOS), data for dietary intake and physical activity was collected. The relation between plasma levels of metabolites and dietary intake and physical activity was examined.

Figure S2. MS/MS spectrum of m/z 202.1192.



MS/MS fragmentation of 202.1194 was performed at a collision energy of 20V and isolation width of 1.3 m/z generated to major fragments: 71.0601 and 70.0650.

Figure S3. Manhattan plot of the GWAS of DMGV in Malmö Offspring Study and Malmö Preventive Project.



Genetic variants associating with plasma dimethylguanidino valerate (DMGV) levels. Genome-wide significant SNPs (P<5e-8) are denoted as green. Above = Malmö Preventive Project (MPP), Below = Malmö Offspring Study (MOS).

Figure S4. Variability of DMGV measurements in quality control samples analyzed in the Malmö Diet and Cancer – Cardiovascular Cohort (MDC) and Malmö Preventive Project (MPP).

Coefficients of variation (CV) are presented in the figure.