Estimated GFR Is Biased by Non-Traditional Cardiovascular Risk Factors

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Key Words

GFR estimation · L-arginine · Dimethylarginines ·
Insulin resistance · Cardiovascular disease risk factors ·
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Abstract

Background: Estimated glomerular filtration rate (eGFR) based on either cystatin C or creatinine performs similarly in estimating measured GFR, but associate differently with cardiovascular disease (CVD) and mortality. This could be due to confounding by non-GFR-related traits associated with cystatin C and creatinine levels. We investigated non-GFR-related associations between eGFR and two types of nontraditional risk factors for CVD and death: L-arginine/dimethylarginine metabolism and insulin resistance. **Methods:** GFR was measured via iothexol clearance in a cross-sectional study of 1,624 middle-aged persons from the general population without CVD, diabetes or chronic kidney disease. The dimethylarginines were measured using liquid chromatography tandem mass spectrometry (LC-MSMS). Insulin resistance was determined by the homeostasis model assessment (HOMA-IR). **Results:** Asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), the L-arginine/ADMA ratio and insulin resistance were associated with creatinine-based eGFR after accounting for measured GFR in

multivariable adjusted analyses. The cystatin C-based eGFR showed a similar residual association with SDMA; an oppositely directed, borderline significant association with ADMA; and a stronger residual association with insulin resistance compared with eGFR based on creatinine. **Conclusion:** Both creatinine- and cystatin C-based eGFR are influenced by nontraditional risk factors, which may bias risk prediction by eGFR in longitudinal studies.

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Introduction

An estimated glomerular filtration rate (eGFR) below 60 ml/min/1.73 m² is an important risk factor for end-stage renal disease, cardiovascular disease (CVD) and death. Epidemiological studies have found that eGFR measurements based on cystatin C (eGFR_{cys}) show a stronger association with renal and CVD outcomes than GFR estimated according to the creatinine level (eGFR_{cre}) [1, 2]. Because there is little evidence indicating that cystatin C is more effective for estimating GFR compared to creatinine, this superior risk prediction by eGFR_{cys} may be caused by the confounding of non-GFR determinants of cystatin C and creatinine [3, 4]. Several studies have shown eGFR to be influenced by traditional

CVD risk factors independently of the measured GFR (mGFR). In particular, cystatin C seems to be associated with obesity, smoking, HDL-cholesterol, triglycerides, hypertension and C-reactive protein (CRP) [5–7].

These findings indicate that the estimated risk associated with reduced eGFR in longitudinal studies may be confounded, particularly when eGFR_{cys} is used. However, because most epidemiological studies reduce confounding by adjusting their survival analysis for these traditional CVD risk factors, this may not explain the large difference between eGFR_{cys} and eGFR_{cre} risk estimates. Novel CVD risk factors are more difficult to measure in population studies and therefore not commonly used for adjustment in survival analysis. Accordingly, confounding by these risk factors may contribute to different risk predictions generated according to creatinine- and cystatin C-based eGFR.

In this study, we investigated the association between eGFR based on the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equations [4] and two types of variables representing nontraditional CVD risk factors: arginine/dimethylarginine metabolism and insulin resistance. Asymmetric dimethylarginine (ADMA), its isomer symmetric dimethylarginine (SDMA), and the L-arginine/ADMA ratio have recently emerged as strong and independent risk factors for CVD and death, both in the general population and in high-risk patients [8–13]. Insulin resistance (IR) also predicts CVD and death in community cohorts [14–18], and IR and dimethylarginines are hypothesized to interact in a vicious cycle leading to endothelial dysfunction and vascular disease [19–22].

In the Renal Iohexol Clearance Survey in Tromsø 6 (RENIS-T6), we measured GFR by iohexol clearance as well as the levels of L-arginine, ADMA, SDMA and IR in a middle-aged cohort from the general population. Using a cross-sectional design, we aimed to explore whether these novel risk factors are determinants of eGFR, after accounting for mGFR. In addition, we sought to study the relationship between mGFR and dimethylarginines. The relationship between mGFR and IR has been reported previously [23].

Subjects and Methods

Participants

The RENIS-T6 is a substudy of the sixth population-based Tromsø study (Tromsø 6). Tromsø 6 included an age-stratified representative sample of 12,984 inhabitants of the municipality of Tromsø in Northern Norway [24]. Forty percent of all inhabitants between 50 and 59 years of age and all inhabitants between 60 and

62 years of age were invited to participate in Tromsø 6. In these age groups, 3,564 subjects (65%) completed the main part of the Tromsø 6 survey; of these, we excluded 739 who reported previous myocardial infarction, angina pectoris, stroke, diabetes mellitus or renal disease (fig. 1). The remaining 2,825 individuals were invited to participate in RENIS-T6, and 2,107 (75%) responded positively. A detailed description of the study participants and methods of the RENIS-T6 has been published elsewhere [25]. Briefly, 77 individuals were excluded because of allergies to contrast media and 48 individuals did not appear at their appointments. Among the remaining 1,982 individuals, we included 1,632 individuals according to a predetermined target. Five participants were excluded because of technical failure in the GFR measurements, leaving 1,627 persons in the RENIS-T6 cohort (fig. 1). The characteristics of the RENIS-T6 cohort were comparable to the total group of eligible subjects (n = 2,825) [26]. In the present investigation, we excluded 3 individuals because of methylarginine measurement failure.

The study adhered to the Declaration of Helsinki and was approved by the Regional Ethics Committee of Northern Norway. All participants provided informed written consent.

Measurements

All study participants met between 8:00 and 10:00 a.m. in the Clinical Research Unit at the University Hospital of Northern Norway after an overnight fast. Blood pressure and blood samples were obtained after the participants had been resting for at least 5 min. Serum samples for glucose, creatinine, triglycerides and cholesterol were measured the same day. Serum samples used for measuring the levels of ADMA, SDMA, L-arginine and insulin were stored at –80°C and thawed at the time of analysis.

Additional information about CVD risk factors was obtained using a questionnaire. Smoking status was divided into current smokers and non-smokers. A family history of early myocardial infarction was defined as a first-degree relative with myocardial infarction before the age of 60.

Iohexol Clearance

GFR was measured as the single-sample plasma clearance of iohexol, as previously described in detail [25]. This method has been validated against gold standard methods [27, 28]. Briefly, 5 ml of iohexol (Omnipaque, 300 mg I/ml, Amersham Health, London, UK) was injected intravenously. The exact time for measuring the iohexol concentration was calculated with Jacobsson's method based on the eGFR_{cre} [29]. The iohexol concentration was measured using high-performance liquid chromatography. The between-run coefficient of variation (CV) during the study period was 3.0%. GFR was calculated using the formulas described by Jacobsson [29].

L-Arginine, ADMA and SDMA Measurement

ADMA, SDMA and L-arginine were analyzed by LC-MS/MS using the Waters Acquity™ UPLC system with an auto sampler and a binary solvent delivery system (Waters, Milford, Mass., USA) interfaced to the Waters Xevo TQ-S benchtop tandem quadrupole mass spectrometer (Waters, Manchester, UK). Chromatography was performed on a 1.7 µm, 2.1 × 100 mm Waters Acquity UPLC BEH Amide column maintained at 50°C. The column was eluted isocratically using 10 mM ammonium formate with 0.1% formic acid in water-acetonitrile at 0.5 ml/min. Reference standards for ADMA, SDMA and L-arginine were purchased from Sig-

Fig. 1. Flowchart of the study population.

ma-Aldrich (St. Louis, Mo., USA). The stable isotope-labeled internal standards D₇-ADMA hydrochloride and L-arginine-d₇ hydrochloride were obtained from Cambridge Isotope Laboratories (Andover, Mass., USA). All other chemicals were of an analytical grade or better.

Standard samples, quality control samples and 50 µl of unknown serum samples were mixed with 450 µl of the internal standard/precipitation solution in a Waters 96-well PP sample collection plate (Waters, Milford, Mass., USA). The plate was sealed and placed at 4°C for 30 min before centrifugation at 300 × g for 10 min. The plate was then put back on the liquid handler, and 50 µl of the clear supernatant was transferred to a second Waters 96-well collection plate and diluted with 200 µl water, sealed and placed in the UPLC autosampler for analysis.

The between-day CV was <8% for ADMA, SDMA and L-arginine at three different days. Intraday CV were all <5%. The limit

of quantification for ADMA and SDMA was 0.01 µM, and that for L-arginine was 0.1 µM. The linear dynamic range ($r^2 > 0.99$) for ADMA and SDMA was 0.01–10 µM, and that for L-arginine was 0.2–320 µM.

Other Measurements

We analyzed plasma creatinine using a standardized enzymatic assay (CREA Plus, Roche Diagnostics, GmbH, Mannheim, Germany). External quality assessment was provided by Labquality, Helsinki, Finland. The inter-assay CV in the study period was 2.3%. Cystatin C was measured with a particle-enhanced turbidimetric immunoassay using reagents from Gentian (Gentian, Moss, Norway) and a Modular E analyzer (Roche Diagnostics). The inter-assay CV during the study was 3.1%. External quality control was provided by Equalis (www.equalis.se). In 2013, we reanalyzed 300 randomly selected samples with the same assay using a Cobas

8000 (Roche Diagnostics) and calibrated the cystatin C measurements to the international reference standard [30] (online suppl. Appendix 1; for all online suppl. material, see www.karger.com/doi/10.1159/000371557).

The insulin serum samples were thawed and measured with an ELISA kit (DRG instruments, Marburg, Germany). The intra- and inter-assay CVs were 4.7 and 6.3%, respectively. Insulin resistance was expressed using the HOMA-IR, which was calculated by multiplying the fasting glucose (mmol/l) by the fasting insulin (mU/l) divided by 22.5 [31].

Three samples of first-void morning spot urine were collected on separate days. Urinary albumin and creatinine were measured with commercial kits as previously described [32]. ACR was calculated for each urine specimen, and the mean ACR value was used in the analyses.

Ambulatory blood pressure (ABP) was measured with a Spacelab 90207 device (Redmond, WA, USA) at 20-min intervals from 08:00 to 22:00. The daytime mean systolic and diastolic ABPs were calculated as the weighted mean of the measurements from 10.00 to 20.00. Details of the ABP measurements have been published elsewhere [33].

Statistical Analysis

Means (SD), or median (IQR) values in cases of skewed data, were calculated for baseline characteristics and presented for men and women separately. For 19 individuals with missing data on ambulatory diastolic blood pressure (DBP), we used values obtained from in-office DBP measurements.

Bias and precision of the estimating equations were calculated as the median and interquartile range of eGFR–mGFR, respectively. Accuracy was calculated as the percentage of eGFR values within 30% of the mGFR.

Multiple linear regression analyses were used to explore the association between mGFR and eGFR as dependent variables and L-arginine, the dimethylarginines, L-arginine/ADMA ratio, insulin and HOMA-IR as independent variables. We adjusted for the following known determinants of GFR or factors that influence GFR estimation: age, gender, use of angiotensin converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARB), BMI, DBP, HDL- and LDL- cholesterol, triglycerides, fasting glucose, daily smoking (Y/N), ACR, and having a first-degree relative with myocardial infarction at <60 years of age.

Because the statistical method for analyzing non-GFR-related determinants used in previous studies has been questioned [7], we used a multivariable approach with general estimating equations (GEE) to assess the residual associations between L-arginine, dimethylarginines, insulin, the HOMA-IR and eGFR after accounting for mGFR. This statistical method has been described by Rule et al. [7]. In these analyses, mGFR, eGFRcre, eGFRcys and eGFRcrecys were regressed simultaneously on each independent variable after adjustment for the same variables as described earlier. To detect a significant deviation of the risk factor's association with eGFR compared to mGFR, we analyzed the interaction between each independent variable and the eGFR method relative to mGFR. In these analyses, we also adjusted for the interaction between eGFR method and all other independent variables. A statistically significant interaction was regarded as a non-GFR-related association with eGFR. Finally, we used the same method as described earlier to reanalyze the non-GFR-related associations between traditional CVD factors and eGFR previously reported from the RENIS-cohort [6].

Table 1. Study population characteristics

	Women (n = 823)	Men (n = 801)
Age, years	58.1 (3.9)	58.0 (3.8)
Body mass index, kg/m ²	26.8 (4.4)	27.8 (3.5)
Systolic blood pressure, mm Hg	125.4 (17.3)	134.0 (16.8)
Diastolic blood pressure, mm Hg	80.8 (9.3)	86.2 (9.5)
HDL-cholesterol, mmol/l	1.67 (0.41)	1.39 (0.38)
LDL-cholesterol, mmol/l	3.63 (0.87)	3.71 (0.84)
Fasting glucose, mmol/l	5.20 (0.51)	5.51 (0.55)
Daily smoking (y/n), %	23	19
Triglycerides, mmol/l	1.0 (0.7–1.3)	1.1 (0.8–1.6)
Albumin/creatinine ratio, mg/mmol	0.37 (0.22–0.65)	0.29 (0.15–0.53)
L-arginine, μmol/l	92.4 (17.0)	95.1 (16.6)
ADMA, μmol/l	0.43 (0.06)	0.43 (0.06)
SDMA, μmol/l	0.61 (0.10)	0.64 (0.10)
Arginine to ADMA ratio	215.4 (37.9)	225.4 (40.2)
Fasting Insulin, μU/ml	7.8 (5.5–11.1)	9.5 (6.6–13.2)
HOMA-IR, index	1.8 (1.2–2.6)	2.3 (1.6–3.3)
Measured GFR, ml/min/1.73 m ²	87.8 (14.0)	95.7 (13.7)
GFR estimated by CKD-EPI equations		
eGFRcre, ml/min/1.73 m ²	94.4 (10.0)	95.3 (9.0)
eGFRcys, ml/min/1.73 m ²	102.2 (12.1)	108.6 (11.7)
eGFRcrecys, ml/min/1.73 m ²	101.4 (11.9)	104.6 (10.7)

The statistical analyses were run using STATA 12 (StataCorp, College Station, Tex., USA), and statistical significance was defined as $p < 0.05$.

Results

The RENIS-T6 cohort included 1627 persons, aged 50–62, without self-reported diabetes, CVD, or kidney disease (fig. 1). Study population characteristics are shown in table 1.

The bias (precision), calculated as the median and interquartile range of eGFR–mGFR, was 14.0 (16.1) ml/min/1.73 m² for eGFRcys and 11.4 (13.7) ml/min/1.73 m² for eGFRcrecys. The percentage of eGFR values within 30% of the mGFR (accuracy) was 84% for eGFRcys and 91% for eGFRcrecys. Bias (precision) and accuracy for eGFRcre in the RENIS-T6 cohort were 2.9 (15.4) and 95%, as previously published [25].

Multiple linear regression analyses with mGFR or eGFR as the dependent variable and L-arginine, ADMA, SDMA, fasting insulin and the homeostasis model assessment of IR (HOMA-IR) as the independent variables are shown in table 2. In analyses with multivariable adjustment, increasing ADMA levels were associated with lower mGFR, eGFRcys, GFRcre and eGFRcrecys, but the as-

Table 2. Multiple linear regression analyses of GFR by different method, and L-arginine, methylarginines, insulin and HOMA-IR

Independent variable	Model 1: Adjusted for age, gender and use of ACE-i or ARB			Model 2: As model 1 and multivariable adjustment ^a		
	estimate ml/min/1.73 m ²	95% CI	p	estimate ml/min/1.73 m ²	95% CI	p
Measured GFR						
L-arginine per SD increase	0.33	-0.33 to 0.99	0.33	0.14	-0.53 to 0.81	0.68
ADMA per SD increase	-2.24	-2.91 to -1.58	<0.001	-2.31	-3.00 to -1.66	<0.001
L-arginin to ADMA ratio per SD	1.95	1.28 to 2.61	<0.001	1.86	1.19 to 2.54	<0.001
SDMA per SD increase	-6.72	-7.31 to -6.13	<0.001	6.67 ^b	-7.27 to -6.07	<0.001
Insulin per SD increase	0.28	-0.39 to 0.96	0.41	0.66	-0.70 to 0.97	0.12
HOMA-IR per SD increase	0.58	-0.10 to 1.26	0.10	1.05	0.23 to 1.87	0.01
eGFRcre						
L-arginine per SD increase	-0.32	-0.76 to 0.12	0.16	-0.48	-0.93 to -0.03	0.04
ADMA per SD increase	-0.67	-0.79 to -0.55	<0.001	-0.76	-1.21 to -0.31	0.001
L-arginin to ADMA ratio per SD	0.14	-0.31 to 0.59	0.53	0.07	-0.39 to 0.52	0.77
SDMA per SD increase	-4.47	-4.87 to -4.08	<0.001	-4.57	-4.96 to -4.17	<0.001
Insulin per SD increase	-0.21	-0.67 to 0.24	0.36	0.06	-0.50 to 0.62	0.85
HOMA-IR per SD increase	-0.14	-0.60 to 0.31	0.52	0.25	-0.30 to 0.80	0.37
eGFRcys						
L-arginine per SD increase	-0.70	-1.25 to -0.15	0.01	-0.26	-0.80 to 0.28	0.34
ADMA per SD increase	-3.00	-3.54 to -2.46	<0.001	-2.87	-3.39 to -2.35	<0.001
L-arginin to ADMA ratio per SD	1.44	0.89 to 2.00	<0.001	1.86	1.32 to 2.40	<0.001
SDMA per SD increase	-4.77	-5.28 to -4.26	<0.001	-5.34	-5.82 to -4.86	<0.001
Insulin per SD increase	-1.75	-2.32 to -1.19	<0.001	-0.86	-1.53 to -0.19	0.01
HOMA-IR per SD increase	-1.57	-2.14 to -1.01	<0.001	-0.29	-0.94 to 0.37	0.39
eGFRcrecys						
L-arginine per SD increase	-0.64	-1.17 to -0.11	0.02	-0.46	-0.99 to 0.07	0.09
ADMA per SD increase	-2.29	-2.81 to -1.77	<0.001	-2.26	-2.77 to -1.74	<0.001
L-arginin to ADMA ratio per SD	0.98	0.45 to 1.51	<0.001	1.20	0.67 to 1.73	<0.001
SDMA per SD increase	-5.62	-6.08 to -5.17	<0.001	-6.04	-6.48 to -5.59	<0.001
Insulin per SD increase	-1.26	-1.79 to -0.72	<0.001	-0.55	-1.21 to 0.11	0.10
HOMA-IR per SD increase	-1.10	-1.64 to -0.57	<0.001	-0.07	-0.71 to 0.58	0.84

^a Additional adjusted for body mass index, diastolic blood pressure, HDL-cholesterol, LDL-cholesterol, triglycerides, fasting glucose, daily smoking (Y/N), albumin/creatinine ratio and having a first degree relative with myocardial infarction <60 years (HOMA-IR not adjusted for fasting glucose).

^b Interaction with age, $p = 0.02$. Estimates for the lower, middle and upper age tertiles: -7.7 ($p < 0.001$), -6.5 ($p < 0.001$) and -6.2 ($p < 0.001$).

sociation with eGFRcre was small. A higher L-arginine/ADMA ratio was associated with higher mGFR, eGFRcys and eGFRcrecys, but not eGFRcre (table 2). SDMA was inversely related to GFR using all methods, with the strongest association identified for mGFR and eGFRcrecys. There were no interactions with gender or age, except for a small significant interaction between mGFR and SDMA for age ($p = 0.02$) and eGFRcrecys and SDMA for gender ($p = 0.03$) (table 2). Plots of residuals versus predicted values for all regression analysis were inspected and heteroscedasticity was not detected.

We then assessed the residual associations between the independent variables and eGFR after accounting for mGFR using generalized estimating equations (table 3). Variables representing arginine/dimethylarginine metabolism were significantly associated with eGFRcre after accounting for mGFR in a model adjusted for age, sex and use of ACEi or ARB (table 2) and in the fully adjusted model (table 3). There was also a small residual association between HOMA-IR and eGFRcre in the fully adjusted model. eGFRcys showed a residual association with SDMA, a borderline significant association with ADMA

Table 3. Generalized estimating equations showing residual associations between risk factors and eGFR after accounting for mGFR^a

Independent variable	Model 1: adjusted for age, gender and use of ACEi or ARBs			Model 2: as model 1 and multivariable adjusted ^d		
	estimate ^b (ml/min/1.73 m ²)	95% CI	p	estimate ^b (ml/min/1.73 m ²)	95% CI	p ^c
eGFRcre						
L-arginine per SD increase	-0.65	-1.20 to -0.09	0.02	-0.62	-1.17 to -0.07	0.03
ADMA per SD increase	1.57	1.02 to 2.12	<0.001	1.56	1.00 to 2.11	<0.001
L-arginine to ADMA ratio per SD increase	-1.80	-2.38 to -1.23	<0.001	-1.79	-2.37 to -1.22	<0.001
SDMA per SD increase	2.25	1.71 to 2.79	<0.001	2.10	1.55 to 2.66	<0.001
Insulin per SD increase	-0.50	-1.10 to 0.10	0.10	-0.09	-0.84 to 0.67	0.82
HOMA-IR per SD increase	-0.73	-1.34 to -0.11	0.02	-0.80	-1.57 to -0.04	0.04
eGFRcys						
L-arginine per SD increase	-1.03	-1.61 to -0.45	<0.001	-0.40	-0.94 to 0.13	0.14
ADMA per SD increase	-0.75	-1.35 to -0.15	0.01	-0.55	-1.12 to 0.01	0.06
L-arginine to ADMA ratio per SD increase	-0.51	-1.09 to 0.08	0.09	0.00	-0.55 to 0.54	0.99
SDMA per SD increase	1.95	1.34 to 2.56	<0.001	1.33	0.76 to 1.89	<0.001
Insulin per SD increase	-2.05	-2.70 to -1.41	<0.001	-1.01	-1.78 to -0.24	0.01
HOMA-IR per SD increase	-2.17	-2.81 to -1.52	<0.001	-1.35	-2.11 to -0.59	<0.001
eGFRcrecys						
L-arginine per SD increase	-0.97	-1.48 to -0.46	<0.001	-0.60	-1.09 to -0.11	0.02
ADMA per SD increase	-0.04	-0.56 to 0.48	0.87	0.06	-0.44 to 0.56	0.82
L-arginine to ADMA ratio per SD increase	-0.96	-1.49 to -0.44	<0.001	-0.67	-1.17 to -0.16	0.01
SDMA per SD increase	1.10	0.59 to 1.61	<0.001	0.63	0.13 to 1.13	0.01
Insulin per SD increase	-1.55	-2.12 to -0.98	<0.001	-0.69	-1.40 to 0.01	0.05
HOMA-IR per SD increase	-1.69	-2.27 to -1.11	<0.001	-1.12	-1.83 to -0.42	<0.001

^a Generalized estimating equations with eGFR and mGFR as stacked dependent variables regressed on each independent variable to compare the difference in eGFR and mGFR regression coefficients.

^b Difference between eGFR and mGFR estimates.

^c Statistical significance determined by the statistical interaction between each risk factor and eGFR relative to mGFR.

^d Additionally adjusted for body mass index, diastolic blood pressure, HDL-cholesterol, LDL-cholesterol, triglycerides, fasting glucose, daily smoking (Y/N), albumin/creatinine ratio and having a first degree relative with MI <60 years (HOMA-IR not adjusted for fasting glucose).

in the opposite direction to eGFRcre, and no residual association with the L-arginine/ADMA ratio. However, eGFRcys was significantly associated with both insulin and HOMA-IR (table 3).

The combined eGFRcrecys estimating equation was residually associated with variables representing arginine/dimethylarginine metabolism and HOMA-IR, although the estimates were generally small in magnitude (table 3).

There were no interactions between age or gender, on the residual association between the independent variables and eGFR. Additional adjustment for the use of other antihypertensive medications did not influence the regression estimates.

Among the traditional CVD risk factors, BMI, HDL-cholesterol, triglycerides, fasting glucose, ACR and cur-

rent smoking were residually associated with eGFRcys, whereas only fasting glucose was associated with eGFRcre (online suppl. table 1).

Discussion

We found that dimethylarginines and insulin resistance differed significantly in their associations with eGFR compared to mGFR, indicating non-GFR-related associations between these novel CVD risk factors and eGFR. eGFRcre was influenced by variables representing arginine/dimethylarginine metabolism, while eGFRcys was influenced by SDMA and insulin resistance, even after multivariable adjustment for traditional CVD risk factors. Both arginine/dimethylarginine metabolism and in-

sulin resistance influenced eGFRcrecys, although the associations were weaker.

Non-GFR-related determinants of eGFR, and particularly eGFRcys, have been reported in previous studies [5–7, 34]. In a pooled dataset consisting of patients with CKD, Stevens et al. found that several traditional CVD risk factors influenced cystatin C levels after adjusting for the mGFR according to Cr-EDTA clearance. In contrast, only small non-GFR-related associations were found for creatinine [5]. However, these associations were not analyzed in multiple regression and were adjusted only for age, sex and race. In a previous publication from the RENIS-cohort, Mathisen et al. found that eGFRcre was influenced by smoking, while eGFRcys was influenced by BMI, HDL-cholesterol, triglycerides and smoking after multivariable adjustment [6]. In addition, the residual association between smoking and eGFRcys was large and in the opposite direction compared to the association with eGFRcre.

The statistical method used by Mathisen et al. and other previous studies of non-GFR-related effects, have been questioned [7]. We therefore reanalyzed the non-GFR related associations between eGFR and traditional CVD factors in the RENIS-cohort, and found approximately similar non-GFR related associations as reported by Mathisen et al. The magnitudes of these estimates were comparable to those with IR and dimethylarginines in the current study (online suppl. table 1).

In line with the study of Mathisen, Rule et al. found that eGFRcys was more influenced by traditional CVD risk factors compared to eGFRcre. In the multivariable adjusted model using GEE Rule et al. found BMI, hypertension, and CRP to be determinants of eGFRcys, while only urinary creatinine excretion influenced eGFRcre along the non-GFR-related pathway [7].

In accordance with these data, the authors have argued that eGFRcre is a better proxy for mGFR and provides less biased outcomes compared to eGFRcys or eGFRcrecys [5–7]. However, our findings challenge the general validity of this conclusion. In particular, the dependency of eGFRcre on arginine/dimethylarginine metabolism indicates that a confounding relationship exists also between eGFRcre and the risk of CVD and death. Neither these metabolites nor insulin resistance is usually measured in longitudinal studies of eGFR; accordingly, they cannot be adjusted for to reduce bias in studies of eGFR, CVD and death.

It is well known that eGFRcre may be falsely high due to reduced muscle mass during chronic disease, which may explain the association between high eGFRcre and

increased mortality in several studies [1, 28]. The non-GFR-related effect of ADMA and the L-arginine/ADMA ratio found in the current study may bias risk predictions caused by changes in eGFRcre levels in the same direction as those observed in reduced muscle mass (increased risk of high eGFR and attenuated risk of low eGFR). ADMA inhibits nitric oxide (NO) production from L-arginine, causes endothelial dysfunction, and has been shown to predict CKD progression as well as CVD [35, 36]. In the present study, a non-GFR-related effect of 1.5 ml/min/1.73 m² in eGFRcre was found per one SD increase in ADMA. This result corresponds to an adjusted hazard ratio of 1.3 for mortality in non-diabetic subjects in the Framingham offspring study, indicating a considerable increase in risk associated with a small difference in eGFRcre [8]. Moreover, the non-GFR-related associations of eGFRcre and eGFRcys were opposite for ADMA, which may contribute to the different risk-estimates associated with eGFRcre vs. eGFRcys [2].

We found that eGFRcrecys was less biased by ADMA and SDMA (in the fully adjusted model; table 3) compared to either eGFRcre or eGFRcys; however, eGFRcrecys was still significantly associated with the L-arginine/ADMA ratio, SDMA and insulin resistance. Two previous studies that evaluated classic CVD risk factors found that eGFRcre was less affected than eGFRcys and eGFRcrecys [6, 7]. Our results show that eGFRcre may have similar problems in relation to nontraditional CVD risk factors and that neither equations are clearly superior, despite the fact that eGFRcrecys is more precise for estimating mGFR.

The non-GFR influence of L-arginine (and the L-arginine/ADMA ratio) on eGFRcre could in part be driven by its dependence on homoarginine and/or its involvement in creatinine production. Homoarginine, another CVD risk factor, has been associated with both L-arginine and eGFRcre [37]. Furthermore, formation of homoarginine from L-arginine, via the enzyme arginine-glycine amidonotransferase (AGAT), catalyzes synthesis of guadinioacetate, which is subsequently transformed to creatin [38].

Similarly, the influence of IR on eGFRcys could relate to metabolic pathways that affect cystatin C production, like growth hormone, insulin growth factor 1 and obesity [39], or through metabolic parameters such as acylcarnitines, which are shown to be associated with obesity, IR and decreased eGFR [40].

This study had several strengths. First, GFR was measured according to iohexol clearance in a large representative sample of the general population. Plasma iohexol

clearance has been shown to be an accurate method for measuring GFR [27, 28]. In addition, the dimethylarginine level was determined using LC-MSMS; creatinine and cystatin C assays were both calibrated to international standards; and we were able to adjust our analyses for the most important confounders. The cross-sectional design was appropriate to study non-GFR-related determinants of eGFR.

Nonetheless, there were also weaknesses in this study. Only middle-aged Caucasian individuals participated, which may have limited the generalizability. In addition, insulin resistance was not measured with the gold standard euglycemic clamp method. However, HOMA-IR has been shown to correlate well with results obtained using the euglycemic clamp technique and remains the preferred method in epidemiological studies [41].

We conclude that novel CVD risk factors, including ADMA, SDMA, and the L-arginine/ADMA ratio, represent non-GFR determinants of eGFR_{cre}, while SDMA and IR serve as determinants of eGFR_{cys}. Both eGFR_{cre} and eGFR_{cys} may be influenced by non-GFR-related

factors, although not necessarily in the same direction, which may lead to different risk prediction in longitudinal studies.

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Disclosure Statement

None of the authors have any conflicts of interest. The results presented in this paper have not been published in whole or part, except in abstract format.

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