Estimated GFR is biased by non-traditional cardiovascular risk factors

Toralf Melsom MD PhD[1,3], Ole Martin Fuskevåg PhD[2], Ulla Dorte Mathisen MD PhD[1,3], Harald Strand MSc[2], Jørgen Schei MD[1,3], Trond Jenssen MD PhD[3,4], Marit D. Solbu MD PhD[1,3], and Bjørn O. Eriksen MD PhD[1,3].

[1]Department of Nephrology, [2]Department of Laboratory Medicine, University Hospital of North Norway, UNN, Norway, [3]Metabolic and Renal Research Group, UiT The Arctic University of Norway and [4]Department of Nephrology, Oslo University Hospital, Rikshospitalet, Oslo, Norway

Word count for manuscript: 4830 including abstract, references and acknowledgement

Running Head: Non-traditional risk factors affect GFR estimation

Key words: GFR estimation, L-arginine, dimethylarginines, insulin resistance, cardiovascular disease risk factors, epidemiology, creatinine and cystatin C

Conflicts of interest statement: None of the authors have any conflicts of interest

Corresponding author: Toralf Melsom, MD

Section of Nephrology, University Hospital of North Norway

N-9038 Tromsø, Norway, mailto: toralf.melsom@unn.no

Phone: +47 776 26 511, +47 99534892. Fax: +4777628060

Abstract

Background: Estimated glomerular filtration rate (eGFR) based on either cystatin C or creatinine perform 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 non-traditional risk factors for CVD and death: L-arginine/dimethylarginine metabolism and insulin resistance.

Methods: GFR was measured via iohexol 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 non-traditional risk factors, which may bias risk prediction by eGFR in longitudinal studies.

Introduction

An estimated glomerular filtration rate (eGFR) below 60 ml/min/1.73 m^2 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 (eGFRcys) show a stronger association with renal and CVD outcomes than GFR estimated according to the creatinine level (eGFRcre) [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 eGFRcys may be caused by 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 eGFRcys 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 eGFRcys and eGFRcre 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 non-traditional 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 50–59 years of age and all inhabitants between 60–62 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 (Figure 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 e.g. 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 (Figure 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 8:00–10:00 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 at least 5 minutes. 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 years.

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, U.K.) was injected intravenously. The exact time for measuring the iohexol concentration was calculated with

Jacobsson's method based on the eGFRcre [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-MSMS using the Waters Acquity^{TM} UPLC system with an auto sampler and a binary solvent delivery system (Waters, Milford, MA) 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 x 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 Sigma–Aldrich (St. Louis, MO, USA). The stable isotope-labeled internal standards D_7-ADMA hydrochloride and L-arginine-d7 hydrochloride were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). All other chemicals were of 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, MA). 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] (Supplementary Appendix 1).

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 above. 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 above to reanalyze the non-GFR related associations between traditional CVD factors and eGFR previously reported from the RENIS-cohort [6].

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

Results

The RENIS-T6 cohort included 1627 persons, aged 50-62 years, without self-reported diabetes, CVD, or kidney disease (Figure 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 m^2 for eGFRcys and 11.4 (13.7) ml/min/1.73 m^2 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 association 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 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 current smoking was residually associated with eGFRcys, whereas only fasting glucose associated with eGFRcre (Supplemental 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 insulin 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 only adjusted 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 (Supplemental 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 m^2 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 an 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 non-traditional 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 homoargine from L-arginine, via the enzyme arginine-glycine amidonotransferase (AGAT), catalyzes synthesis of guaidinioacetate, 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 eGFRcre, while SDMA and IR serve as determinants of eGFRcys. Both eGFRcre and eGFRcys 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.

Acknowledgments

We would like to thank Britt-Ann Winter Eilertsen, Bjørg Skog Høgset, Saskia van Heusden, and the rest of the staff at the Clinical Research Unit (University Hospital of North Norwa y, UNN) for their assistance in planning the study, performing the procedures, and collecting data according to the GCP standard. We also thank the staff at the Laboratory of Medicine (UNN) for the analyses of creatinine and cystatin C, as well as Inger Sperstad and Ingrid D. Sandstad (Clinical Research Centre, UNN) for database support. Above all, we thank all the participants in the RENIS-T6 cohort for making this study possible.

Conflicts of interest 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.

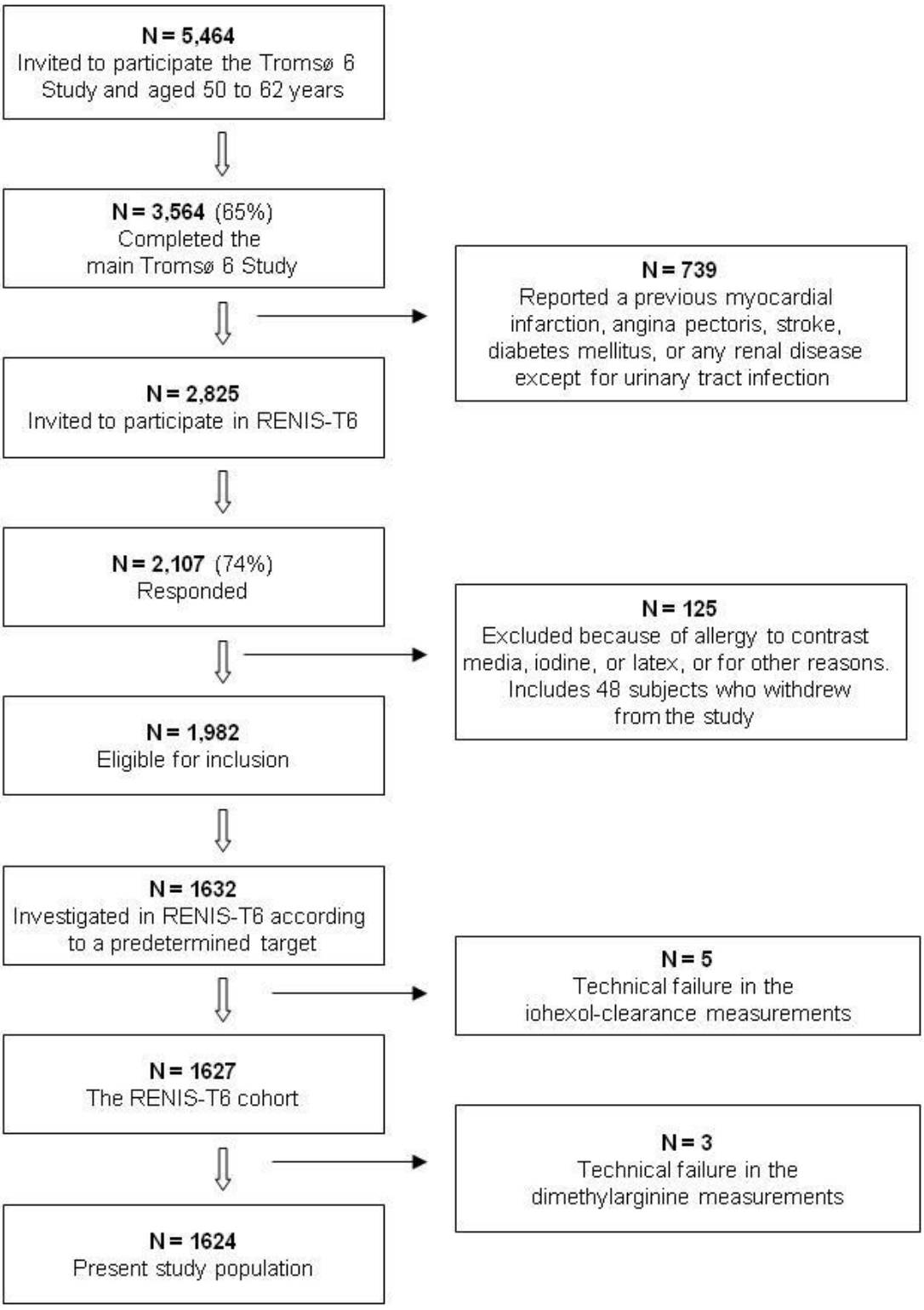

Figure 1 Flowchart of the study population

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, mmHg	125.4	(17.3)	134.0	(16.8)
Diastolic blood pressure, mmHg	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/1,73m^2	87.8	(14.0)	95.7	(13.7)
GFR estimated by CKD-EPI equations				
eGFRcre, ml/min/1,73m^2	94.4	(10.0)	95.3	(9.0)
eGFRcys, ml/min/1,73m^2	102.2	(12.1)	108.6	(11.7)
eGFRcrecys, ml/min/1,73m^2	101.4	(11.9)	104.6	(10.7)

Numbers are means (SD), precentages or medians (IQR)

ADMA; asymmetric dimethylarginine, SDMA; symmetric dimethylarginine, HOMA-IR; homeostasis model assessment of insulin resistance.

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/1,73m^2)	(95 % CI)	P	Estimate (ml/ min/1,73m^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)

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

Dependent variable	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[c]	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).

Headings and Legends

Table 1. Study population characteristics

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

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

Figure 1 Flowchart of the study population

Supplemental table 1: Generalized estimating equations showing residual associations between risk factors and eGFR after accounting for mGFR[a].

REFERENCES

1 Astor BC, Levey AS, Stevens LA, Van Lente F, Selvin E, Coresh J: Method of glomerular filtration rate estimation affects prediction of mortality risk. JAmSocNephrol 2009;20:2214-2222.

2 Shlipak MG, Matsushita K, Arnlov J, Inker LA, Katz R, Polkinghorne KR, Rothenbacher D, Sarnak MJ, Astor BC, Coresh J, Levey AS, Gansevoort RT: Cystatin c versus creatinine in determining risk based on kidney function. NEnglJMed 2013;369:932-943.

3 Inker LA, Okparavero A: Cystatin c as a marker of glomerular filtration rate: Prospects and limitations. Current opinion in nephrology and hypertension 2011;20:631-639.

4 Inker LA, Schmid CH, Tighiouart H, Eckfeldt JH, Feldman HI, Greene T, Kusek JW, Manzi J, Van LF, Zhang YL, Coresh J, Levey AS: Estimating glomerular filtration rate from serum creatinine and cystatin c. NEnglJMed 2012;367:20-29.

5 Stevens LA, Schmid CH, Greene T, Li L, Beck GJ, Joffe MM, Froissart M, Kusek JW, Zhang YL, Coresh J, Levey AS: Factors other than glomerular filtration rate affect serum cystatin c levels. Kidney Int 2009;75:652-660.

6 Mathisen UD, Melsom T, Ingebretsen OC, Jenssen T, Njolstad I, Solbu MD, Toft I, Eriksen BO: Estimated gfr associates with cardiovascular risk factors independently of measured gfr. JAmSocNephrol 2011;22:927-937.

7 Rule AD, Bailey KR, Lieske JC, Peyser PA, Turner ST: Estimating the glomerular filtration rate from serum creatinine is better than from cystatin c for evaluating risk factors associated with chronic kidney disease. Kidney Int 2013;83:1169-1176.

8 Boger RH, Sullivan LM, Schwedhelm E, Wang TJ, Maas R, Benjamin EJ, Schulze F, Xanthakis V, Benndorf RA, Vasan RS: Plasma asymmetric dimethylarginine and incidence of cardiovascular disease and death in the community. Circulation 2009;119:1592-1600.

9 Boger RH, Maas R, Schulze F, Schwedhelm E: Asymmetric dimethylarginine (adma) as a prospective marker of cardiovascular disease and mortality--an update on patient populations with a wide range of cardiovascular risk. PharmacolRes 2009;60:481-487.

10 Kiechl S, Lee T, Santer P, Thompson G, Tsimikas S, Egger G, Holt DW, Willeit J, Xu Q, Mayr M: Asymmetric and symmetric dimethylarginines are of similar predictive value for cardiovascular risk in the general population. Atherosclerosis 2008

11 Pizzarelli F, Maas R, Dattolo P, Tripepi G, Michelassi S, D'Arrigo G, Mieth M, Bandinelli S, Ferrucci L, Zoccali C: Asymmetric dimethylarginine predicts survival in the elderly. Age (Dordr) 2013;35:2465-2475.

12 Schulze F, Carter AM, Schwedhelm E, Ajjan R, Maas R, von Holten RA, Atzler D, Grant PJ, Boger RH: Symmetric dimethylarginine predicts all-cause mortality following ischemic stroke. Atherosclerosis 2010;208:518-523.

13 Meinitzer A, Kielstein JT, Pilz S, Drechsler C, Ritz E, Boehm BO, Winkelmann BR, Marz W: Symmetrical and asymmetrical dimethylarginine as predictors for mortality in patients referred for coronary angiography: The ludwigshafen risk and cardiovascular health study. Clinical Chemistry 2011;57:112-121.

14 Bonora E, Kiechl S, Willeit J, Oberhollenzer F, Egger G, Meigs JB, Bonadonna RC, Muggeo M: Insulin resistance as estimated by homeostasis model assessment predicts incident symptomatic cardiovascular disease in caucasian subjects from the general population: The bruneck study. Diabetes Care 2007;30:318-324.

15 Jeppesen J, Hansen TW, Rasmussen S, Ibsen H, Torp-Pedersen C, Madsbad S: Insulin resistance, the metabolic syndrome, and risk of incident cardiovascular disease: A population-based study. JAmCollCardiol 2007;49:2112-2119.

16 Despres JP, Lamarche B, Mauriege P, Cantin B, Dagenais GR, Moorjani S, Lupien PJ: Hyperinsulinemia as an independent risk factor for ischemic heart disease. NEnglJMed 1996;334:952-957.

17 de Boer IH, Katz R, Chonchol MB, Fried LF, Ix JH, Kestenbaum B, Mukamal KJ, Peralta CA, Siscovick DS: Insulin resistance, cystatin c, and mortality among older adults. Diabetes Care 2012;35:1355-1360.

18 Zethelius B, Lithell H, Hales CN, Berne C: Insulin sensitivity, proinsulin and i Insulin as predictors of coronary heart disease. A population-based 10-year, follow-up study in 70-year old men using the euglycaemic insulin clamp. Diabetologia 2005;48:862-867.

19 Perticone F, Sciacqua A, Maio R, Perticone M, Galiano Leone G, Bruni R, Di Cello S, Pascale A, Talarico G, Greco L, Andreozzi F, Sesti G: Endothelial dysfunction, adma and insulin resistance in essential hypertension. International journal of cardiology 2010;142:236-241.

20 Siervo M, Bluck LJ: In vivo nitric oxide synthesis, insulin sensitivity, and asymmetric dimethylarginine in obese subjects without and with metabolic syndrome. Metabolism 2012;61:680-688.

21 Andreozzi F, Presta I, Mannino GC, Scarpelli D, Di Silvestre S, Di Pietro N, Succurro E, Sciacqua A, Pandolfi A, Consoli A, Hribal ML, Perticone F, Sesti G: A functional variant of the dimethylarginine dimethylaminohydrolase-2 gene is associated with insulin sensitivity. PLoS One 2012;7:e36224.

22 Toda N, Okamura T: Obesity impairs vasodilatation and blood flow increase mediated by endothelial nitric oxide: An overview. Journal of clinical pharmacology 2013;53:1228-1239.

23 Melsom T, Mathisen UD, Ingebretsen OC, Jenssen TG, Njolstad I, Solbu MD, Toft I, Eriksen BO: Impaired fasting glucose is associated with renal hyperfiltration in the general population. Diabetes Care 2011;34:1546-1551.

24 Jacobsen BK, Eggen AE, Mathiesen EB, Wilsgaard T, Njolstad I: Cohort profile: The tromso study. IntJEpidemiol 2011

25 Eriksen BO, Mathisen UD, Melsom T, Ingebretsen OC, Jenssen TG, Njolstad I, Solbu MD, Toft I: Cystatin c is not a better estimator of gfr than plasma creatinine in the general population. Kidney Int 2010;78:1305-1311.

26 Melsom T, Mathisen UD, Eilertsen BAW, Ingebretsen OC, Jenssen T, Njølstad I, Solbu MD, Toft I, Eriksen BO: Physical exercise, fasting glucose, and renal hyperfiltration in the general population: The renal iohexol clearance survey in tromsø 6 (renis-t6). Clinical Journal of the American Society of Nephrology 2012;7:1801-1810.

27 Bird NJ, Peters C, Michell AR, Peters AM: Comparison of gfr measurements assessed from single versus multiple samples. AmJKidney Dis 2009;54:278-288.

28 Stevens LA, Levey AS: Measured gfr as a confirmatory test for estimated gfr. JAmSocNephrol 2009;20:2305-2313.

29 Jacobsson L: A method for the calculation of renal clearance based on a single plasma sample. ClinPhysiol 1983;3:297-305.

30 Grubb A, Blirup-Jensen S, Lindstrom V, Schmidt C, Althaus H, Zegers I, C IWGoSoC: First certified reference material for cystatin c in human serum erm-da471/ifcc. Clinical chemistry and laboratory medicine : CCLM / FESCC 2010;48:1619-1621.

31 Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC: Homeostasis model assessment: Insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412-419.

32 Solbu MD, Kronborg J, Eriksen BO, Jenssen TG, Toft I: Cardiovascular risk-factors predict progression of urinary albumin-excretion in a general, non-diabetic population: A gender-specific follow-up study. Atherosclerosis 2008;201:398-406.

33 Mathisen UD, Melsom T, Ingebretsen OC, Jenssen TG, Njolstad I, Solbu MD, Toft I, Eriksen BO: Ambulatory blood pressure is associated with measured glomerular filtration rate in the general middle-aged population. Journal of hypertension 2012;30:497-504.

34 Knight E, Verhave J, Spiegelman D: Factors influencing serum cystatin c levels other than renal function and the impact on renal function measurement. Kidney Int 2004;65:1416-1421.

35 Fliser D, Kronenberg F, Kielstein JT, Morath C, Bode-Boger SM, Haller H, Ritz E: Asymmetric dimethylarginine and progression of chronic kidney disease: The mild to moderate kidney disease study. JAmSocNephrol 2005;16:2456-2461.

36 Ravani P, Tripepi G, Malberti F, Testa S, Mallamaci F, Zoccali C: Asymmetrical dimethylarginine predicts progression to dialysis and death in patients with chronic kidney disease: A competing risks modeling approach. JAmSocNephrol 2005;16:2449-2455.

37 Tomaschitz A, Meinitzer A, Pilz S, Rus-Machan J, Genser B, Drechsler C, Grammer T, Krane V, Ritz E, Kleber ME, Pieske B, Kraigher-Krainer E, Fahrleitner-Pammer A, Wanner C, Boehm BO, Marz W: Homoarginine, kidney

function and cardiovascular mortality risk. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association 2014;29:663-671.

38 Michel T: R is for arginine: Metabolism of arginine takes off again, in new directions. Circulation 2013;128:1400-1404.

39 Sze L, Bernays RL, Zwimpfer C, Wiesli P, Brandle M, Schmid C: Impact of growth hormone on cystatin c. Nephron extra 2013;3:118-124.

40 Goek ON, Doring A, Gieger C, Heier M, Koenig W, Prehn C, Romisch-Margl W, Wang-Sattler R, Illig T, Suhre K, Sekula P, Zhai G, Adamski J, Kottgen A, Meisinger C: Serum metabolite concentrations and decreased gfr in the general population. Am J Kidney Dis 2012;60:197-206.

41 Bonora E, Saggiani F, Targher G, Zenere MB, Alberiche M, Monauni T, Bonadonna RC, Muggeo M: Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity - studies in subjects with various degrees of glucose tolerance and insulin sensitivity. Diabetes Care 2000;23:57-63.