UiT The Arctic University of Norway

Faculty of Health Sciences

Department of Clinical Medicine

Novel serum biomarkers and their association with measured and estimated GFR decline in the general population

The Renal Iohexol Clearance Survey (RENIS)

Inger Therese Tønsberg Enoksen

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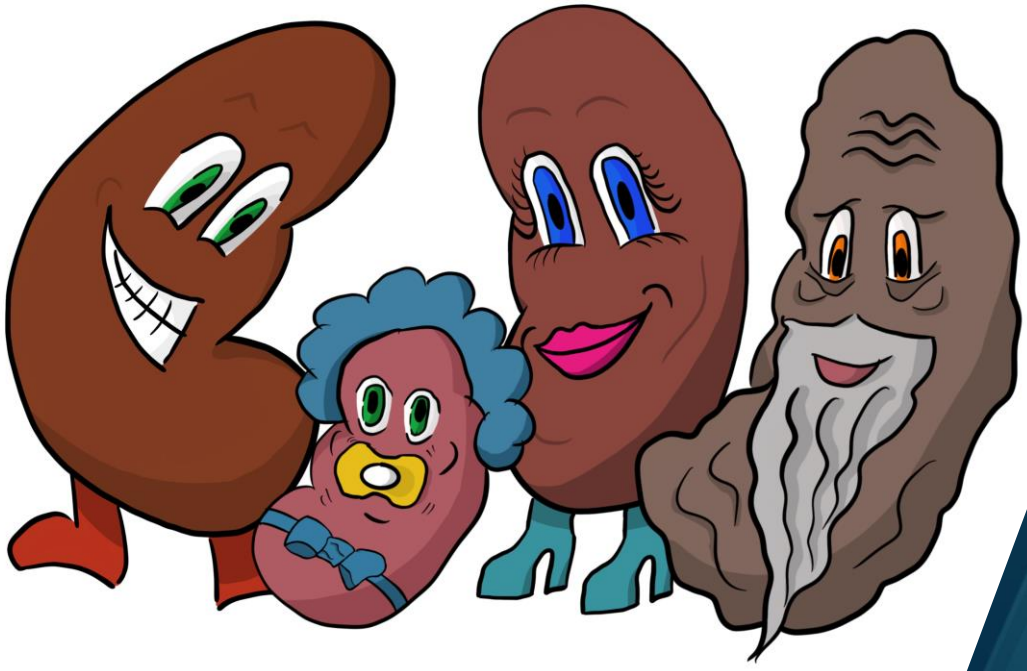

Table of Contents

List of Tables

List of Figures

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Abbreviations

ADMA	Asymmetric dimethylarginine
AKI	Acute kidney injury
BP	Blood pressure
CD40Lig	CD40 ligand
CKD	Chronic kidney disease
CKD-EPI	Chronic kidney disease epidemiology collaboration
CVD	Cardiovascular disease
DM1	Diabetes mellitus type 1
DM2	Diabetes mellitus type 2
eGFR	Estimated glomerular filtration rate
eGFRcre/cys/cyscre	Estimated GFR based on creatinine, cystatin C, or both
ESKD	End stage kidney disease
FABP4	Fatty acid binding protein 4
Gal-3	Galectin-3
GDF-15	Growth/differentiation factor 15
GFR	Glomerular filtration rate
HT	Hypertension
IF	Interstitial fibrosis
KDIGO	Kidney disease improving global outcomes
KDOQI	Kidney disease outcomes quality initiative
MCP-1	Monocyte chemoattractant protein-1
mGFR	Measured glomerular filtration rate
RENIS-T6	The Renal Iohexol Clearance Survey in Tromsø 6
RENIS-FU	The RENIS follow-up
RRT	Renal replacement therapy
SDMA	Symmetric dimethylarginine
snGFR	Single nephron glomerular filtration rate
suPAR	Soluble urokinase-type plasminogen activator receptor
T6	The Tromsø study: Tromsø 6
Tie-2	TEK tyrosine kinase/TEK
TIMP1	Tissue inhibitor of metalloproteinase 1
TNFR2	Tumor necrosis factor receptor 2
TRAIL-R2	TNF-related apoptosis-inducing ligand receptor 2
uACR	Urinary albumin to creatinine ratio
Umod	Uromodulin
UUO	Unilateral ureteral obstruction
MMP2	Matrix metalloproteinase 2
MMP7	Matrix metalloproteinase 7
YLL	Years of life lost

List of Papers

Paper 1

Enoksen IT, Svistounov D, Norvik JV, Stefansson VTN, Solbu MD, Eriksen BO, Melsom T. **Serum matrix metalloproteinase 7 and accelerated glomerular filtration rate decline in a general non-diabetic population.** *Nephrology Dialysis Transplantation. 2022 Vol. 37 Issue 9 Pages 1657-1667. doi:10.1093/ndt/gfab251*

Paper 2

Enoksen IT, Rinde NB, Svistounov D, Norvik JV, Stefansson VTN, Solbu MD, Eriksen BO, Melsom T. **Validation of eGFR for Detecting Associations Between Serum Protein Biomarkers and Subsequent GFR Decline**. (Article in press. Journal of the American Society of Nephrology. Published online ahead of print April 24th, 2023: doi: 10.1681/ASN.0000000000000147)

Paper 3

Rinde NB, Enoksen IT, Melsom T, Fuskevåg OM, Eriksen BO, Norvik JV. **Nitric-oxide precursors and dimethylarginines as risk factors for accelerated GFR decline in the nondiabetic general population**. *Kidney International Reports. 2023 Vol. 8 Issue 4 Pages 818-826. doi: 10.1016/j.ekir.2023.01.015*

SUMMARY

During the last decades, the prevalence and health burden of chronic kidney disease (CKD) have increased globally, with an overrepresentation among older individuals. Since 1990, the death rate of CKD has increased by approximately 40% worldwide, and the need for renal replacement therapy (RRT) has risen by 138%. Globally, only 27-53% of those in need of RRT received treatment in 2010, indicating that healthcare systems in many countries are not equipped to handle this growing burden.

The age-related loss of kidney function, as assessed by the glomerular filtration rate (GFR), is a significant contributor to the increasing prevalence of CKD. However, the age-related decline in GFR varies significantly between people, even in healthy individuals, after accounting for known risk factors for CKD. This indicates a potential for preventive measures.

The mechanisms underlying age-related GFR decline remain unclear, and current kidney function biomarkers lack predictive abilities for future GFR decline. Knowledge is lacking partly because previous studies relied on estimated GFR (eGFR), which is imprecise in the near-normal range and biased by non-GFR-related factors. Accordingly, in the prospective Renal Iohexol Clearance Survey (RENIS), we measured the GFR using plasma iohexol clearance (mGFR). In this thesis, we investigated the relationship between 18 proposed serum biomarkers representative of central renal pathophysiological mechanisms and age-related mGFR decline. We also investigated whether estimated GFR can be used as a surrogate endpoint for measured GFR when searching for biomarkers associated with GFR decline.

At baseline, 1627 individuals without diabetes, cardiovascular disease (CVD), or kidney disease had their GFR measured. Over 11 years, two follow-up visits were conducted, resulting in 1410 individuals with baseline mGFR and one or more follow-up GFR measurements. We used a linear mixed model to estimate the GFR slope and logistic regression or interval-censored Cox regression to assess the risk of accelerated GFR decline (defined as the 10% steepest GFR decline rates) and incident CKD.

We found that higher baseline concentrations of the pro-fibrotic biomarker MMP7 were independently associated with all mGFR decline outcomes and improved the risk prediction of accelerated GFR decline and incident CKD compared to a model of conventional risk factors. The other biomarkers were either not associated with any mGFR outcome or showed variable associations depending on the different GFR outcomes and models used for covariate adjustment. Some biomarkers exhibited different associations depending on whether mGFR or eGFR based on either creatinine or cystatin C (cys C) was used. Thus, for some biomarkers, associations with eGFR decline were not reproducible with mGFR. We conclude that results from studies focusing on risk markers for GFR decline, using eGFR and particularly eGFR from cys C, should be interpreted with caution.

1 INTRODUCTION

Chronic kidney disease (CKD) is a growing global health problem, with an increasing prevalence worldwide, affecting approximately 8-13% of the world population.[1-7] CKD is associated with an increased risk of cardiovascular disease (CVD), end-stage kidney disease (ESKD), and death, and is predicted to become the fifth most common cause of years of life lost (YLL) by 2040.[3,8-10]

An aging world population drives the burden of CKD-related health issues because kidney function, assessed by the glomerular filtration rate (GFR), declines with age, even in healthy individuals. This age-related decline in GFR leads to a significantly higher prevalence of CKD with increasing age, with over 40% of people above 70 years of age having CKD.[11-13] The underlying mechanisms are only partly understood, and the rates of GFR decline differ significantly between individuals, regardless of traditional risk factors such as hypertension (HT) and diabetes (DM).[14] This suggests the potential for preventive measures. However, to initiate effective measures, it is necessary to identify individuals at risk of CKD and to investigate the underlying mechanisms of accelerated age-related GFR decline.

Several studies have therefore examined blood and urinary biomarkers involved in various pathophysiological pathways for GFR decline and CKD.[15-27] However, these biomarkers have yet to be incorporated into clinical practice. These studies used estimated GFR (eGFR) based on creatinine and/or cystatin C to assess GFR change rates, which lack precision in the near-normal range - where preventive measures need to be applied.[8,9,28-34] Additionally, eGFR is influences by non-GFR-related factors and may introduce confounding and spurious associations between the biomarkers and renal outcomes.[35-40] Most studies also included individuals with underlying diseases and comorbidities, which could potentially increase the confounding issue.

In this thesis, we investigated the association between baseline concentrations of 18 proposed serum biomarkers and their relationship with longitudinal changes in measured GFR decline and incident CKD, using iohexol clearance, in a healthy general middle-aged population during 5.6 and 11 years of follow-up.

The biomarkers were selected through a literature review of biomarkers associated with GFR loss, CKD development, and renal aging in previous studies using eGFR. We also explored the limitations of using eGFR versus (vs.) mGFR in the search for promising biomarkers and kidney function outcomes.

2 BACKGROUND

2.1 Chronic kidney disease - prevalence and health burden

The global prevalence and health burden of chronic kidney disease (CKD) is increasing, affecting approximately 8-13% of the world population, with older adults being overrepresented.[1-7] In one study, the overall lifetime risk of CKD was 41%, at 50 years of age.[4] In individuals without the CKD risk factors HT, DM, and obesity the risk was lower but still high at 34%. The age-standardized rate of disability-adjusted life years (DALYs) for CKD is highest in low (and middle) socio-demographic countries, where there is a large gap between the healthcare provided and the burden of CKD.[3]

The global burden of disease study predicts that by 2040, CKD will become the fifth most common cause of YYLs.[3,41] This increase is mainly due to the ageing world population, as kidney function declines with age.[12,41-43] In 2017, CKD was the cause of death for 1.2 million people globally, and this number is projected to increase to 2.2-4.0 million deaths by 2040. [3,41] CKD leads to increased morbidity and mortality with reduced life expectancy and quality of life.[3,8-10] Although older adults with mild to moderately reduced kidney function and no proteinuria might have a minimal increased risk of premature death, their risk of developing end-stage kidney disease (ESKD) is increased.[44] Worsening kidney function and progression to ESKD also pose a financial burden on the healthcare system due to expenses related to renal replacement therapy (RRT) and CVD complications.[45]

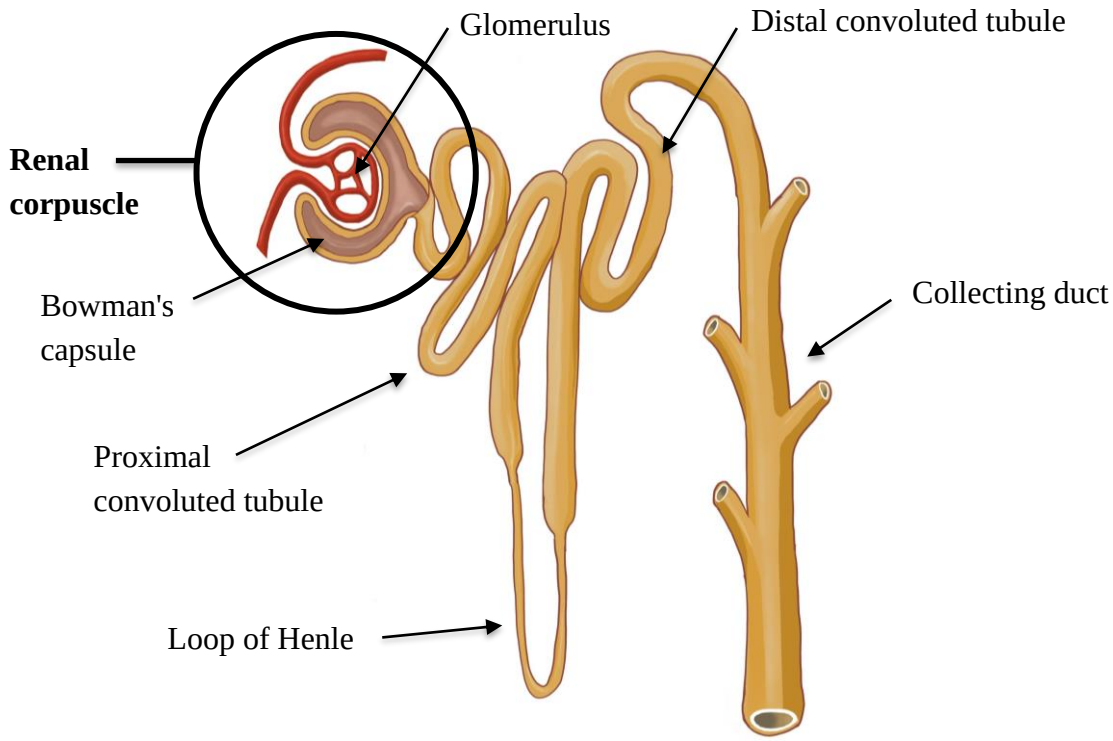
Currently, more than 2.6 million people worldwide receive RRT.[46] Over the last decades, the number of people in need of RRT has increased by 138% globally (1.5 million people from 1990-2010) and by 366% in Norway (1990-2018). [46,47] This increase is projected to continue worldwide, particularly in developing countries, due to better survival and an aging population. By 2030, the number of people in need of RRT is expected to reach 5.4 million (a 200% increase).[46] One study estimated that in 2010, only 27-53 % of people in need of RRT received treatment, and as many as 96-98% in low-income countries did not receive necessary RRT.[3,46]

2.2 Kidney function – the glomerular filtration rate (GFR)

The kidneys play a crucial role in maintaining homeostasis in the body through their excretory-, endocrine-, and metabolic functions.[48] Although the kidneys execute many functions, the glomerular filtration rate (GFR), one of its excretory functions, is accepted as the best overall indicator of kidney function.[14] It reflects the kidneys' ability to filter waste products from the blood.

The nephron is the functional unit of the kidney (Figure 1).[48] At birth, each kidney contains approximately 1-1.5 million nephrons.[49] However, the total number of nephrons decreases with age and disease, resulting in significant variation among individuals of different ages, with an average nephron number of approximately 900.000 to 1.0 million in each kidney.[49]

Figure 1: The Nephron



The GFR is defined as the rate at which fluid is filtered from the glomerular capillaries into the Bowman's capsule, measured in milliliters per minute (mL/min). The total kidney GFR is determined by two factors: the filtration rate of each nephron, known as the single nephron GFR (snGFR), and the number of functioning glomeruli.[50] However, individual glomeruli have compensatory abilities and can increase the snGFR when needed, utilizing the kidneys' functional reserve.[30,51] For example, if half the functional nephron mass is lost (e.g. in kidney transplantation donor), one would expect a 50% decrease in GFR. However, due to compensatory mechanisms (increased snGFR), the actual decrease in GFR is only around 20-30% when measured after the postoperative period. [52-54]

An ideal filtration marker will have clearance identical to the GFR and is defined as a marker that is freely filtrated in the glomerulus, not protein bound, not secreted or reabsorbed in the tubules, that does not affect kidney function and is not metabolized or synthesized by the kidneys.[52] Biomarkers that are water-soluble and have a molecular mass below 40 kDa are typically freely or nearly freely filtered in the glomeruli.[55] The concentration of these biomarkers in blood or urine varies depending on the GFR, tubular reabsorption or secretion, extra-renal metabolism, and, for endogenous substances, the production rate of the biomarker in the body.

The "gold standard" method for measuring the total kidney GFR is urinary inulin clearance, which uses an exogenous ideal filtration marker.[52] However, this method is challenging, expensive, time-consuming, and not routinely used in research and clinical practice.[56,57] Approximations to this method include urinary creatinine clearance (CrCl) and measurements

of GFR (mGFR) using other exogenous substances such as iohexol, iothalamate, or radioactive isotopes.[58] Estimations of GFR (eGFR) based on circulating endogenous substances (creatinine and cystatin C), which are freely filtered in the kidneys and have minimal extra-renal clearance, are widely used in clinical practice.[52]

2.3 Assessment of the GFR

2.3.1 Iohexol (measured GFR)

Iohexol is a non-radioactive contrast agent that is exclusively eliminated from plasma through glomerular filtration. It has a molecular mass of 0.82 kDa, is distributed into the extracellular space, has less than 2% plasma protein binding, and is not metabolized by the kidneys.[52,59,60] Iohexol plasma clearance can be used to measure kidney function, either with single or multiple samples, and demonstrates a strong correlation with urinary inulin clearance, making it a sufficiently accurate method.[57,59,61] The single sample iohexol clearance method is just as reliable as the multiple sample method.[62,63] The plasma clearance of iohexol is calculated using equations proposed by Jacobsson.[52,64] To minimize the error from imprecise estimates of extracellular volume (ECV) in these equations, Jacobsson developed an equation for calculating the optimal sampling time based on a patient's expected GFR. [57,65] Plasma iohexol clearance is used in clinical practice and research to get exact measurements of the GFR. It is often preferred over radioactive substances (such as 125I-iothalamate and 99mTc-diethylenetriaminepenta-acetic acid: DTPA) to avoid exposing individuals to radiation.[52,61]

2.3.2 Creatinine and cystatin C (estimated GFR)

Creatinine is a metabolite with a molecular mass of 0.113 kilodaltons (kDa) that is continuously released from muscle tissue as a byproduct of protein metabolism. It is exclusively eliminated by the kidneys through free filtration in the glomeruli, with a small fraction undergoing proximal tubular secretion. Creatinine is not absorbed or metabolized by the tubules. Therefore, if the GFR declines, the blood levels of creatinine will increase.

Cystatin C (cys C) is another minor metabolite with a molecular mass of 13 kDa. It is produced by all nucleated cells in the body and is freely filtrated in the glomeruli, similar to creatinine.[66-68] However, cystatin C differs in that it is catabolized and almost entirely reabsorbed in the proximal tubule, thus not subject to tubular secretion.[52,69]

Estimating GFR from blood levels of creatinine (eGFRcre), cys C (eGFRcys), or both (eGFRcyscre), using separate GFR estimation equations (including variables such as age, sex, and race) is routine in clinical practice.[28,70] Several GFR estimation equations have been developed over the years.[28,33,71-73] The most commonly used equation is the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation, which was developed in CKD patients and healthy individuals.[28,74] The CKD-EPI equation performs better in the normal range of GFR compared to older equations, and international guidelines have recommended its use for GFR estimation.[75,76] There is an ongoing debate on whether race should be included in the eGFR equation, as race is a social construct and not a biological factor.

Accordingly, a new CKD-EPI equation (2021) has been proposed and adopted in the USA.[77,78] However, eGFR based on creatinine levels remains imprecise in the normal and near-normal GFR range, potentially leading to delayed detection of early CKD development.[32,33] It is important to note that although the mean eGFR may closely approximate the mean mGFR at a population level, eGFR exhibit significant bias at the individual level or within specific subgroups of people due to the influence of non-GFR related factors.

2.3.3 Non-GFR related factors

Factors other than the GFR that influence endogenous filtration markers are referred to as non-GFR related factors or determinants. Both creatinine and cys C levels can be influenced by non-GFR related factors such as muscle mass (age-related muscle wasting (sarcopenia)), diet, smoking, medication (such as steroids), and other endogenous substances (adipose tissue, cancer, thyroid disease, low-grade inflammation, and variations in tubular secretion and extra-renal creatinine excretion).[35-39] These factors can lead to inaccurate estimates, either overestimating or underestimating kidney function.

Some studies have found that cys C has certain advantages over creatinine in estimating GFR. Cys C is found to be less influenced by diet, muscle mass, age, ethnicity, and sex compared to creatinine.[68,79,80] As a result, cys C may be more useful in detecting kidney disease in children and older individuals.[68] However, cys C is still influenced by other factors such as inflammation (e.g., CRP), obesity, smoking, and albumin.[81,82] These factors should be taken into consideration when interpreting serum levels in clinical practice. It is important to note that other studies, including publications from RENIS, have found that cys C is not a better estimator of GFR than creatinine in the general population and may even perform worse.[65 82]

2.4 Kidney damage markers

Cystatin C and creatinine are filtration markers that primarily reflect the filtrating function of the glomeruli. Alongside these biomarkers, albuminuria, a marker of glomerular injury, is used for risk prediction and stratification of CKD stage in combination with eGFR.[14] Kidney damage, indicated by albuminuria, often precedes GFR decline and significantly influences disease progression and outcome.[1,34] However, albuminuria may occur after established kidney damage or not occur at all, depending on the underlying pathology of the kidney disease.[32,55]

Current markers of kidney function and damage (GFR and uACR) primarily reflect glomerular health and not the other parts of the nephron.[83-85] Although tubular disease has been associated with GFR decline over time, eGFR cannot fully detect tubular disease, and tubulointerstitial damage and fibrosis are invisible without a kidney biopsy.[85-87]

In recent years, several research projects have therefore investigated the role of different biomarkers in identifying kidney damage prior to GFR decline.[15,21,88] These studies have proposed novel biomarkers for both acute kidney injury (AKI) and CKD progression,[32,55]

including biomarkers of filtration and proximal tubular damage, such as kidney injury molecule 1 (KIM-1) and monocyte chemoattractant protein-1 (MCP-1).[89-91] Several researchers have investigated markers like uromodulin (Umod), which represents tubular dysfunction in the loop of Henle, and markers associated with parenchymal damage, endothelial damage, fibrosis, and inflammation, such as TNF-alpha receptors, Galactin-3, asymmetric dimethylarginine (ADMA), and their association with GFR.[23,32,55,92-95] However, many of these studies relied on eGFR to examine the associations between these biomarkers and GFR decline. It remains unexplored whether these associations remain consistent when using mGFR, which is not influenced by non-GFR related factors.

2.5 Chronic kidney disease and age-related GFR decline

The first definition and classification of CKD occurred in 2002 with the *Kidney Disease Outcomes Quality Initiative* (KDOQI) guidelines.[14,96] In the *Kidney Disease Improving Global Outcomes* (KDIGO) guidelines, CKD is defined as; "*A reduction in GFR <60 mL/min/1.73 m^2 and/or markers of kidney damage (albuminuria: uACR ≥3 mg/mmol), which is present for >3 months"*.[14,76,97] This GFR cut-of value represents slightly less than half of the normal GFR in young adults (125 mL/min/1.73 m^2).[14] CGA (Cause, GFR, Albuminuria) staging is employed to further classify CKD into six GFR categories (five CKD stages) and three uACR categories to determine the degree of kidney damage (
Figure ***2***).[14]

Clinical management and prognosis of CKD progression are based on this classification, and patients are divided into four risk groups for CKD progression: low, moderate, high, and very high risk. These risk groups determine the frequency of yearly monitoring and the need for referral (
Figure ***2***).[14] However, the risk associations of GFR and uACR appear to be independent of each other, and neither parameter alone can capture the overall prognosis.[14,76] In the general population, CKD stage G3a and G3b are the most common, accounting for 7.7% in the United Stated (1999-2004) and 3.9% worldwide (stage 1-2: 5.0%).[1,3]

Major risk factors for CKD include diabetes, hypertension, obesity, smoking, and autoimmune diseases.[3,4,47,98,99] However, aging is the primary driving force behind CKD development, defined as a GFR < 60 mL/min/1.73 m^2.[42] Even in healthy individuals, GFR starts to decline after the age of 40.[12,13,100] Therefore, approximately 40-50% of individuals beyond 70 years of age have developed CKD.[1 4] However, the extent of age-related GFR decline varies substantially among individuals within a population.[12-14,43,85,101,102] In the RENIS study, the only longitudinal population study using measured GFR, the mean GFR decline rate in healthy middle-aged individuals was 0.7 mL/min/1.73 m^2/year. While women exhibited an almost linear decline rate with aging, men experienced a steeper GFR decline with increasing age, reaching -1.5 mL/min/1.73 m^2/year at the age of 72 (Figure 3).[13]

Prognosis of CKD by GFR and albuminuria categories: KDIGO 2012

GFR categories (mL/min/1.73m^2) Description and range				Persistent albuminuria categories Description and range		
				A1	**A2**	**A3**
				Normal to mildly increased	Moderately increased*	Severely increased**
				<30 mg/g <3 mg/mmol	30-300 mg/g 3-30 mg/mmol	>300 mg/g >30 mg/mmol
	G1	Normal or high	≥ 90			
	G2	Mildly decreased*	60-89			
	G3a	Mildly to moderately decreased	45-59			
	G3b	Moderately to severely decreased	30-44			
	G4	Severely decreased	15-29			
	G5	Kidney failure	< 15			

Figure 2: Prognosis of CKD by GFR and albuminuria category:

Green: low risk (if no other markers of kidney disease, no CKD); Yellow, moderately increased risk; Orange, high risk; Red, very high risk. CKD, chronic kidney disease; GFR, Glomerular filtration rate; KDIGO, kidney Disease: Improving Global Outcomes.
* Relative to young adult level
** Including nephrotic syndrome (Albumin excretion usually >2200 mg/24 hours [ACR >2200mg/g; >220 mg/mmol]).
In the absence of evidence of kidney damage, neither GFR category G1 nor G2 fulfill the criteria for CKD.
Figure Adapted from the KDIGO Guidelines 2012[14]

Studies have shown that healthy adults experience a loss of nearly half of their nephrons from young adulthood (18-29 years) to old age (70-75 years), amounting to approximately 6200 nephrons per year.[101] The precise mechanism underlying age-related GFR decline and nephron loss remains unknown. Macroscopically a reduction in kidney size is observed, while microscopically, there is a decrease in functional nephrons and an increase in interstitial fibrosis.[51,85,101,103,104] The age-related histological changes, collectively known as senile-nephrosclerosis, are characterized by glomerulosclerosis, tubular atrophy, cortical and interstitial fibrosis (IF), and arteriosclerosis.[85,98,104-106] These chronic changes tend to increase with decreasing kidney function but do not necessarily correlate with the GFR-based staging of CKD.[85,106,107]

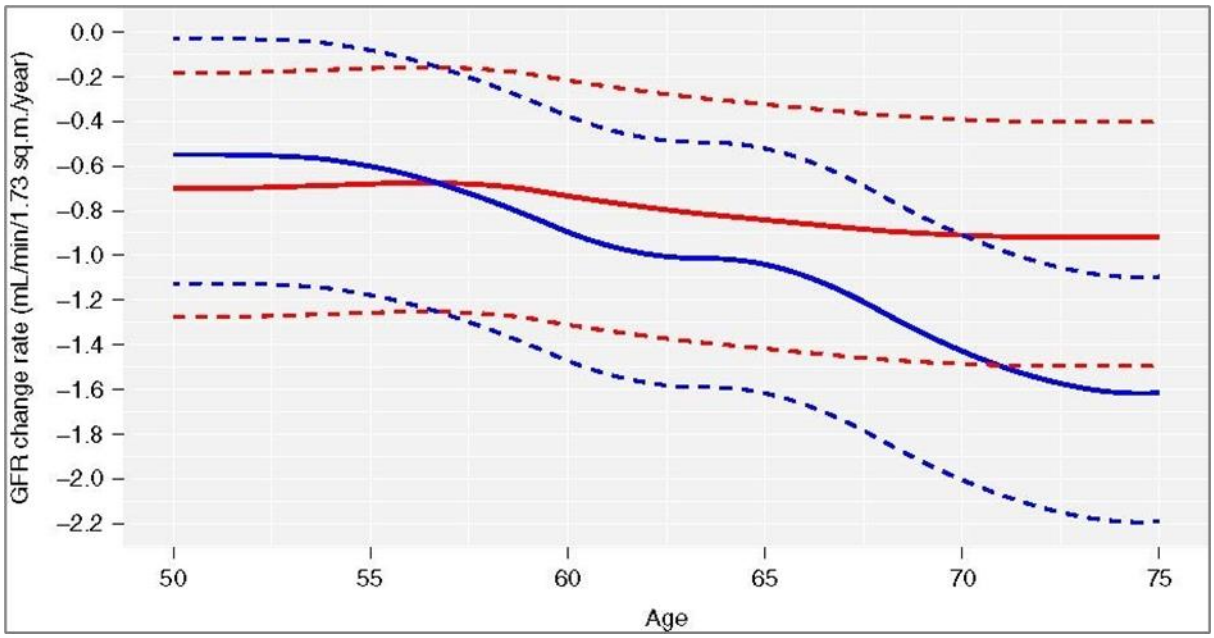


Figure 3: Sex-specific GFR change rates for healthy women and men as a function of age with 95% reference intervals.

The solid lines represent the GFR change rates for women (red) and men (blue). The dashed lines represent the 95% reference intervals.
Figure reused with permission from Journal of the American Society of Nephrology 33(10):1891-1902, October 2022.[13]

The loss in nephron number and progression of glomerulosclerosis are correlate with the age-related GFR decline.[101] However, nephrosclerosis alone does not explain the age-related decline in GFR, and the increasing prevalence of nephrosclerosis with age cannot be solely attributed to CKD risk factors.[85,98,103] The snGFR is found to be stable (not to increase) during healthy aging, but if nephrosclerosis exceeds that expected for age, and/or the metabolic demand increases due to factors such as obesity or hyperglycemia, snGFR and nephron size may increase.[50,51,101,108] A recent study reported that larger nephrons, nephrosclerosis and a higher degree of IF are predictive of progressive CKD.[109]

The question of whether kidney aging is a physiological process or a result of pathological mechanisms has been extensively debated.[103,104,110] Some researchers argue that the current definition of CKD leads to overdiagnosis among the elderly, as it fails to consider the age-related nephron loss and GFR decline observed in healthy individuals.[103] Thus, an age-adapted definition of CKD was proposed.[44,50,111-116] This age-adapted definition would reduce the prevalence of CKD stage 3a in healthy elderly individuals, alleviate unnecessary patient anxiety, and reduce healthcare expenditures.[13] Also, by adjusting the CKD threshold for the younger populations based on age-adapted reference limits, earlier detection and management of rapidly progressing CKD may be possible.[44]

The current KDIGO guidelines (2012) do not support this suggestion due to the increased mortality and risk of adverse kidney and CVD outcomes observed when GFR falls below 60

mL/min/1.73 m^2 or uACR increases, even in older individuals.[14] However, a more comprehensive analysis of the data underlying this assertion has revealed that mortality risk for the elderly with normal uACR is negligible until GFR falls below 45 mL/min/1.73 m^2, while the risk increases for the youngest age group (18-54 years) if GFR falls below 75 mL/min/1.73 m^2.[44,111,113,117] As a result, some advocates propose using these thresholds in otherwise healthy individuals without elevated uACR.[44]

2.6 Fibrosis and kidney function

Fibrosis development is proposed to be a crucial factor linked to the decline of GFR, which occurs both with age and in various diseases.

2.6.1 Matrix metalloproteinases 7 (MMP7) and 2 (MMP2)

MMP7 and MMP2, members of the MMP family, are involved in extracellular matrix (ECM) turnover and remodulation.[118,119] They contribute to epithelial-mesenchymal transition (EMT), which is pro-fibrotic in the kidney. [120,121] TGF-α and TGF-β/Smad activation stimulate MMP2 production, inducing EMT, and Wnt4 and the Wnt/β-catenin pathway regulate MMP7 expression in the tubulointerstitium after renal injury.[120,122,123] MMP7 is an important pathogenic mediator of renal fibrosis, activating β-catenin through a Wnt-independent pathway,[124] which further induces MMP7 expression, establishing a vicious cycle.[125] MMP7 also upregulates the Col1α2 and Col3α1 genes, leading to increased collagen accumulation.[126] Consequently, MMP7 levels correlate directly with Wnt/β-catenin activity and are upregulated in renal fibrosis, making MMP7 a potential biomarker of pro-fibrotic signaling and/or fibrosis in the kidney.[120,123-128]

MMP7 is commonly expressed in epithelial cells, but only minimally expressed in healthy human or mouse kidneys under normal physiological conditions.[123,126,129] However, MMP7 expression increases in various kidney diseases, including AKI, focal segmental glomerulosclerosis (FSGS), CKD, IgA nephritis, and diabetic kidney disease, particularly in the distal nephron as part of the epithelial response to injury.[120,123,124,129-133] Furthermore, MMP7 has been proposed to be a therapeutic target for treating fibrosis development in the kidney.[124,127] However, whether MMP7 is associated with GFR decline in the general population has not been investigated.

Similarly, MMP2 is expressed at low levels in the proximal convoluted tubules and glomerulus under normal kidney conditions.[134] However, during CKD development, MMP2 is rapidly upregulated and serves as a critical mediator of fibrosis formation by inducing EMT.[134-137] Studies using murine unilateral ureteral obstruction (UUO) models have shown increased MMP2 expression, enhanced EMT activity, tubular atrophy, fibrosis, and kidney failure.[135,138,139] Two knock-out (KO) mice studies have demonstrated that MMP2 deficiency protects against fibrosis development and kidney damage.[135,140] Additionally, a study using an aging rat model found an increase in MMP2 concentration with age.[141] While a few studies in

patients with diabetes or CKD have identified associations between MMP2 and future GFR decline or CKD development,[21,26] general population studies on MMP2 are lacking.

2.6.2 Tissue inhibitor of metalloproteinase 1 (TIMP1)

Only a limited number of studies have investigated the associations between TIMP1, one of the four MMP inhibitors, and baseline GFR, kidney function decline, and fibrosis development. In murine models and human kidney proximal tubular epithelial cell lines, increased TIMP1 concentration promotes age-related fibrosis by enhancing inflammation.[142-144] Neutralization of TIMP1 has hindered fibrosis development in genetically modified mice, and UUO models have shown increased TIMP1 expression, indicating genetic alterations in TIMP1 prior to fibrosis development.[145] Cross-sectional population studies have found a positive association between TMIP1 levels and lower baseline GFR or CKD stage.[137,146-148] We are aware of only one study that investigated the relationship between plasma TIMP1 levels and GFR decline in the general population. This study found a positive association between higher TIMP1 levels and incident CKD.[22]

2.6.3 Other proposed pro-fibrotic markers

Studies using UUO models in rats and mice have demonstrated that fatty acid binding protein 4 (FABP4) contributes to interstitial renal fibrosis by mediating macrophage-to-myofibroblast transition (MMT), inflammation, and lipid metabolism.[149-152] Additionally, inhibiting FABP4 could be a potential treatment to attenuate fibrotic kidney disease and kidney damaged.[149-152]

Galectin 3 (Gal-3) has been found to be upregulated in kidney fibrosis, as well as fibrosis in the liver, lung, and heart.[95,153-157] Animal models have shown that mice lacking Gal-3 exhibit little to no fibrosis development compared to control mice, and Gal-3 inhibitors have shown potential for treating fibrosis.[95,153-155,158-160] In addition to being a pro-fibrotic mediator of kidney fibrosis in murine models, Gal-3 has demonstrated promising findings regarding kidney function decline and fibrosis in population studies.[95,158,161-165]

In 1997, a study found that MCP-1 is involved in the development of interstitial kidney fibrosis in a murine glomerulonephritis model.[166] Inhibition of MCP-1 reduced macrophage and T-cell infiltration, crescent formation, fibrosis, and renal impairment.[166] Subsequent populations studies have shown MCP-1 to be a promising biomarker and predictor for GFR decline in both diabetic and general populations.[15,26,167,168]

The interaction between CD40 Ligand and the CD40 receptor mediates fibrosis in ischemic renal disease. [169-171] In a mouse model, blocking the CD40-CD40 Ligand interaction has been shown to protect against renal structural and functional changes in chronic proteinuric renal disease.[169,170]

2.7 Nitric oxide (NO) and kidney function

Nitric oxide (NO) plays an important role in blood pressure (BP) regulation.[172,173] NO deficiency is associated with endothelial dysfunction in CKD, and it is believed to be

involved in kidney function impairment. In rat models, inhibition of NO leads to progressive kidney damage and HT, while elevated NO-production has protective effects.[173-177]

Arginine, citrulline, ornithine, ADMA and symmetric dimethylarginine (SDMA) are components of the urea cycle (also known as the ornithine cycle) and enhance or inhibit NO formation (Figure 4). Therefore, they can be regarded as markers of the NO metabolism. Increased levels of NO synthase inhibitors (such as ADMA) or limitations in the substrate (arginine) may lead to NO-deficiency.[178] ADMA is by far the most studied of these biomarkers in relation to kidney function, but SDMA has received more attention in recent years. Higher concentration of the amino acids arginine, citrulline, and ornithine have also been associated with lower baseline eGFR and CKD prevalence at baseline.[179]

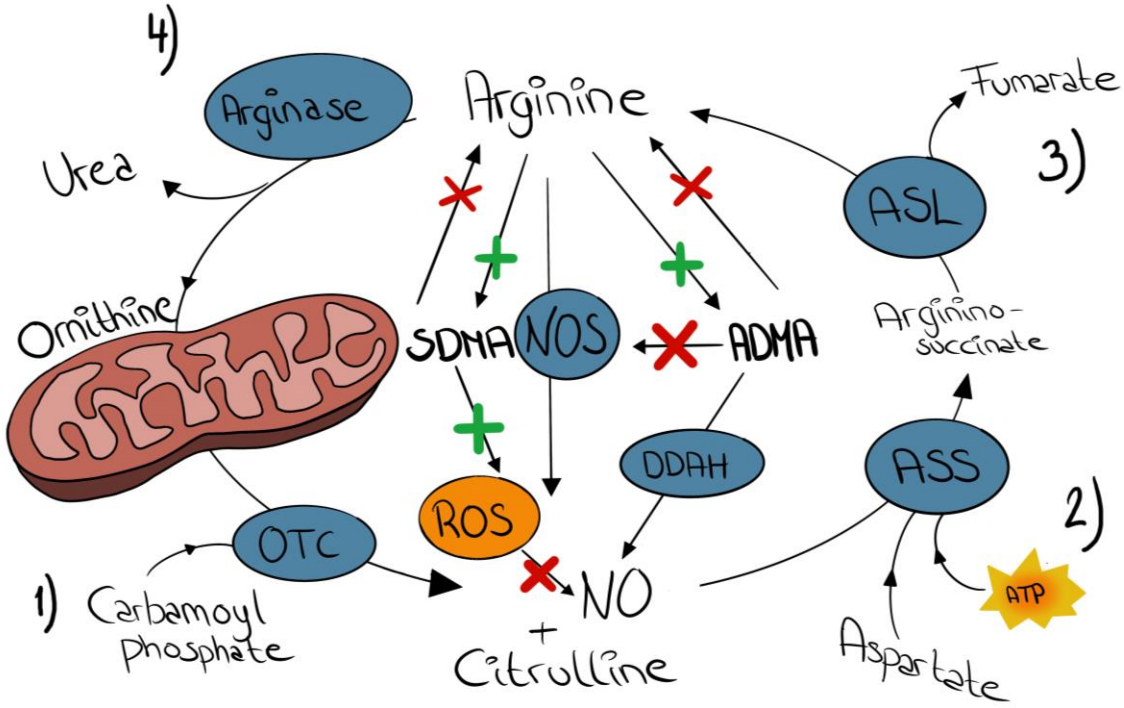


Figure 4: The Urea cycle

1) Carbamoyl phosphate is converted to citrulline with catalysis by ornithine transcarbamoylase (OTC).
2) An ATP dependent condensation reaction catalyzed by argininosuccinate synthetase (ASS), between aspartate and citrulline produces argininosuccinate.
3) Argininosuccinate undergoes cleavage by arginosuccinate lyase (ASL) and form arginine and fumarate.
4) Arginine is cleaved into urea and ornithine. Ornithine is then transported back to the mitochondria to begin the cycle again.

Nitric oxide synthase (NOS) converts arginine to citrulline and NO. Symmetric dimethylarginine (SDMA) stimulates reactive oxygen species (ROS), which inhibits nitric oxide (NO). dimethylarginine dimethylaminohydrolase (DDAH) converts asymmetric dimethylarginine (ADMA) to citrulline and dimethylamine.

2.7.1 ADMA

ADMA is a close relative of arginine and a metabolic by-product of the urea cycle. It naturally occurs in plasma and competes with arginine in the production of NO by inhibiting the NO reaction by binding to nitric oxide synthase (NOS).[172,180] ADMA is metabolized by dimethylaminohydrolase (DDAH) but can also be eliminated unchanged by the kidneys through urine.[172,180] ADMA is a strong marker of atherosclerosis and a risk factor for CVD.[181,182] In individuals with CKD, ADMA levels increase due to impaired DDAH enzymatic activity and reduced renal elimination, leading to inhibition of NO-metabolism.[172,183] ADMA has therefore been proposed to contribute to HT and immune dysfunction in kidney failure.[174] Higher baseline ADMA levels have been found to predict progressive non-diabetic CKD, ESKD, and death.[184,185] Thus, reducing ADMA levels has been suggested as a novel preventive treatment target for CKD progression,[178,184] which could restore NO production and reduce CVD complications.[186] However, we are not aware of any studies that have investigated ADMA as a risk factor for early GFR decline or incident CKD.

2.7.2 SDMA

SDMA is an exogenous isomer of ADMA which stimulates the production of reactive oxygen species (ROS), which in turn inhibits NO production. However, it does not directly inhibit NOS activity. SDMA is exclusively eliminated by the kidneys and shows a stronger correlation with GFR compared to ADMA.[172,180] SDMA levels increase with kidney insufficiency and have been found to predict progression to ESKD in patients with CKD.[187] Additionally, studies have investigated whether SDMA can serve as an endogenous filtration marker of renal function assessment. Strong correlations have been observed between SDMA and inulin clearance as well as creatinine levels.[188] However, further research is needed to determine if SDMA can improve the assessment of renal function beyond the current endogenous filtration markers. The potential role of SDMA as a risk factor for decline in GFR and incident CKD in the general population has not been investigated.

2.7.3 Arginine

Arginine is classified as one of the six conditionally essential amino acids in humans. It can be obtained from protein-containing foods or synthesized in the body through the conversion of glutamine to citrulline in the urea cycle.[189,190] Arginine serves as a precursor to NO, thus important in blood pressure regulation.[191] The availability of arginine is a limiting factor in NO production.[190] The kidneys are probably only a minor route for arginine elimination since plasma concentrations are found to decrease with kidney failure.[172] Supplementation with arginine has shown promising results in restoring NO production, improving renal function, normalizing the Arginine/ADMA ratio, enhancing endothelial function, and reducing inflammation in both kidney transplant mice and patients.[192,193]

2.7.4 Citrulline and ornithine

Citrulline is an important intermediate in the urea cycle and can be formed from ornithine through the breakdown of proline or glutamate/glutamine, as well as from ADMA through the

action of DDAH. Furthermore, citrulline is a byproduct in NO production from arginine, catalyzed by nitric oxide synthase (NOS), and it can be converted back to arginine, if the enzymes arginosuccinate synthase (ASS) or arginosuccinate lyase (ASL) are present within the same cell.[190] Similar to the effects observed with arginine supplementation in kidney transplant patients, citrulline supplementation has been shown to increased arginine availability.[194] Additionally, citrulline has demonstrated renoprotective effects in diabetic mice.[195]

Ornithine plays a centrale role in the urea cycle and is one of the products generated when arginase breaks down arginine to form urea. Ornithine is then recycled within the urea cycle. To the best of our knowledge, only two studies have investigated the role of citrulline in the risk of incident CKD,[179,196] and one study examined ornithine.[179] These studies found that citrulline, but not ornithine, was associated with incident CKD. Furthermore, one study reported that citrulline and ornithine improved the prediction of incident CKD when included in a multi-marker panel.[196]

2.8 Inflammation and kidney function

Markers of inflammation have been linked to a more rapid decline in kidney function with age,[197] and it is suggested that low-grade inflammation plays a role in the development of fibrotic and histological changes in CKD.[198] Several pro-inflammatory biomarkers, including CRP, tumor necrosis factor receptor 1 (TNFR1), and TNFR2, have been associated with GFR decline and CKD development.[15,17,23-25,88,168,198-206] However, studies examining the relationship between inflammatory biomarkers and risk of eGFR decline may be confounded by non-GFR-related factors that affect both creatinine- and cys C-based eGFR.[40,207,208] Thus, future studies investigating inflammatory biomarkers and mGFR decline are necessary to validate these findings.

2.8.1 TNFR1 and TNFR2

TNFR2, also known as tumor necrosis factor receptor superfamily member 1B (TNFRSF1B), is a membrane-bound receptor that binds to tumor necrosis factor alpha (TNFα).[209,210] While TNFR1 is expressed in all cells of the body, TNFR2 is primarily expressed in immune cells but can also be found in endothelial cells, cardiomyocytes, and glia cells. TNFR2 is not expressed in healthy kidneys.[211-213]

Increasing serum concentrations of both TNFR1 and TNFR2 correlate with various glomerular structural lesions observed in kidney biopsies of patients with DM2, suggesting their involvement in early diabetic nephropathy.[198] Plasma TNFR2 and TNFR1 have been associated with increased risk of incident CKD in diabetes and ESKD in diabetic CKD patients.[200,204]

Furthermore, longitudinal increases in both TNFR1 and TNFR2 have been linked to a greater risk of progressive CKD, independent of baseline biomarker level and kidney function.[23] Similarly, in general population cohorts, TNFR2 was associated with both accelerated eGFR

decline and incident CKD based on eGFR.[15,17,205,206] However, in a previous RENIS study, TNFR2 was not associated with a steeper change in mGFR during a median follow-up period of 5.6 years; instead, it was associated with a slower rate of change.[207] Several explanations may account for these discrepant results, however, we speculate that TNFR2 may be associated with a phase of hyperfiltration lasting several years in relatively healthy individuals, such as those with prediabetes or metabolic syndrome. Alternatively, the results from previous studies may have been influenced by non-GFR related factors.[40]

2.9 Prediction of GFR decline

Current kidney function biomarkers, such as eGFR and ACR are suboptimal prediction tools for early GFR decline when GFR is still in the normal range (>60 mL/min/1.73 m^2).[33,34] However, the risk of CVD and ESRD increases when GFR drops < 60 mL/min/1.73 m^2, particularly < 45 mL/min/1.73 m^2.[10] A steeper GFR decline per se has also been associated with an increased risk of CVD, ESRD, and premature death.[34,214]Thus, novel biomarkers are needed to identify underlying mechanisms and individuals at risk of accelerated GFR decline for early preventive measures.

3 AIM OF THE STUDY

1) Primary aim
To investigate the association of novel serum protein biomarkers with accelerated mGFR decline or incident CKD in a general population without pre-existing diabetes, CVD, or kidney disease. The biomarkers of interest were identified from previous studies of eGFR decline and were representative of central pathophysiological mechanism of renal aging and CKD development.

2) Secondary aim
To assess the validity of associations between biomarkers and eGFR decline relative to decline in mGFR.

4 METHOD

4.1 Study population: The RENIS cohort

4.1.1 The Tromsø study: Tromsø 6 (T6)

The Renal Iohexol Clearance Survey in Tromsø 6 (RENIS-T6) originated as a sub study of the sixth wave of the Tromsø Study, Tromsø 6 (T6).[215,216] A total of 19.762 residents in Tromsø county aged 30-87 were invited to participate in T6. The invited participants included all residents aged 60-87, and a 40% random sample of those aged 43-59.

4.1.2 The Renal Iohexol Clearance Survey in Tromsø 6 (RENIS-T6)

The baseline study RENIS-T6 was conducted between November 2007 and June 2009. The target sample size was calculated based on the power to demonstrate an association between baseline GFR and incident CVD.

All participants invited to T6 aged 50-62 (n=5464) who completed the two parts of T6 (65%, n=3564) and did not report a previous diagnosis of diabetes, CVD, or kidney disease (except urinary tract infections) (n=739), were invited (n=2825). The age-group 50-62 years was chosen to provide a relatively healthy cohort (without high comorbidity and medication use) with a high attendance rate, but with sufficient risk of CKD, CVD, and age-related GFR decline for later end-point studies.

A total of 2107 (75%) responded positively; however, 48 withdrew or did not attend their appointment, and 77 reported allergies to contrast, iodine, latex, or other reasons, leaving 1989 eligible individuals (Figure 5). A total of 1632 participants were included in random order (based on the target sample size) stratified by sex and age. Five of the investigated participants experienced technical failure with the iohexol measurement, resulting in 1627 individuals with GFR measurements at baseline in the RENIS-T6 cohort (representing 82% of all eligible participants) (Figure 5).

4.1.3 The RENIS follow-up (RENIS-FU)

All participants in the baseline cohort, who had not died (n=23) or experienced a possible adverse reaction to iohexol at RENIS-T6 (n=7) were invited to the RENIS follow-up (RENIS-FU) conducted from 2013 to 2015 (n=1597). The median follow-up period was 5.6 years (interquartile range (IQR): 4.3-7.0). In total, 86% (n=1368) responded positively. However, 39 individuals did not attend their appointments, and 5 participants had unsuccessful vein cannulation, resulting in 1324 (97%) positive responding participants with GFR measurements in the RENIS-FU. To measure the intra-individual day-to-day variation of the GFR measurements, a random sample of 6% (n=88) was invited back for a second follow-up GFR measurement within 2-8 weeks after the initial measurement (median 35 days, IQR 22-49).

4.1.4 RENIS-3

The third RENIS examination (RENIS-3) took place from September 2018 to October 2020, with a median follow-up time of 11.0 years (IQR: 10.6-11.5) from RENIS-T6. To minimize possible selection bias, we invited all participants who were eligible at baseline, regardless of whether they were included in the RENIS-T6 or not, except those who had experienced possible adverse reactions to iohexol, had withdrawn in previous rounds, had moved to other parts of the country, or had died (n=1846 invited). A total of 1528 (83%) responded positively, and of these, 91% (n=1384) completed RENIS-3, including 210 participants without previous participation in RENIS (Figure 5). Data collection in RENIS-3 was interrupted on the 12th of March 2020 due to the corona pandemic after 1201 participants had completed examinations. The remaining participants were examined between the 14th of May and 8th of October 2020. Thus, 183 participants were examined during the pandemic.

A total of 1837 individuals have had their kidney function measured at one or several times during the span of the RENIS surveys, resulting in a total of 4423 GFR measurements. Among all the 1837 participants, 73 had four GFR measurements (among those 88 with a repeat GFR in RENIS-FU), 1030 had three, 307 had two, and 427 had one GFR measurement.

4.1.5 Study population selection: papers

In paper 1, which was initiated before data from RENIS-3 were available, the study population consists of participants who have baseline and follow-up measurements after a median of 5.6 years (RENIS-T6 and -FU) (n=1324).

In paper 2, the study population comprises participants with one or more follow-up GFR measurements (n=1410) during 11 years of follow-up. One participant with an error in the cys C measurement was excluded, resulting in 1409 participants included in the study.

In paper 3, only participants with at least one follow-up GFR measurements and available baseline biomarker measurements (missing, n=3) were included, resulting in a final sample size of 1407 participants. For the incident CKD analysis, participants with baseline CKD were excluded, leaving a sample size of n=1382.

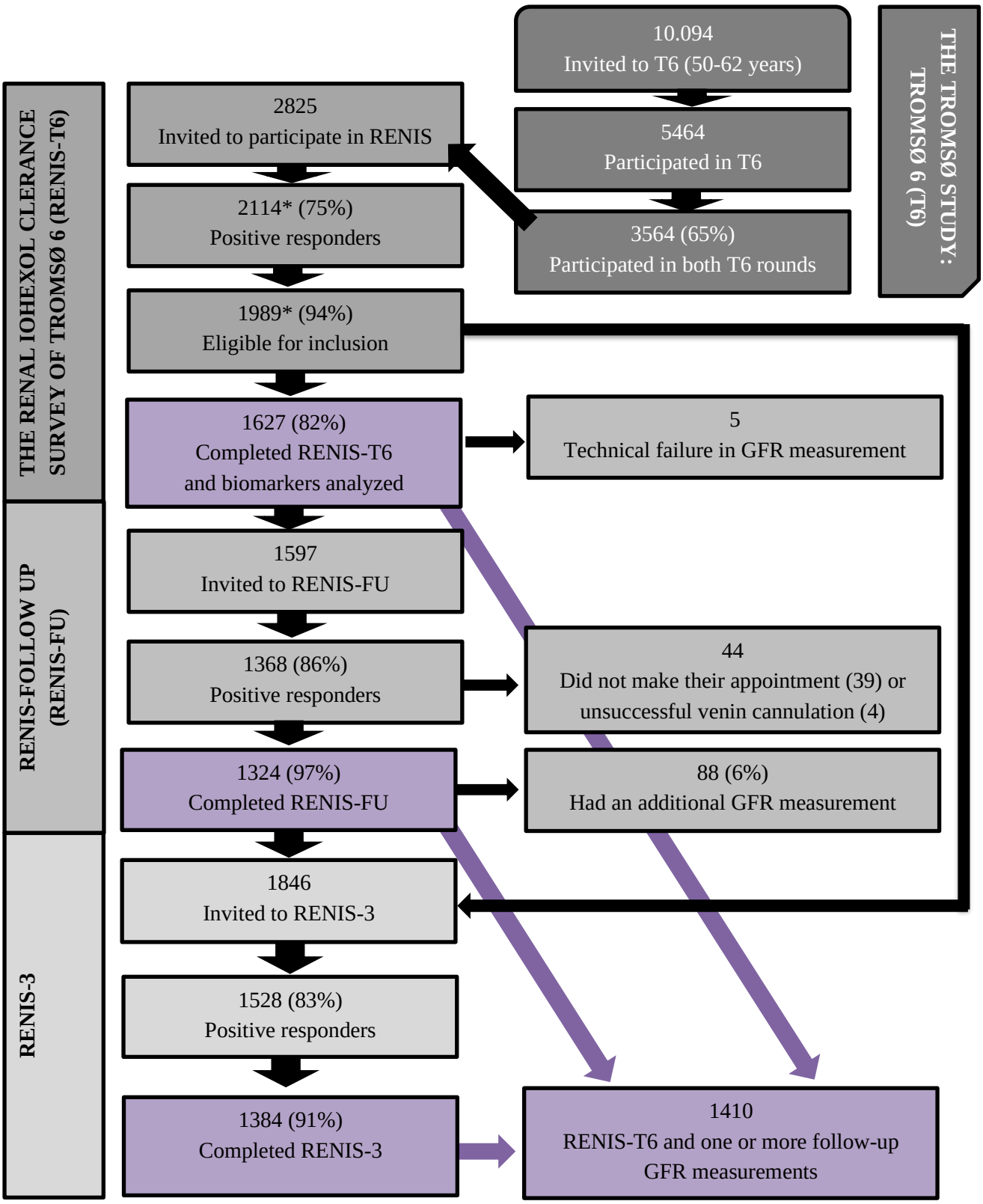


Figure 5: Flowchart of the RENIS-T6, RENIS-FU and RENIS-3 studies.

Including the number (N) of participants invited to T6 and reasons for exclusion/drop-out.
*Miscount in previous publications (Earlier numbers are 2107 and 1982, the correct numbers are 2114 and 1989, respectively).

4.2 Data collection and measurements

All three waves of RENIS were conducted by trained study nurses at the Clinical Research Unit at the University Hospital of Northern Norway (UNN). The protocol for iohexol clearance measurements was identical in all three rounds, except that the iohexol assay was changed from HPLC to LC/MS between RENIS-FU and RENIS 3. Participants met in the morning after an overnight fast and abstained from nicotine for the last 12 hours. They were instructed to drink two glasses of water in the morning before arrival and to avoid consuming large meat meals (>200g) and non-steroid anti-inflammatory drugs (NSADIs) in the two days preceding the examination. If participants showed any symptoms of acute illness on the day of the examination, a new appointment was scheduled. Upon arrival at RENIS-FU and RENIS-3, participants completed a questionnaire that included information on current medication use, past illnesses, smoking and alcohol habits, physical activity, occupation, and diet. The same questionnaire was answered during T6 approximately 2 months prior to RENIS-T6, with the exception of medication use, which was collected at arrival.

All measurements and examinations were conducted between 08:00 and 10:00 am. Fasting blood samples were drawn from a Teflon catheter in an antecubital vein. Weight and height were measured, and the BMI (kg/m^2) was calculated. Blood pressure (BP) was measured three times by a study nurse using an automated device (model UA 799; A&D, Tokyo, Japan) with one-minute intervals after two minutes of rest in a seated position. The average of the last two measurements was recorded. Hypertension was defined as either a systolic BP (sBP) ≥140 mmHg, a diastolic BP (dBP) ≥90 mmHg, or the use of antihypertensive medication. Smoking was categorized as previous, never, or current daily smoker, as defined in paper 2. In paper 3, a dichotomized variable indicating current smoker (yes/no) was used, while in paper 1, a dichotomized variable combining previous and current smoker as ever daily smokers and never smokers was used, in accordance with the smoking variable used in Nelsons CKD risk prediction equation.[217]

Three fresh first-void urine samples were collected on consecutive days in the morning for measurement of urinary albumin to creatinine ratio (uACR mg/mmol) using commercial kits.[218] The urinary creatinine concentration was measured using colorimetric methods (Jaffe`s reaction), and the urinary albumin concentration was measured using an immunoturbidimetric method, both conducted with an autoanalyzer (ABX PENTRA, Horiba ABX, Montpellier, France). The uACR in RENIS-T6 was measured in the main part of Tromsø 6. The uACR measurements in RENIS-FU and RENIS-3 followed the same protocol, but in RENIS-3, the urinary albumin and creatinine concentration were measured using the modular P800 (Roche diagnostics). For each participant, the ACR in each urine sample was determined, and the mean value used in the analysis.

Serum glucose and cholesterol levels were measured using a Modular P800 (Roche diagnostics). Serum creatinine was measured using an enzymatic assay standardized to the isotope dilution mass spectrometry method (CREA Plus, Roche Diagnostocs GmbH,

Mannheim, Germany). Serum cys C was measured using a particle-enhanced turbidimetric immunoassay with reagents from Gentian (Gentian, Moss, Norway) and a Modular E analyzer (Roche Diagnostics). The interassay coefficient of variation for cys C during the study period was 3.1%, and external quality control was provided by Equalis (www.equalis.se). Baseline cys C measurements were recalibrated to the international reference ERM-DA471/IFcC using Cobas 8000 (Roche Diagnostics) in 2013,[219] due to the absence of an established standard during RENIS-T6.[65,220] This standard was established and utilized during data collection in RENIS-FU and RENIS-3. To estimate GFR based on creatinine and/or cys C, the CKD-EPI equations using creatinine, cys C, or both, were used.[28,70]

4.2.1 Iohexol clearance measurement

GFR was measured with single sample plasma clearance of iohexol (Omnipaque, 300 mgI/ml; Amersham Health, London, UK) in all three surveys. A Teflon catheter was inserted into an antecubital vein, and a null sample was drawn. Subsequently, 5 ml of Iohexol (Omipaque 300 mgI/ml; Amersham Health, London, UK), was injected through the catheter. To ensure accurate delivery of the Iohexol dose, the syringe was weighed before and after injection. The catheter was flushed with 30 ml of isotonic saline. Participants were then allowed to have a light breakfast before the iohexol blood sample was draw from the catheter. For baseline and RENIS-FU, the optimal time for measuring Iohexol concentration after injection was calculated in minutes using Jacobsson's method, based on the GFR estimated by creatinine measured in T6 for each individual.[64] In RENIS-3, the optimal time point was calculated based on the individual predicted GFR derived from a mixed model analysis using data from RENIS-T6 and RENIS-FU. The time was recorded using a stopwatch for each participant. The shortest sampling time was set to 180 minutes to ensure complete distribution of Iohexol in the extracellular fluid volume. The extracellular fluid volume (distribution volume) was estimated using the Granerus equation.[221] The minimal extrarenal Iohexol clearance was ignored, in accordance with previous studies. The mGFR was calculated by solving the three equations of Jacobsson using a numerical method, with a small correction for nonimmediate mixing and for non-uniform distribution of the tracer. The GFR was then normalized to a body surface area of 1.73 m² using Dubois's equation.[64,222] The single sample method has been shown to be unbiased compared to multiple sample methods.[62,63,223]

In RENIS-T6 and -FU, serum Iohexol concentration was measured using high-performance liquid chromatography (HPLC), as described in more detail previously.[65,224] The coefficient of variation was 3.0% in RENIS-T6 and 3.1% in RENIS-FU. The mean intra-individual coefficient of variation in GFR measurements, based on the random 5.0% sample (n=88) with two GFRs from RENIS-FU, was 4.2% (95% CI: 3.4-4.9%). To account for potential drift in the iohexol GFR analysis and between the two surveys (RENIS-T6 and FU), a random 6% sample of blood collected during RENIS-T6 was reanalyzed during RENIS-FU. A mean difference of 2.28 mL/min/1.73 m^2 (95% CI: 1.05-3.51) was found between the analysis in

RENIS-T6 and -FU. Accordingly, all the baseline GFR measurements were adjusted by adding this difference.

In RENIS-3 the Iohexol concentration was measured using Liquid Chromatography Mass Spectrometry (LC-MS). To account for potential difference between the two methods, a calibration equation was developed to convert results between HPLC and LC-MS. This was done by reanalyzing a random sample of 300 participants (22%) from RENIS-FU using the LC-MS method.[13] As a result, all Iohexol measurements in the RENIS surveys were calibrated and made comparable.

4.3 Selection of biomarkers

Fifteen of the biomarkers in the study were selected by two researchers (ITTE and TM) based on a literature search conducted on the PubMed database from March to April 2018. Three slightly different searches were performed to broaden the search (Table-S1 Paper 2), focusing on blood protein biomarkers related to GFR decline, CKD development, and renal aging in general populations and patients with diabetes mellitus type 2 (DM2). The searches were limited to articles published within the last five years. The three searches yielded 391, 307 and 975 articles, with some overlap. After reviewing the titles and abstracts and excluding studies involving DM1 and AKI patients, 32, 10 and 47 articles were selected for detailed reading from the three searches, respectively. Both original research articles and review articles were included in the review process. In total, 72 different proteins were identified. Each researcher then compiled a list of 20 proteins based on their relevance in findings. The final selection was made by comparing the lists, considering which proteins could be analyzed using the Luminex method, and whether some had already been analyzed in previous RENIS projects.

The literature search led to the selection of the following 15 proteins, which could be analyzed using the Luminex method: MMP2, MMP7, Gal-3, TNF-related apoptosis-inducing ligand receptor 2 (TRAIL-R2), FABP4, CD40Lig, Growth/Differentiation factor 15 (GDF-15), TEK Tyrosine kinase/TEK (Tie2), soluble urokinase-type plasminogen activator receptor (suPAR), MCP-1, Umod, TIMP1, KIM-1, TNFR1 and TNFR2. TNFR1 and TNFR2 are strongly correlated,[23] thus we selected TNFR2 which had already been analyzed in serum RENIS samples in 2015 using a quantitative sandwich ELISA with a QuantiKine kit from R&D systems, Inc (Minneapolis, MN). TNFR2 was not reanalyzed using the Luminex method.

ADMA, SDMA, arginine, citrulline, and ornithine were previously analyzed as part of RENIS-T6 because they have been proposed to play a significant role in endothelial dysfunction and CKD development.[208] Briefly, the serum levels of these biomarkers were measured using an LC-MS/MS system consisting of a Waters Acquity UPLC/-Class FTN system, using an autosampler and a binary solvent delivery system (Waters, Milfrd, MA), interfaced to Waters Xevo TQ-X benchtop tandem quadrupole mass spectrometer (Waters, Manchester, UK). ADMA, SDMA, arginine, citrulline, and ornithine were obtained from

Sigma-Aldrich (St. Louis, Mo., USA), and labelled versions were purchased from TRC (Toronto Research Chemicals, Ontario, Canada). Their inter-day CV over three different days was <8%. The association of each biomarker with GFR decline was investigated separately in paper 3.
Table 1 provides an overview of the biomarkers included in the thesis, including identification number for the Luminex proteins and main function of each biomarker.

4.3.1 Biomarkers included in the thesis

Table 1: Overview of the biomarkers.

Proteins selected by the literature search and biomarkers of the NO metabolism included in the thesis/papers. Abbreviation, full name, identification number, function, and method used analyze concentration and year analyzed.

Abbreviation	Biomarker name	Protein ID		Function	Method (year analyzed)
		Uniprot	Entrezid		
MMP7	Matrix Metalloproteinase 7	A5GZ72	4316	Fibrosis, matrix remodulation, wound healing.	Luminex multiplex (2018-2019)
MMP2	Matrix Metalloproteinase 2	P08253	4313	Fibrosis, matrix remodulation. Neural system.	Luminex multiplex (2018-2019)
TIMP1*	Metalloproteinase inhibitor 1	P01033	7076	Matrix remodulation, wound healing, pregnancy. Cell proliferation, anti-apoptotic.	Luminex multiplex (2018-2019)
TNFR2	Tumor Necrosis Factor Receptor 2			Inflammation, anti-apoptotic	ELISA (2015)
TRAIL-R2	TNF-Related Apoptosis Inducing Ligand Receptor 2	O14763	8795	Apoptosis, inflammation	Luminex multiplex (2018-2019)
FABP4	Fatty Acid Binding Protein 4	P15090	2167	Binds long-chain fatty acids, fat absorption, transportation, and metabolism, inflammation.	Luminex multiplex (2018-2019)
Gal-3	Galectin-3	P17931	3958	Carbohydrate binding protein, apoptosis, immunity, antimicrobial	Luminex multiplex (2018-2019)

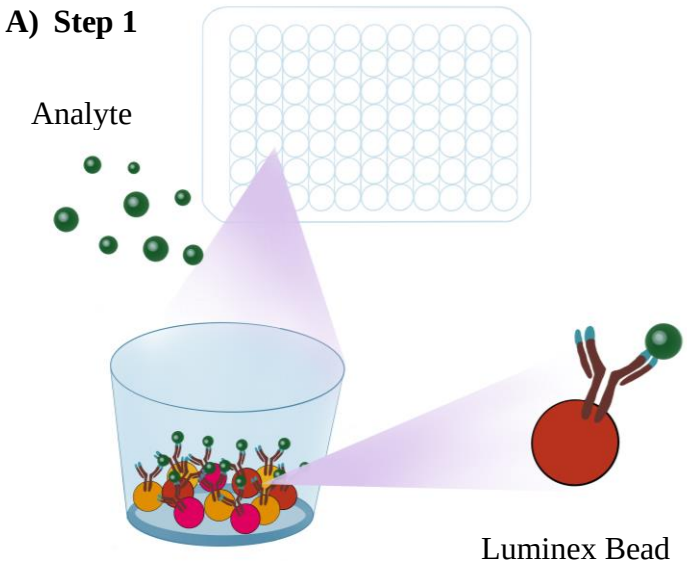
suPAR	Soluble urokinase-type Plasminogen Activator Receptor	Q03405	5329	Phosphate metabolism, apoptosis, inflammation.	Luminex multiplex (2018-2019)
GDF-15	Growth/Differentiation Factor 15	Q99988	9518	Cell damage, inflammation, apoptosis	Luminex multiplex (2018-2019)
MCP-1 (CCL2)	Monocyte Chemoattractant Protein 1	P13500	6347	Inflammation, immunity	Luminex multiplex (2018-2019)
CD40Lig	CD40 receptor Ligand	P29965	959	Immunity.	Luminex multiplex (2018-2019)
Tie2	TEK Tyrosine Kinase	Q02763	7010	Embryonic angiogenesis, immunity, anti-inflammatory.	Luminex multiplex (2018-2019)
Umod	Uromodulin	P07911	7369	Immunity.	Luminex multiplex (2018-2019)
Arginine	Arginine	-	-	Amino acid, cell division, wound healing, immunity. NO-metabolism (blood pressure), urea cycle	LC-MS/MS (20xx)
Citrulline	Citrulline	-	-	Amino acid, urea cycle. Byproduct of NO. Intestinal functionality.	LC-MS/MS (2011)
Ornithine	Ornithine	-	-	Amino acid, urea cycle. NO-metabolism.	LC-MS/MS (2011)
ADMA	Asymmetric Dimethylarginine	-	-	Inhibitor of NO-metabolism. Cytoplasm of all cells.	LC-MS/MS (2011)
SDMA	Symmetric Dimethylarginine	-	-	Exogenous isomer of ADMA. Inhibitor of NO-metabolism.	LC-MS/MS (2011)
KIM-1 (TIM1)	Kidney Injury Molecule-1	Q96D42	26762	Kidney damage marker (proximal tubule), allergies, immunity	Luminex multiplex (2018-2019)

Uniprot: Unique protein ID used to find/identify a protein (https://www.uniprot.org/)

4.3.2 Luminex Multiplex Immunoassays and the Bio-Plex 200 machine

Luminex multiplex assays are a type of immunoassay capable of measuring multiple analytes in one sample simultaneously using color-coded superparamagnetic beads, in a three-step approach. The beads are internally dyed with different proportions of infrared and red fluorophores, which create distinct spectral signatures corresponding to specific bead regions.[225]

First, the sample of blood (or urine) is incubated with beads specific to the analyte(s) of interest, along with their corresponding capture antibodies, forming a complex of bead-antibody-analyte (Figure 6, A). Second, any unbound material is washed away, and biotinylated detection antibodies specific to the analyte(s) of interest are added, forming an antibody-antigen sandwich (Figure 6, B). Third, PE-conjugated streptavidin is introduced, which binds to the biotinylated detection antibodies (Figure 6, C). As a result, multiple analytes of interest can be quantified in a single sample using a dual-laser flow-based detection instrument, such as the Bio-Plex 200.[226]



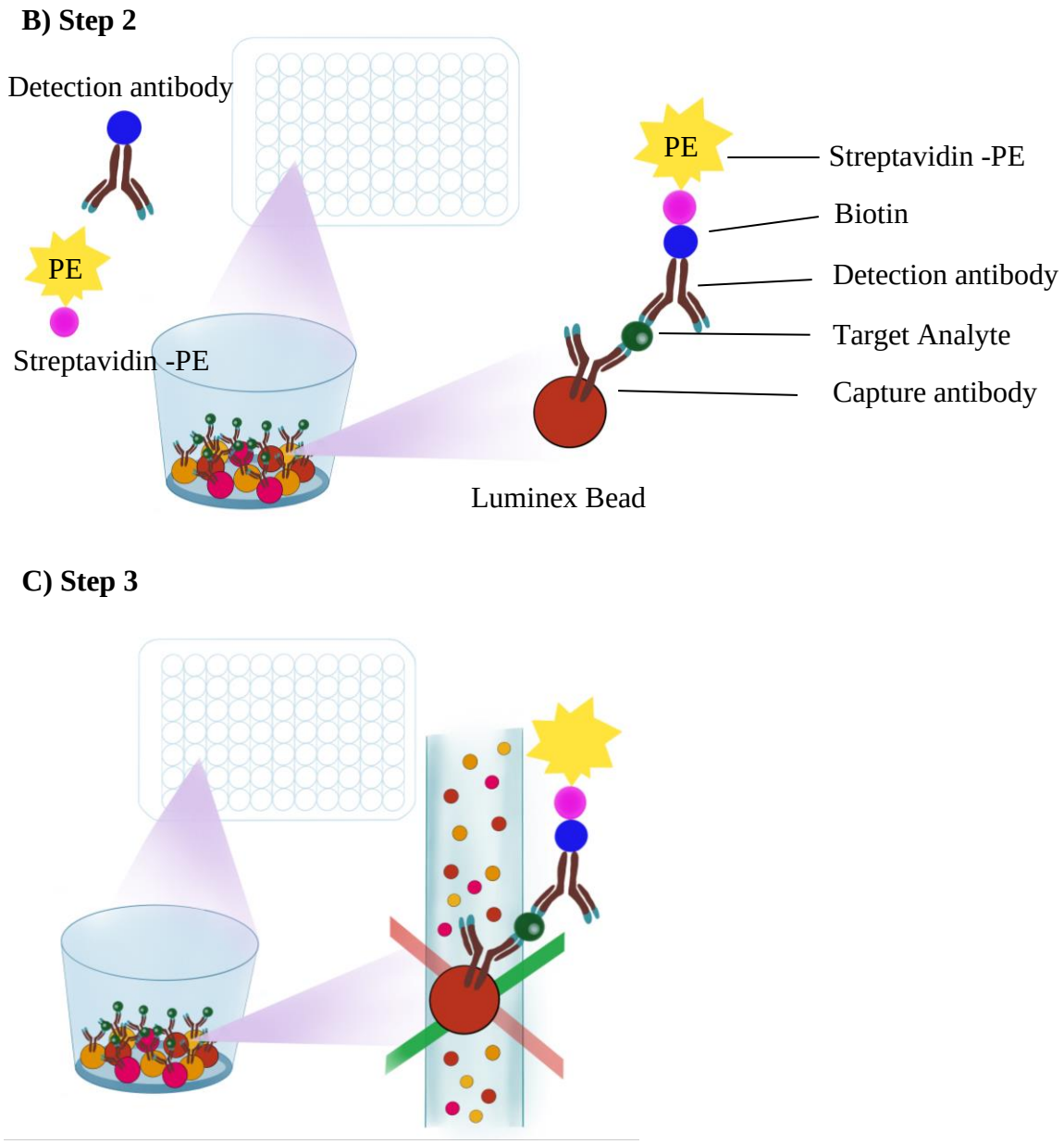


Figure 6: The Luminex Multiplex Method.

The illustrations are adapted from the illustrations on the R&D systems website. [225,226]

The Bio-Plex 200 machine operates based on the principles of fluorescent flow cytometry and utilizes bead-based multiplex assays to measure and distinguish up to 100 different color-coded beads simultaneously in a single sample, using very small sample volumes (<50 µl).[225] Due to the low sample volume, and ability to measure several proteins in the same sample, the Luminex method was ideal for our study, since baseline serum samples from RENIS-T6 is sparce, and we wanted to analyze several predetermined proteins.

4.3.3 Luminex multiplex analysis

The Luminex multiplex analysis was performed at the lab of the Metabolic and Renal Research Group, UiT – The Arctic University of Norway, from June 2018 to January 2019, using the Luminex xMAP multiplex (immunoassay) technology (Bio-Plex 200 systems, BIO-RAD) and human magnetic bead-based assays from R&D Systems (Bio-Teche, Minneapolis, Minnesota, USA). The assays consisted of microplates with 96 wells, standards, and magnetic antibody-coated beads.

Fasting serum samples collected at baseline (n=1627) were frozen and stored at -80 degrees Celsius. Prior to measurement, the samples were thawed in a water bath at approximately 10-15 degrees Celsius. Two internal controls were prepared to measure the day-to-day coefficient of variation (CV) and were treated in the same manner as the baseline samples. However, sampling of the controls was done in 2018, resulting in a shorter storage duration.

Since the proteins required different dilutions of serum samples before analysis, and due to the limited shelf life of the kits after preparation by the manufacturer, the analysis was conducted in two phases using different batches. Nine proteins were analyzed with a 1:2 dilution factor, while four proteins with a 1:50 dilution factor. The same serum samples were used for both analyses. Hence, four proteins (Umod, Gal-3, TIMP1, MMP2) had undergone one freeze-thaw cycle prior to analysis. However, previous studies have shown that these four proteins remain stable even after two to five freeze-thaw cycles.[227-230] To minimize batch-differences, all the microplates and standard solutions used during each analysis phase were from the same batch. All the equipment was kept at room temperature before analysis, and the analysis was performed according to the manufacturer's instructions. A single lab technician conducted the analysis.

Samples were analyzed in a random order. Standards were prepared, and the samples underwent three different steps, involving the addition of magnetic beads, Biotin antibody, and Streptavidin-PE. During each step, the samples were incubated at room temperature on a shaker, washed, and then proceeded to the next step. To enhance precision, nearly all pipetting was performed using a pipetting machine (epMotion 5070).

All samples, including standards and blank, were analyzed in duplicates on the same plate. In the case of any errors during the analysis, the readings were immediately rerun after completing the initial reading. Protein levels were calculated using a five-parameter logistic (5-PL) standard curve, and concentrations were measured in picogram (pg)/ml, displayed as the mean of the duplicate measurements.

4.4 Statistical analysis

4.4.1 Outcome definitions

Incident CKD:
In paper 1, incident CKD was defined as new-onset GFR <60 mL/min/1.73 m^2 and/or ACR ≥3.0 mg/mmol at FU, according to the definition proposed by KDIGO.[14] In paper 2 and 3, as well as in the secondary analysis in paper 1, incident CKD was defined as new-onset GFR <60 mL/min/1.73 m^2 (incident CKD stage 3), as the main objective was to assess GFR decline without the influence of associations between biomarker and uACR.

Two additional, more stringent definitions of CKD were used in paper 1: incident CKD (GFR<60 mL/min/1.73 m^2 and/or ACR ≥3.0 mg/mmol) with a baseline GFR >70 mL/min/1.73 m^2, and incident CKD accompanied by a GFR decline ≥25% from baseline to follow-up, as suggested by others.[231,232] These definitions were used to avoid classifying incident CKD solely due to the expected age-related GFR decline.

Accelerated GFR decline:
Accelerated GFR decline was defined as the 10% of participants in the study population with the steepest annual GFR decline slope.[19] This definition was chosen to ensure a reasonable number of individuals with accelerated GFR decline in this relatively healthy population. Each individual's mean annual GFR change rate was calculated using a linear mixed model regression analysis with a random intercept and slope, and an unstructured covariance matrix. The analysis was adjusted for baseline age, sex, and GFR decline risk factors (as described in each paper), using a within-person centered time-variable. Therefore, all available GFR measurements were included, as the method allows for missing variables at more than one point.[233] The respective cut-off values for the 10% with the steepest annual GFR decline slopes in paper 1, 2 and 3 were: -1.78 mL/min/1.73 m^2/year (n = 131), -1.61 mL/min/1.73 m^2/year (n=140), and -1.62 mL/min/1.73 m^2/year (n=141).

In supplementary analyses in paper 1, accelerated GFR decline was defined as the 25% with the steepest GFR decline using the slope from the mixed model (-1.33 mL/min/1.73 m^2/year, n=325) and two other definitions of accelerated GFR decline used by others. These definitions were, calculated by subtracting GFR at follow-up from baseline GFR and dividing by the follow-up time in years; an annual GFR decline of >3mL/min/1.73 m^2/year (n=138) and annual GFR decline twice the cohort mean (-1.68 mL/min/1.73 m^2/year, n=409).[234,235]

Mean annual GFR change rate:
The annual change in mGFR was calculated using a linear mixed model regression analysis with a random intercept and slope, and an unstructured covariance matrix.[233] (For paper 1, a random sample of 88 participants had three GFR measurements, which allowed for the use of linear mixed models. In paper 2 and 3, the majority of the included persons had three measurements).

Power calculations:
Power calculations for RENIS-3 (paper 2 and 3) was performed by a simulation of 2000 iterations of the linear mixed regression model based on data from RENIS-T6 and RENIS-FU. We assumed a total participation of 1550 individuals in RENIS-3. The power to detect an effect of -0.10 mL/min/1.73 m^2/year or less in GFR decline per SD increase of a risk marker, assuming a normal distribution in the study population, was calculated. With α=0.05, the power was found to be 0.83. Similar power calculations had been conducted previously for RENIS-T6 and RENIS-FU.

4.4.2 Regression analyses

In all regression analyses, skewed biomarker variables were log2 transformed. The associations between serum biomarker concentrations and baseline GFR (and other baseline characteristics) were explored using Spearman's correlation coefficient, scatterplots, linear regression (paper 1 and 3), and linear mixed models (Thesis), and multiple linear regression analyses (paper 3).

The associations between increasing baseline serum biomarker concentrations and mean annual GFR change rates were investigated using linear mixed model regression with a random intercept and slope in all three papers. A negative rate of change signifies a steeper annual GFR change rate with increasing baseline biomarker concentration, and a positive rate of change indicates a slower annual GFR change rate.

The risk of accelerated GFR decline was examined using multiple logistic regression analysis in all three papers. The risk of incident CKD was examined using logistic regression analysis in paper 1 and interval censored Cox regression analysis in paper 2 and 3. Logistic regression was used in paper 1 since we only had two measurements. In paper 2 and 3 we used interval-censored Cox regression analysis, a method that is better suited to determine the risk when the exact time of the outcome is not known but is known to have occurred before, after, or between two points in time (observations). Participants with the outcome at baseline were excluded from the logistic regression analyses.

All regression analyses were adjusted for known or possible confounders of GFR decline chosen *a priori* in different models. These included sex, age, BMI, smoking habits, sBP, BP medication, uACR, and fasting glucose in paper 1 and 3. In paper 3, we also included CRP as an adjustment for inflammation. In paper 2, we adjusted for factors commonly included in biomarkers studies that may be related to creatinine and/or cys C production (non-GFR related factors influencing creatinine or cys C), such as sex, age, BMI, and smoking habits. Adjustment for baseline GFR was included in the logistic and Cox regression analysis, as it could reduce confounding of baseline eGFRcre or eGFRcys due to unmeasured non-GFR related factors.

Non-linear associations between the biomarkers and outcomes were investigated by including interaction terms between biomarker, biomarker, and time (mixed model analysis), and

between biomarker and biomarker in the other regression analyses. If the interaction was statistically significant, it was further explored using quartiles of biomarker concentration.

To test for effect modification by *a priori* selected independent variables on the association between the biomarker and all outcomes of interest, we included interaction terms for these variables in the fully adjusted models. If a significant interaction was found, the analysis was run again, stratified by the modifying variable. In Paper 1, we tested for effect modification by sex, age, smoke, BMI, HT, baseline GFR and uACR. In paper 3, only effect modification by sex was investigated.

In paper 1 and 3, all analyses were rerun using eGFR to see if the results remained the same when using estimate GFR compared to measured GFR. In paper 2, we tested for statistically significant differences between the GFR methods used (mGFR vs each eGFR separately or between eGFR from creatinine and cys C) in terms of the protein's associations with accelerated GFR decline and incident CKD (a detailed description of the methods is given in paper 2).

In paper 1, we evaluated if the addition of a protein biomarker (MMP7) to the statistical model improved the prediction of the outcome (incident CKD and accelerated GFR decline). Using logistic regression, improvement of risk prediction was assessed by comparing the aera under the receiver operator characteristics curve (AUC), continuous net reclassification index (cNRI), and integrated discrimination index (IDI) in a model with the biomarker of interest compared to a model without it, using the likelihood test.

All statistical analysis were performed using STATA version 16.1 (StataCorp, College Station, TX, USA) in paper 1 and version 17.0 in paper 2 and 3. For some analysis, R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria; www.r-project.org) was used.

5 RESULTS

In paper 1, our focus was on the pro-fibrotic metalloproteinases, while in paper 3, we investigated biomarkers representing the NO and arginine metabolism. In paper 2, we examined whether associations between protein biomarkers and eGFR decline are reproducible when using mGFR decline as the outcome.

5.1 Paper 1

The median annual change in GFR for the study population during 5.6 years of follow-up was -0.87 mL/min/1.73 m^2 (IQR -1.33 to -0.41). Concentrations of MMP2 and TIMP1 showed no associations with any of the outcomes. However, higher baseline levels of MMP7 were independently associated with all GFR endpoints after full adjustment. We observed a faster annual GFR decline per SD increase in baseline MMP7 of -0.23 (95% CI: -0.34 to -0.12) ml/ min/1.73m^2 per year, and an increased risk of accelerated GFR decline (n=131) and incident CKD (n=37) with odds ratios (95% CI) of 1.58 (1.30-1.93) and 1.62 (1.19-2.19), respectively.

The association between baseline MMP7 and accelerated GFR decline was non-linear, with an increased risk when baseline concentrations exceeded approximately 2000 pg/ml (close to the median of 1900 pg/ml). A stronger association between MMP7 and accelerated GFR decline was found in individuals with hypertension at baseline and a baseline GFR below 94 mL/min/1.73 m^2 (p for interaction = 0.003) Including MMP7 in the model improved the prediction of accelerated GFR decline, with the AUC increasing by 0.03 ($p<0.001$). According to the NRI, the model improved prediction by correctly reclassifying individuals to the lower-risk category. Similar results were obtained when using eGFR instead of mGFR in the analysis.

5.2 Paper 2

The mean annual GFR change rate for the 1409 participants with at least one follow-up from RENIS-T6 to RENIS 3 was -1.07 mL/min/1.73 m^2 in a linear mixed model adjusted for baseline age, sex, BMI, smoking, blood pressure medication, sBP, and fasting glucose, using a within-person centered time-variable. Out of the 13 selected proteins for paper 2, one (KIM-1) was excluded because most participants had undetectable levels.

Among the 12 protein biomarkers included in paper 2, higher concentrations of four were associated with accelerated mGFR decline, five with eGFRcre decline, and six with eGFRcys and eGFRcyscre decline. Statistically significant differences were observed between eGFRcys and mGFR decline for four proteins (TRAIL-R2, FABP4, TNFR2 and Umod), and between mGFR decline and eGFRcyscre for one protein (Gal-3). The associations with eGFRcre decline differed statistically from eGFRcys for three proteins (TNFR2, Tie2 and Umod).

Most associations and differences between the GFR methods remained significant after adjustment for age, sex, BMI, and smoking (model 1). However, in model 2, which included baseline GFR, only one out of four proteins for eGFRcre and one out of five proteins for

eGFRcys (which showed significant associations in model 1) remained associated with eGFR decline, while three out of three remained associated with mGFR decline. Five proteins showed statistically significant different results between eGFR and mGFR in model 1, and two proteins in model 2.

As with accelerated GFR decline, most associations between proteins and incident CKD, as well as the different results compared to mGFR, remained similar after adjustment for age, sex, and BMI, and were attenuated after additional adjustment with baseline GFR.

Several biomarkers generally exhibited different associations with eGFR decline compared to mGFR decline. For eGFR, most associations were found in analysis using cys C in the estimation equation compared to creatinine. More biomarkers showed statistically significant different associations with eGFRcys decline than with eGFRcre decline, compared to mGFR. More proteins showed associations in models that did not include adjustment for baseline GFR. Only MMP7 was associated with GFR decline using both mGFR and eGFR in fully adjusted models for both outcomes.

5.3 Paper 3

All participants from RENIS-T6, with available baseline serum levels of the endogenous inhibitors of NO (ADMA and SDMA) and the NO precursors (arginine, citrulline, and ornithine), and at least one follow-up GFR measurement, were included in the study (n=1407). The median follow-up time was 11 years.

Each SD increase in baseline ornithine concentration was associated with a steeper annual GFR change rate of -0.07 mL/min/1.73 m² (95% CI: -0.13 to -0.001) after full adjustment. SDMA was associated with a less steep annual GFR change rate of 0.07 mL/min/1.73 m² (95% CI: 0.002 to 0.13). Higher baseline citrulline and ornithine were associated with accelerated GFR decline, with OR of 1.43 (95% CI: 1.16 to 1.76) and 1.23 (95% CI: 1.01 to 1.49) per SD increase in baseline concentration, respectively. Additionally, increasing baseline citrulline was associated with incident CKD, with a HR of 1.33 (95% CI: 1.07 – 1.66).

In supplementary analysis using eGFRcre instead of measured GFR, the association between Citrulline and accelerated GFR decline was attenuated (OR: 1.24 (95% CI: 1.03 - 1.49), while the HR for incident CKD was similar at 1.34 (1.03 – 1.73). SDMA was associated with accelerated GFR decline using eGFR (OR:1.41 (95% CI: 1.16 – 1.73), but not when mGFR was used. Neither ADMA nor arginine showed any associations with either estimated or measured GFR.

5.4 Additional analysis

In addition to the analyses presented in paper 2, we investigated the association of each protein biomarker with baseline mGFR and the annual mGFR change rate using linear mixed model regression analysis. Most proteins showed a negative association with baseline GFR, except for Umod, where higher concentrations were associated with higher baseline GFR. Three proteins did not show a significant association with baseline GFR after adjustment (

Table *2*).

The results for the annual GFR change rate are provided in Table 3; however, they were not included in paper 2 due to reviewer feedback urging us to limit the number of outcomes and results. In Table 3,Table 4 we summarize the associations of all 18 biomarkers included in this thesis with the mean annual GFR change rate, the risk of accelerated GFR decline, and incident CKD using mGFR.

Table 2: Baseline association between the biomarkers and measured GFR (mGFR)

Protein	Crude analysis β (95% CI) P-value	Model 1 β (95% CI) P-value	Model 2 β (95% CI) P-value
MCP-1*	-1.09 (-2.44 to 0.26) 0.11	-1.18 (-2.44 to 0.09) 0.07	-1.36 (-2.62 to -0.11) **0.033**
TRAIL-R2*	-3.76 (-4.99 to -2.53) **<0.001**	-3.28 (-4.45 to -2.12) **<0.001**	-3.82 (-4.99 to -2.66) **<0.001**
FABP4*	-5.17 (-5.89 to -4.46) **<0.001**	-3.75 (-4.49 to -3.01) **<0.001**	-4.32 (-5.10 to -3.53) **<0.001**
TNFR2*	-6.37 (-7.38 to -5.36) **<0.001**	-6.56 (-7.51 to -5.61) **<0.001**	-7.06 (-8.02 to -6.10) **<0.001**
CD40Lig	0.46 (-0.23 to 1.16) 0.19	0.46 (-0.19 to 1.11) 0.16	0.37 (-0.28 to 1.02) 0.26
GDF-15	-1.27 (-1.96 to 0.57) **<0.001**	-1.31 (-1.97 to -0.66) **<0.001**	-1.61 (-2.28 to -0.94) **<0.001**
Tie2	0.23 (-0.47 to 0.92) 0.52	-0.50 (-1.16 to 0.15) 0.13	-0.51 (-1.16 to 0.15) 0.13
MMP7	-2.22 (-2.85 to -1.59) **<0.001**	-1.80 (-2.39 to -1.20) **<0.001**	-2.05 (-2.66 to -1.45) **<0.001**
suPAR	-0.96 (-1.66 to -0.26) **0.007**	-0.40 (-1.05 to 0.26) 0.24	-0.49 (-1.14 to 0.16) 0.14
MMP2	-1.42 (-2.10 to -0.74) **<0.001**	-0.85 (-1.50 to -0.21) **0.009**	-0.66 (-1.30 to -0.2) **0.043**
Umod	0.97 (0.28 to 1.66) **0.006**	1.87 (1-21 to 2.52) **<0.001**	1.97 (1.31 to 2.63) **<0.001**
Gal-3	-2.98 (-3.65 to -2.32) **<0.001**	-1.80 (-2.46 to -1.14) **<0.001**	-1.92 (-2.58 to -1.25) **<0.001**

* Log2 transformed, the others are per SD increase

Model 1: Sex, age.

Model 2: Model 1 + systolic blood pressure (sBP), body mass index (BMI), smoking (now, previously, newer), glucose, blood pressure medication and uACR.

5.5 Summary of results

Table 3: Summary of results using mGFR.

BIOMARKER	OUTCOME			PAPER	YEARS OF FU
	Annual mGFR change rate[1]	Accelerated mGFR decline (10 %)[2]	Incident CKD* (mGFR)[2]		
	mL/min/1.73 m^2	OR (95% CI)	OR/HR (95% CI)		
MMP7	**-0.23 (-0.34 to -0.12)**	**1.58 (1.30 to 1.93)**	**1.62 (1.19 to 2.19)**	1	5.6[3]
	-0.08 (-0.15 to -0.01)	**1.74 (1.44 to 2.09)**	**1.53 (1.34 to 1.75)**	2	11.0
MMP2	1.01 (0.83 to 1.23)	0.96 (0.79 to 1.16)	1.10 (0.79 to 1.52)	1	5.6[3]
	0.01 (-0.05 to 0.07)	0.97 (0.82 to 1.16)	1.08(0.91 to 1.30)	2	11.0
TIMP1	0.95 (0.77 to 1.17)	1.03 (0.84 to 1.26)	0.72 (0.46 to 1.11)	1	5.6[3]
TNFR2	0.04 (-0.06 to 0.14)	**1.28 (1.07 to 1.53)**	**1.15 (1.05 to 1.26)**	2	11.0
TRAIL-R2	-0.04 (-0.15 to 0.08)	1.07 (0.75 to 1.53)	**1.48 (1.18 to 1.87)**	2	11.0
FABP4	**0.13 (0.05 to 0.20)**	1.07 (0.84 to 1.37)	1.10 (0.82 to 1.48)	2	11.0
Gal-3	0.02 (-0.04 to 0.09)	1.09 (0.91 to 1.21)	1.09 (0.90 to 1.31)	2	11.0
suPAR	-0.003 (-0.07 to 0.06)	0.98 (0.82 to 1.17)	1.11 (0.98 to 1.25)	2	11.0
GDF-15	0.01 (-0.06 to 0.07)	**1.13 (1.01 to 1.26)**	1.25 (0.90 to 1.74)	2	11.0
MCP-1	0.07 (-0.06 to 0.19)	1.08 (0.77 to 1.52)	0.81 (0.52 to 1.27)	2	11.0
CD40Lig	-0.03 (-0.09 to 0.03)	1.00 (0.83 to 1.19)	1.04 (0.89 to 1.23)	2	11.0
Tie2	0.06 (-0.001 to 0.12)	0.87 (0.74 to 1.04)	0.95 (0.77 to 1.17)	2	11.0
Umod	-0.05 (-0.11 to 0.01) 0.13	0.92 (0.76 to 1.12)	0.82 (0.64 to 1.06)	2	11.0
Arginine	-0.05 (-0.12 to 0.01)	1.07 (0.88 to 1.30)	1.07 (0.87 to 1.31)	3	11.0[3]
Citrulline	-0.04 (-0.10 to 0.03)	**1.43 (1.16 to 1.76)**	**1.33 (1.07 to 1.66)**	3	11.0[3]
Ornithine	**-0.07 (-0.13 to -0.001)**	**1.23 (1.01 to 1.49)**	1.17 (0.93 to 1.47)	3	11.0[3]
ADMA	0.00 (-0.06 to 0.07)	1.11 (0.91 to 1.36)	1.03 (0.84 to 1.28)	3	11.0[3]
SDMA	**0.07 (0.002 to 0.13)**	1.14 (0.91 to 1.41)	1.23 (0.98 to 1.54)	3	11.0[3]

Results from fully adjusted analysis, per SD or doubling in baseline biomarker concentration using mGFR.
*Defined as new-onset GFR <60 mL/min/1.73 m^2 and/or uACR >3 in paper 1, and the risk is expressed as OR. Defined as new-onset GFR <60 mL/min/1.73 m^2 in Paper 2 and 3, and the risk is expressed as Hazard Ratios (HR).

[1]Adjusted for: sex, age, sBP, BMI, smoking, glucose, blood pressure medication, and uACR.

[2]Adjusted for: sex, age, BMI, smoking, and baseline GFR.

[3]Adjusted as [1] in addition to baseline GFR for incident CKD and accelerated GFR decline, and CRP for the dimethylarginines.

Table 4: Baseline differences between participants with one GFR measurement and those participating in all three surveys.

RENIS study population characteristics	Only 1 GFR (RENIS-T6 or RENIS-3)	3 GFRs (All RENIS surveys)	P-value for difference
Participants (n)	427	1103	
Male sex (n)	**167 (39%)**	**544 (49%)**	**<0.001**
Age (years)	**60.1 (56.4 - 62.0)**	**58.5 (54.6 - 61.4)**	**<0.001**
Height (cm)	**169.1 (9.1)**	**170.7 (8.5)**	**<0.001**
Weight (kg)	78.2 (15.3)	79.4 (13.7)	0.11
BMI (kg/m^2)	27.3 (4.4)	27.2 (3.8)	0.58
mGFR (mL/min/1.73 m^2)	93.7 (15.2) *	94.0 (14.0)	0.74
eGFRcre (mL/min/1.73 m^2)	94.5 (10.7) *	94.7 (9.3)	0.72
eGFRcys (mL/min/1.73 m^2)	**103.4 (13.9) ***	**105.8 (12.2)**	**0.002**
eGFRcyscre (mL/min/1.73 m^2)	**101.7 (12.8) ***	**103.1 (11.2)**	**<0.001**
Urinary ACR (mg/mmol)	0.25 (0.1 - 0.6)	0.22 (0.1 - 0.5)	0.02
Systolic BP (mmHg)	132 (19)	129 (18)	0.01
Diastolic BP (mmHg)	**80 (11)**	**83 (10)**	**<0.001**
BP medication (n)	**111 (26%)**	**186 (17%)**	**<0.001**
Fasting blood glucose (mmol/l)	5.4 (0.7)	5.4 (0.5)	0.03
LDL cholesterol (mmol/l)	3.7 (0.9)	3.7 (0.8)	0.88
HDL cholesterol (mmol/l)	1.5 (0.4)	1.6 (0.4)	0.58
Triglycerides (mmol/l)	1.1 (0.8 - 1.5)	1.0 (0.7 - 1.4)	0.08
Ever daily smoker (n)	**310 (73%)**	**730 (66%)**	**0.006**
Current smoker (n)	**106 (25%)**	**203 (18%)**	**<0.001**
Previously smoker (n)	204 (48%)	527 (48%)	-
Never smoker (n)	112 (26%)	369 (34%)	-

Abbreviations: RENIS: The Renal Iohexol Clearance Survey, BMI: body mass index, mGFR: measured glomerular filtration rate, eGFRcre/cys/cyscre: estimated GFR based on the CKD-EPI equation for creatinine, cystatin C or both, ACR: albumin to creatinine ratio, BP: blood pressure, LDL: low density lipoprotein, HDL: high density lipoprotein.

Mean (SD) for normally distributed variables, median (IQR) for skewed variables, and number and percentages (%) for categorical variables.

Missing baseline variables n=427 (n): ACR (1), triglycerides (1), smoke (5).

Missing baseline variables n=1103 (n): ACR (4), triglycerides (2), smoke (4).

*Only 210 at baseline.

6 DISCUSSION

6.1 Methodological discussion

6.1.1 Design

The RENIS is an epidemiological prospective cohort study that represents a healthy general population. Participants with previous CVD, kidney disease, or diabetes at baseline were excluded, resulting in a participant pool assumed to be healthier than the general population. Originally, RENIS-T6 was designed to investigate the longitudinal associations between baseline GFR and incident CVD using measured GFR. The follow-up studies, RENIS-FU and RENIS-3 were conducted to explore risk factors for age-related GFR decline. As the study has an observational design, definitive conclusions regarding causality cannot be drawn.

6.1.2 Random error: Sampling error

In epidemiology, random error arises due to the random variability that may occur when recruiting participants to a study from a reference population meant to be represented, known as sampling error. The sample estimates may differ from the true parameters due to random error, leading to results with poor precision.[236]

6.1.3 Systematic error: Bias

Any systematic error in study design or the conduct of a study that results in an incorrect estimate of the association between exposure and outcome is referred to as "bias".[236] Bias is generally categorized into two main groups: selection bias and information bias.

6.1.4 Selection bias

Selection bias is present when there is a systematic difference in the probability of individuals being included, participating, or continuing in a study based on the exposure and outcome of interest.[236]

The response rates for the RENIS studies were high (75%, 86% and 83%). Overall, 26% of the participants invited to RENIS-T6 did not participate, and 19% were lost from baseline to the first follow-up study (RENIS-FU). Prior to this, approximately 34% of the participants invited to T6 did not participate in T6.[216] These non-responders, or losses to follow-up, could differ from the responders in terms of the exposure and outcomes of interest.[237] Thus, selection bias in the form of non-responder bias and loss to follow-up may be present in the RENIS study. However, the 1627 participants examined at baseline were randomly selected from all eligible individuals. Also, to mitigate internal selection bias, all eligible participants at baseline (including those not initially investigated) were invited to the last follow-up study (RENIS-3), resulting in 210 new individuals undergoing their first examination in RENIS-3.

To assess the presence of selection bias in a study, it is necessary to compare the characteristics of the responders with those of the non-responders and those lost to follow-up. We do know that the non-attendees in T6 and The Tromsø studies, in general, were more

likely to be men (a consistent finding across all age groups).[215,216] However, in RENIS-T6, an equal number of participants from each sex were invited, and an equal number from each sex attended. Nevertheless, we lack information about the motivation of those invited to participate or not. Nonetheless, studies have reported that poor physical health can contribute to non-participation and that research participants have higher education and lower mortality rates than non-participants.[215,216,238,239] Taken together, this may indicate that participants, on average, are healthier than non-participants or that participation in a study influences individuals to adopt healthier behaviors. [215,216,238-240]

While we do not possess data on non-responders in T6, we do have data on all individuals eligible for RENIS-T6 (n=2825) and those who participated in RENIS-T6, RENIS-FU, and RENIS-3.[13] There were only minor differences in age, BMI, dBP, and the three eGFRs between those who participated at baseline and those who did not.[13,65] The differences were similar between those eligible and those who participated in RENIS-FU, except for BMI and eGFR based on creatinine. For RENIS-3 participants, the baseline differences were even smaller, with only the proportion of smokers differing. Furthermore, we compared those lost to RENIS-FU with those included in RENIS-FU, and the differences were small, except for the proportion of current smokers (28% vs. 18%).[241]

In this thesis, baseline characteristics of participants included in all three surveys (with 3 or 4 GFR measurements, n=1103), were compared to one-time participants (baseline n= 217 or RENIS-3 only n=210; total n=427) (Table 4). One-time participants had a higher proportion of women (61% vs. 51%), were older (60.1 vs 58.5 years), had higher sBP and lower dBP (132/80 vs. 129/83), and a higher proportion used BP medication (26% vs. 17%). Additionally, there were more daily smokers (25% vs. 18%) and persons who had ever been daily smokers (73% vs. 66%).

6.1.5 Information bias

Information bias encompasses factors that can impact the accuracy of the information obtained from participants, whether through questionnaires, interviews, or laboratory and clinical measurements.[236]

In the current study, participants completed a questionnaire either before or on the day of the examination. When recalling past lifestyle-related behaviors, there is a possibility of recall bias, where different subgroups may overestimate or underestimate frequencies (reporting bias) depending on their characteristics (e.g., gender) and the specific question being asked. For instance, individuals may have underestimated their smoking habits.[236,237] Although smoking is an established risk factor for GFR decline, smoking was only associated with four proteins and used as an adjustment variable in the analyses. Hence, the results in this paper would probably not be affected by this.

Other sources of information bias could arise from imprecise and inaccurate measurement values due to poorly calibrated instruments with low precision and validity. In the RENIS

study, several measures were implemented to mitigate information bias. The study was conducted at the Clinical Research Unit at the university hospital of North Norway by trained study nurses. A consistent and detailed research protocol was followed across all three surveys. All participants met in the morning after a 24-hour fasting period and had received instructions on dietary restrictions and behavior during the 48 hours preceding the examination (Methods 4.2). These measures minimized the influence of factors that could potentially influence GFR, and other measurements taken on the examination day.

Instruments and equipment utilized in the study, such as laboratory instruments, blood pressure devices, and weights, were of high quality and calibrated. Whenever possible, the same manufacturer was preferred for consistency across surveys. Additionally, random blood samples from previous examinations were reanalyzed for Iohexol during follow-up examinations to account for any drift in the analysis over time and enable direct comparison between the two measurement methods (HPLC and LC-MS). As a result, all GFR measurements in the RENIS study were comparable (Methods 4.2.1).

Information bias or measurement error may also be present in the Luminex analysis. Most of the pipetting during analysis was automated, reducing the risk of day-to-day variation due to human error. However, it is still possible for the automated machine to introduce error. If such error were to occur, it would likely be systematic and affecting all samples and standards to the same extent. Manual steps in the analysis process are more susceptible to error. The Bio-Plex 200 machine provided notifications in case of calculation error during concentration determination. In such cases, appropriate measures were taken, and the samples were immediately reanalyzed. Additionally, protein stability during prolonged storage and freeze-thaw cycles could potentially impact protein concentrations. Thus, measurement error or bias in the protein analysis could have been a concern in papers 1 and 2 in this thesis. The LC-MSMS method employed in paper 3 is considered accurate and reliable.[242]

6.1.6 Confounding and mediation

Confounding occurs when the relationship between an exposure and outcome variable is influenced by a third variable (Figure 7).[243] There is limited knowledge about the expression of the proteins studied in the kidneys, the mechanisms driving or inhibiting their expression, and their impact on GFR. Thus, we adjusted for known or potential determinants of GFR decline that were chosen a priori. However, residual confounding may still be present due to unknown mechanisms involved in kidney aging, CKD development and decline in kidney function.

Distinguishing between a confounder and an intermediate (mediator) in a relationship can be challenging (Figure 7). For example, we adjusted for albuminuria, which may serve as both a confounder and an intermediate factor on the causal pathway between the risk factor (biomarker) and outcome. Thus, we included uACR in the final models for papers 2 and 3. However, in paper 1, which focused on risk prediction, uACR was included in the initial model.

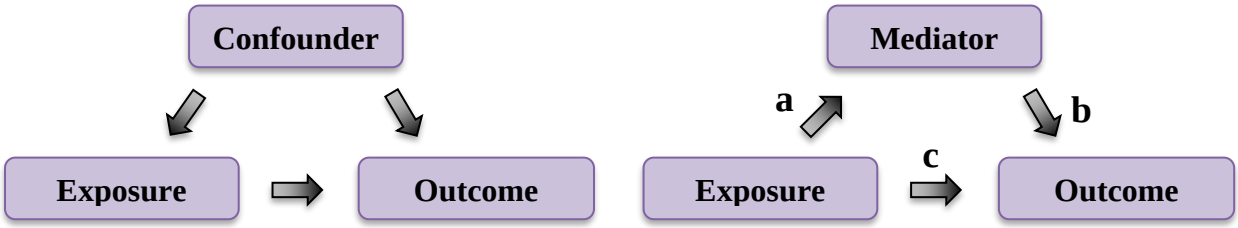


Figure 7: Confounding and Mediation:

A confounder is a variable that influences both the exposure and outcome variable. A mediator explains the relationship between an exposure variable and outcome variable either partly or fully. The strength of the relationship between exposure and outcome is reduced by including the mediator in the statistical analysis.

In this thesis, we hypothesize that the biomarkers, or more likely the underlying mechanism they represent, causally contribute to kidney disease. However, we cannot exclude the possibility of reverse causality, wherein kidney disease or damage leads to an increase in these biomarkers. It is also possible that a biomarker can represent both renoprotective and pathological mechanisms, for example, by exhibiting a dual role in kidney disease development depending on the duration of kidney damage or concentration of the biomarker. This has been suggested for MMP7, which has been found to have reno-protective effects after AKI, but is also associated with fibrosis and CKD development.[131] Similarly, MMP2 tends to exhibit similar behavior, where a threshold level is required to initiate fibrosis.[140]

In conclusion, despite our adjustment, residual confounding remains a concern in this research study due to limited understanding of underlying casual relationships.

6.1.7 Effect modification: Interaction

If the effect of an exposure on the outcome varies in different subgroups or levels of a third variable, it indicates the presence of effect modification (Figure 8).[243]

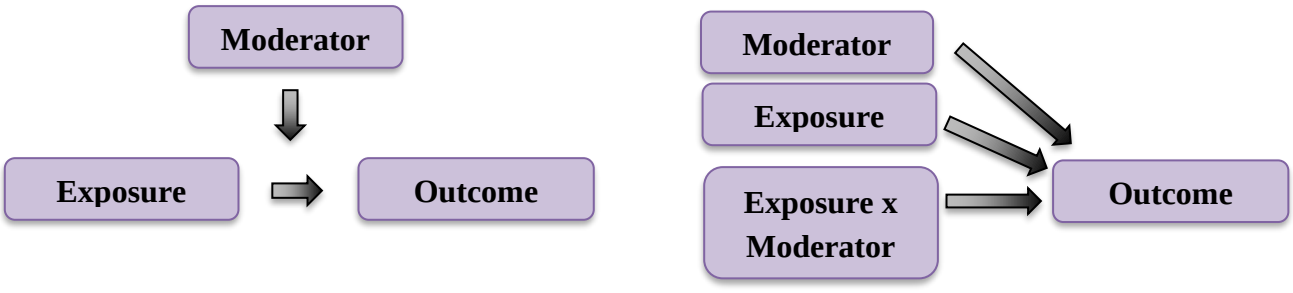


Figure 8: Effect modification:

A variable that affects the direction and/or strength of the relationship between exposure and outcome is a modifier; a significant interaction is present in the analysis.

Considering that sex or gender disparities have been observed in various diseases and disease mechanisms, including CKD development, we conducted tests for interaction by sex. Therefore, we examined effect modification by sex in papers 1 and 3. However, we did not

find any significant interactions with sex. As hypertension has been suggested to mediate fibrosis and is a risk factor for long-term GFR decline, we also tested for effect modification by hypertension in paper 1. Interestingly, we identified a qualitative interaction (effect in one stratum and not the other), where the association between increasing baseline MMP7 and accelerated GFR decline was significant only in those with higher baseline BP (discussed on page 41-42).[243]

6.1.8 Prediction models

In paper 1, we observed that adding MMP7 to a model with known CKD risk factors improved the prediction of accelerated GFR decline and incident CKD when considering metrics such as the AUC, NRI and IDI. These prediction methods offer insight into different aspects of prediction and have distinct strengths and weaknesses. The AUC represents the probability that a given predictive model assigns a higher probability of an event to those who experience the event, thus evaluating the predictive performance of the model.[244,245] Adding MMP7 to our model increased the AUC, indicating improved predictive ability. The NRI is a comparative statistic that assesses the new model's ability to reclassify individuals into the appropriate outcome category or risk group.[244] In the case of MMP7, incorporating it into the model resulted in down-classifying individuals to the non-GFR decline category, correctly identifying more individuals who did not experience accelerated GFR decline. The IDI, based on integrated sensitivity and specificity, measures the difference in discrimination slopes between the models. It can be interpreted as the increase in sensitivity without changes in specificity and, similar to the NRI, distinguishes between events and non-events.[244,246] While the AUC is the primary criterion for determining prediction based on the model, the NRI and IDI should be used to supplement interpretations when assessing effect of the new marker. They provide insight into the marginal strength of the new marker, considering its correlation with the variables in the baseline table.[247]

6.1.9 External validity

The external validity of a study refers to the extent to which the study results can be generalized to other populations or the broader population. It is contingent upon the internal validity of the study. The RENIS study was conducted on a healthy general middle-aged population of North European origin. Therefore, caution should be exercised when extrapolating the findings to other populations with different ethnicities, age distributions, or diseases. Ideally, the results presented in this thesis should have been validated in an independent cohort. However, to our knowledge, no other longitudinal cohort with repeated GFR measurements is available.

The concentrations of the biomarkers under study may vary among different ethnic groups or exhibit different concentrations or effects based on sex, age, or underlying diseases. In the RENIS study, we observed statistically significant differences in baseline concentrations of eight proteins (DGF-15, Tie-2, FABP4, suPAR, Umod, Gal-3, TIMP1, MMP2) between men and women. However, none of the biomarkers in papers 1 and 3 displayed a tendency towards

opposite effects on GFR decline outcomes between sexes. These findings suggest that the baseline differences between sexes generally did not impact the association of the protein with GFR decline.

Experimental studies and population-based studies have established a link between MMP7 and disease progression through its involvement as a mediator in of fibrosis in conditions such as diabetes, various kidney diseases, and aging.[20,124,133] This support our notion that our results obtained from a general population may be applicable to other disease groups as well.

6.1.10 Iohexol clearance

The measured GFR exhibits day-to-day variability due to measurement error and intraindividual variability. This error and variability introduce fluctuations in GFR measurements, making it challenging to accurately capture the true change within and between individuals.[248] Therefore, several measures were implemented to minimize this error, as described earlier (under information bias).

The intra-individual CV, which reflects the day-to-day variability, of the GFR measurements varies depending on the GFR level and the measurement method utilized.[57,248-250] In RENIS, the intra-individual CV was 4.2%, which is lower than what has been reported in most previous studies of measured GFR. When employing the single sample plasma iohexol clearance method, the most significant source of contributing error is the formula used to estimate the distribution volume, which relies on race and gender and is somewhat inaccurate.[57] Additionally, in the RENIS study, a Teflon catheter was utilized for both iohexol injection and blood sampling for iohexol concentration measurement. There is a possibility that a small fraction of iohexol may adhere to the plastic wall of the Teflon catheter after injection. To prevent this, the iohexol injection was followed by flushing with a high volume (30 mL) of sodium chloride (NaCl). Another precautionary measure to avoid contamination was the collection of three discarded samples of 5 mL each from the Teflon catheter before obtaining the single sample for iohexol measurement. However, a study has demonstrated that the technique used in the RENIS study yielded similar results to a procedure employing two Teflon catheters.[223] Ideally, we would have performed a validation in a subset of the RENIS study, involving a different hospital, to mitigate “in-hospital” bias and validate against a gold standard. However, the single-sample method has been validated previously, and conducting validation within the RENIS study would have been costly and inconvenient for the participants.[248]

6.1.11 Definition of GFR outcome variables

To define “accelerated eGFR decline” based on the risk of all-cause mortality and ESKD, the KDIGO workgroup has proposed two definitions using the Alberta Kidney Disease Network (AKDN).[251] The first definition involves a change in the CKD-stage GFR category combined with a >25% decrease in GFR from baseline. The second utilizes a slope analysis, considering both the absolute rate of change (-5 mL/min/1.73 m^2/year) and the percent change (-7% each year or more).[14] Previous population studies investigating GFR decline, which included

individuals at both high and/or low risk of CKD development or CKD progression, have used less stringent definitions such as an annual GFR decline >30%, >5 mL/min/1.73 m^2, or >3 mL/min/1.73 m^2.[234,252,253] However, the RENIS study comprises a healthy general population without diabetes, kidney disease, or CVD at baseline, with a mean annual GFR decline rate of approximately 1 mL/min/1.73 m^2/year. Therefore, to identify a reasonable number of participants with accelerated GFR decline, we opted to define accelerated GFR decline decided as the top 10% with the steepest GFR decline slope in the papers presented in this thesis. The slope obtained from a linear mixed model is more precise and closely associated with the risk of ESKD than a slope based on linear regression, particularly among those with GFR >60 mL/min/1.73 m^2.[254] The annual GFR decline threshold for the top 10% steepest slope obtained with this method after 5.6 years of follow-up was lower, at approximately 1.8 mL/min/1.73 m^2/year (n=131), compared to approximately 3 mL/min/1.73 m^2/year (n=138) when the GFR change rate was calculated using the conventional and less precise method of subtracting the baseline GFR from the GFR at follow-up and dividing by the follow-up time in years. These two methods resulted in approximately the same number of participants with accelerated GFR decline (due to a wider distribution of GFR decline rates using the conventional less precise method).

In the incident CKD analysis in paper 1, we used the KDIGO definition that includes ACR. However, the statistical power in this analysis was low due to a limited number of new-onset CKD events in RENIS-FU (n=37). In papers 2 and 3 based on RENIS 3, which encompassed approximately 11 years of follow-up and included 95 individuals who developed CKD stage 3 (without the uACR criteria), the power of the analysis was higher. Furthermore, in papers 2 and 3, we used interval-censored Cox regression instead of logistic regression. This may account for some discrepancies in the findings concerning TNFR2 in paper 2 compared to a previous RENIS-FU study.[207] Additionally, in papers 2 and 3, we chose not to include the uACR criteria, as our primary focus was on associations with GFR decline. Consequently, protein biomarkers associated with proteinuria would not exhibit the same associations as those with the KDIGO CKD definition (that includes uACR) (Table S3, paper 2).

6.1.12 The Luminex Multiplex method

The Luminex multiplex method allowed us to simultaneously analyze multiple biomarkers using small volumes, which is preferable considering the limited serum volume available from RENIS-T6. However, it is important to note that absolute protein concentrations obtained using the Luminex multiplex method cannot be directly compared to measurements from the same method using different kits or to measurements obtained using different methods such as ELISA. This is because the absolute concentrations detected can vary due to the lack of standardized calibration across different kits and methods. Each manufacturer establishes their own calibration standards, and it is normal for concentrations to differ by a factor of two to five when comparing different methods or different batches of the same method. Nonetheless, the concentrations and results obtained are reliable, and associations between protein levels and, for example, GFR can be compared.

6.2 Discussion of results

6.2.1 Fibrosis and age-related GFR decline

Interstitial fibrosis development plays an important role in various kidney diseases and has been proposed to be a risk factor for age-related GFR decline. Therefore, in paper 1, we examined the relationship between three pro-fibrotic biomarkers in the matrix metalloproteinase family (MMP2, MMP7 and TIMP1) and their association with GFR decline. Our findings revealed that increasing baseline MMP7 concentrations were independently associated with a steeper GFR decline during follow-up, whereas MMP2 and TIMP1 showed no associations with any of the outcomes. The association between MMP7 and GFR decline was confirmed in paper 3, which had a longer follow-up period.

We observed a negative cross-sectional association between increasing MMP7 levels and baseline GFR, which could be attributed to reduced filtration of the marker and subsequent accumulation in the blood as kidney function declines. This is likely due to the small size of MMP7 (28 kDa inactive form and 19 kDa active form).[129] An alternatively explanation for the observed association at baseline could be upregulation of MMP7 during fibrotic development in the kidneys or other organs, or as part of a general fibrotic process in the body for individuals with lower GFR.[255-257] Since we only investigated serum MMP7 levels, the exact source of MMP7 remains unknown. Regardless of the mechanism for higher or lower baseline MMP7 levels, this baseline association may potentially confound the results in the longitudinal analyses.[258] However, the association between MMP7 and GFR decline was consistently observed across various definitions of GFR decline using different statistical methods, even when adjusting for baseline GFR. This argues against confounding and suggests that the associations are not solely due to reduced filtration of the marker.

MMP7 is currently one of the most studied MMPs in kidney disease, alongside MMP2 and MMP9.[118,124,125,259-261] It has been proposed that MMP7 could serve as a marker of renal fibrosis and a potential target for fibrotic treatment.[125,259,261-263] However, to the best of our knowledge, no previous study has assessed the association between MMP7 and GFR decline in the general population. A study investigating urinary levels of MMP7 and its activity (confirmed by mRNA analysis) in patients with CKD compared to non-CKD patients suggested that MMP7 plays an important role in the pathophysiology of CKD.[20] Another recent study further confirmed these findings and provided new insight into the role of MMP7 in CKD development.[264] The study found upregulation of MMP7 in both CKD patients and CKD mouse models compared to controls. Additionally, MMP7 was shown to induced fibrosis and inhibit autophagy in kidney cells.[264]

In paper 1, we observed that individuals with hypertension and GFR < 94 mL/min/1.73 m² at baseline were particularly susceptible to accelerated GFR decline with increasing baseline MMP7 levels. Similar results have been observed in CKD patients, where increasing urinary MMP7 showed a stronger association with lower eGFR (<90).[124] This lead us to speculate that individuals with a low normal GFR accompanied by higher MMP7 levels may represent

a subgroup with early progressive kidney disease. Hypertension itself can also contribute to renal fibrosis,[265] and it is possible that serum MMP7 levels partly reflects this process and help identifies individuals at risk of GFR decline. Although HT is an established risk factor for ESKD and has been associated with CKD,[99,266] we previously reported no association between HT and the mean age-related GFR decline in the RENIS cohort,[241] possibly indicating that additional risk factors or longer follow-up are necessary to develop accelerated GFR decline and incident CKD.

We did not find any associations between MMP2 and GFR decline in the RENIS study, despite previous studies showing higher MMP2 concentrations in CKD patients compared to controls.[267] In a study using least absolute shrinkage and selection operator (LASSO) regression to select biomarkers for prediction improvement of accelerated eGFR decline (-3 mL/min/1.73 m^2/year) in patients with DM2, MMP2 was among the 28 selected biomarkers.[26] Another prediction panel for GFR decline in DM2 patients with different stages of CKD selected nine biomarkers, including MMP2 and MMP7.[21] Increasing plasma MMP2 levels have also been found to improve the prediction of incident CKD and were independently associated with faster eGFRcre decline in non-diabetic patients with coronary artery disease over a mean follow-up of 8.5 years.[268] However, these previous studies mostly focused on patients with underlying disease such as DM or established CKD, which may differ from the age-related GFR decline observed in our healthy cohort. The pathophysiological processes and mechanisms underlying CKD progression in those studies may be different from those involved in age-related GFR decline in our study. Additionally, mouse models have suggested that a threshold level of MMP2 may be necessary to initiate kidney damage and fibrotic development, which may not have been reached in our baseline sample.[140] Therefore, longer follow-up and serial measurements of MMP2 could have provided more detailed information.

Both serum and urinary TIMP1 levels are found to be higher in CKD patients compared to controls, with higher levels observed in serum than urine.[146] Significant correlations between serum and urinary TIMP1 levels and creatinine clearance were also present, suggesting that the retention of TIMP1 is not solely due to declining kidney function.[146] Serum TIMP1 levels have been found to be significantly higher in adults with HT and CKD, as well as in children with CKD, compared to controls, and its levels increase with CKD stages.[137,147,148] A recent study also found a significant association between increasing baseline plasma TIMP1 levels and incident CKD in a middle-aged general population during approximately 20 years of follow-up.[22] However, in the RENIS study, TIMP1 was not associated with GFR decline. A possible reason could be that the follow-up time in RENIS-FU was too short (paper 1) and the study sample to healthy. However, TIMP1 was still not associated with accelerated mGFR decline at follow-up in RENIS-3 (OR: 1.15, 95% CI: 0.97 to 1.36, model 2), not shown in paper 2. Serum TIMP1 levels have been found to increase only in later stages of CKD, and later than MMPs.[148] Indeed, we observed a linear trend of increasing TIMP1 with increasing quartiles of MMP7 concentration and decreasing GFR, indicating a relationship between MMP7 and TIMP1 in the presence of decreasing kidney function.

6.2.2 Novel protein biomarkers for eGFR decline validated by mGFR

Many previous studies have investigated biomarkers and their association with GFR decline using proteomic analysis and advanced statistical methods. In paper 2, we evaluated the association of 13 of these promising biomarkers representing different pathophysiological pathways in the kidney and their association with measured and estimated GFR decline. We found that several proteins showed statistically significant different associations with eGFR decline compared to mGFR decline. These results are discussed in more detail in paragraph 6.2.2.1: "Non-GFR related factors".

Four of the proteins (FABP4, TRAIL-R2, GDF-15, TNFR2) included in our study were among the top six out of 175 biomarkers identified through untargeted proteomic analysis to predict incident CKD in 1098 patients with CVD admitted to a Swedish hospital due to myocardial infarction after a median follow-up of 3.2 years.[88] Another proteomic study investigating kidney function decline in two elderly general population cohorts over a 5-years follow-up identified 20 proteins significantly associated with eGFR decline in both cohorts after adjusting for traditional CKD risk factors.[15] Among these proteins, TRAIL-R2 showed the strongest association with the outcome, and 11 of the proteins also predicted incident CKD. However, none of them predicted annual eGFR decline independently of baseline GFR.

In the present study, we found that TRAIL-R2 (and GDF-15) were associated with incident CKD, supporting their association with CKD development in the general population, not only in CVD patients. Both TRAIL-R2 and GDF-15 are pro-apoptotic proteins expressed in most tissues in the body.[269,270] They are upregulated in the presence of cell death and injury and have been found to be good predictors of mortality.[88,269,270]

Many previous studies aimed at identifying biomarkers associated with or capable of predicting future GFR decline have primarily focused on specific patient cohorts (such as IgA nephritis, DM1, DM2) and CKD populations, and have been limited by using eGFR rather than mGFR. The proteins investigated in this thesis were selected based on promising findings from previous research. However, our results demonstrated that many of the proteins did not associate with either estimated or measured GFR decline in the RENIS study. These discrepancies could be attributed to differences in the study populations, including variations in age distribution, prevalence of underlying diseases and CKD risk factors, as well as variation in follow-up duration and statistical methods employed.

It is important to note that our baseline sample consisted of healthy individuals without diabetes, CVD, or kidney disease. Consequently, our study population was generally healthier than the average general population, and the observed GFR decline in the RENIS study was predominantly driven by the aging process.[13] Therefore, our findings may be considered more as associations with age-related GFR decline rather than associations arising from underlying diseases. The underlying pathological mechanisms contributing to age-related GFR decline may differ from those involved in diabetic kidney disease, hypertensive kidney disease, established CKD, or other specific kidney diseases.

For several of the biomarkers investigated, such as KIM-1, Umod, MMP2, and TIMP1, it is possible that the study population at baseline was too healthy to exhibit pathological increases or decreases in biomarker levels. KIM-1, for instance, was only detectable in 37 individuals and was consequently excluded from the analysis, which suggests that the baseline population had relatively good kidney health.

Six of the 18 biomarkers investigate in this thesis are implicated in inflammation (TNFR2, TRAIL-R2, FABP4, suPAR, GDF-15, MCP-1). The findings from a previous RENIS study and the current study (in which TNFR2 was associated with a slower annual mGFR decline after 5.6 years and a higher long-term risk of accelerated GFR decline after 11.2 years) may suggest an association between TNFR2 (representing low-grade inflammation) and an initial phase of hyperfiltration followed by GFR decline.[207] A recent study indicated that elevated serum levels of TNFRs are more likely a result of systemic production rather than a reflection of reduced GFR (accumulation due to impaired elimination).[271] Therefore, our results could indicate an initial systemic increase in inflammation, which, over the course of several years, leads to kidney damage and subsequent GFR decline, similar to what has been proposed in early diabetes.[272,273]

6.2.2.1 Non-GFR related factors

In paper 2, we found statistically significant differences between the GFR methods used, with regard to the proteins' associations with the GFR decline. This highlights the influence of non-GFR-related factors when evaluating the relationship between proteins and eGFR decline.

Several proteins exhibited associations with accelerated GFR decline and incident CKD in unadjusted models, and there were several differences between the GFR methods used (mGFR vs the eGFRs). The associations and differences remained largely unchanged after adjusting for factors that have previously been associated with non-GFR related influence on creatinine and cys C levels (e.g. age, sex, BMI and smoking: Model1).[81,82] However, certain factors that can affect creatinine and cys C levels, such as fasting insulin levels, muscle mass, and dimethylarginines, were not accounted for in the adjustment, potentially leaving residual non-GFR related confounding when using eGFR.[16] Therefore, it is possible that additional adjustment is required to mitigate the impact of non-GFR-related confounding. Notably, most of the associations and differences observed were attenuated or lost after additional adjustment for baseline GFR. There may be several explanations for this, and one possibility is that the inclusion of baseline eGFR in the model partly blocks the non-GFR related effects on eGFR change, thereby also reducing the longitudinal confounding. Consequently, adjusting for baseline GFR may adjust for some of the confounding factors, allowing eGFR change to better reflect true changes in mGFR.

In general, the majority of differences between mGFR and eGFR were found in analysis using cys C in the estimation equation, indicating a higher propensity for confounding by non-GFR related factors affecting cys C levels. The presence of non-GFR related factors in the

relationship between biomarkers and GFR outcomes could potentially elucidate why many promising biomarkers identified in studies using eGFR did not show associations with mGFR in our study. Given that the RENIS cohort consisted of relatively healthy participants without CVD, CKD, or diabetes at baseline, it is plausible that non-GFR-related associations might be more prominent in other populations.

Several cross-sectional studies have demonstrated that traditional cardiovascular risk factors, including BMI, smoking, lipids, C-reactive protein (CRP), hypertension, and insulin resistance, as well as non-traditional CVD risk factors like ADMA and SDMA, influence eGFR through non-GFR-related pathways.[208,274,275] Consequently, the findings in this thesis highlight the need for caution when interpreting associations between biomarkers and kidney outcomes using eGFR. Even small non-GFR related effects can lead to statistically significant but spurious associations in large epidemiological studies.[40] Therefore, it is preferable to validate promising biomarker studies that employ eGFR with mGFR to ensure robustness.

To address the influence of non-GFR related factors GFR estimation and to obtain more precise GFR estimates, Inker et.al. have proposed the use of a panel of multiple filtration markers (panel GFR). Inker an co-authors argue that a biomarker panel would be less affected by non-GFR related determinants and could potentially be as accurate as mGFR.[35] However, to the best of our knowledge, no biomarker panel has significantly improved the accuracy of GFR estimation compared to eGFR based on creatinine and/or cys C measurements.

6.2.3 NO-metabolism and GFR

Paper 3 is based on the hypothesis that NO deficiency is associated with endothelial dysfunction in CKD and that NO plays a crucial role in kidney function impairment. Therefore, we investigated the endogenous inhibitors of NO (ADMA and SDMA) as well as the NO precursors (arginine, citrulline, and ornithine), and their relationship with mGFR and eGFR decline.

Consistent with previous studies, we found that higher baseline SDMA levels were associated with lower baseline mGFR.[276,277] This association is not surprising considering that 90% of SDMA is eliminated by glomerular filtration in the kidneys.[184] Additionally, increasing baseline SDMA levels were also associated with a slower mean annual mGFR change rate (0.07 mL/min/1.73 m^2/year). It is important to interpret this finding with caution, given the close correlation between SDMA and mGFR. Nonetheless, it suggests a potential association between SDMA (an inflammatory marker) and hyperfiltration, leading to an initial increase in GFR as in participants with conditions such as prediabetes.[272,273]

Higher levels of ADMA and lower levels of arginine have been proposed as risk factors for GFR decline and CKD progression. However, our results indicate that ADMA and Arginine are not risk factors for early GFR decline or incident CKD in the general population.

While the kidneys are considered the main site of net de novo arginine synthesis, a study found that at the whole-body level, net de novo arginine synthesis was maintained in ESKD, possible due to an adaptive increase in citrulline availability and turnover.[278] This suggests that compensatory mechanisms bypass the problem of arginine availability as kidney function declines, even if arginine availability is a rate limiting factor in NO production,[190]. The study also observed increased plasma citrulline concentrations and citrulline turnover, which may be an adaptation enabling a reduced mass of functional nephrons in the kidney to maintain a constant rate of arginine synthesis. [278] Moreover, the production of NO was increased in ESKD, indicating a high turnover of arginine in kidney disease.

Contrary to previous results and hypotheses, that citrulline have reno-protective abilities,[194,195] we found that increasing baseline citrulline levels were associated with accelerated mGFR decline and incident CKD. These results align with another study conducted in the general population, which reported an association between higher citrulline levels and incident CKD over an 8 year follow-up period.[179] Additionally, the same study, along with another, found associations between ornithine and incident CKD,[179,196] consistent with our results showing associations with GFR decline. Collectively, our results are not completely in agreement with earlier biological and epidemiological findings regarding markers of NO metabolism. Therefore, further studies are needed to elucidate the role of the NO metabolism in age-related GFR decline and the development of early kidney disease.

6.2.4 Multi-marker panels: the future in CKD diagnostics?

Several research projects aimed to identify novel risk markers for GFR decline in blood and urine.[15-19,21,25,179,205,234,279-284] However, due to the complex pathophysiology underlying GFR decline and the development of CKD, some researchers have advocated for the use of multi-marker panels to improve risk stratification. [280,285] They argue that a multi-marker panel could provide higher sensitivity and specificity compared to a single marker.[280,285] The involvement of multiple pathophysiological pathways in CKD development and progression makes it unlikely that a single biomarker can fully capture its complexity. Various biomarker panels representing different pathophysiological pathways associated with GFR decline, fibrosis, and CKD development have been tested, but so far, they have not been successful in identifying high-risk individuals for prevention and early treatment.[15,21,26,168,279,285-295]

One promising urinary proteomic panel is the CKD 273 classifier that separates CKD according to renal function and inform on the prognosis of adverse events. It has shown clinical potential and has been validated by others, demonstrating its ability to predict accelerated GFR decline in both CKD and non-CKD patient.[286,292,293,296-300] The panel was developed using urinary proteomic analysis, which identified 273 proteins that differed in the urine of patients with biopsy-verified CKD compared to healthy controls. However, despite its promise, the panel has not been integrated into clinical practice.

If we are to effectively utilize and benefit from the discovery of new and promising biomarkers and panels for disease prediction, it is crucial that the method of analysis is readily

available, affordable, and provides an additional advantage for clinical decision-making. A panel with fewer biomarkers, which is less expensive and more accessible for use, has the potential to be implemented in clinical practice, enabling personalized prevention and treatment strategies for kidney disease.

In our study, we found that MMP7 was associated with all GFR-related outcomes, while TNFR2, TRAIL, Citrulline, and Ornithine showed associations with accelerated GFR decline and/or incident CKD. We did not assess the predictive performance of combining multiple biomarkers into a panel, this may be explored in future studies.

This thesis highlights that the association between certain biomarkers and eGFR decline may not be replicated when using mGFR decline as the outcome measure. Therefore, it is advisable to conduct validation studies of promising biomarkers using mGFR at an early stage in biomarker research.

6.3 Strengths and limitations

The RENIS study has several strengths. Firstly, it utilized a longitudinal design with repeated iohexol clearance measurements, spanning over 11 years, in a relatively large general population with a high participation rate. Furthermore, we investigated the association of biomarkers representing different pathways that may be implicated in kidney function decline and CKD development, using various statistical methods to assess their relationship with multiple kidney outcomes using both mGFR and eGFR. To ensure standardized procedures, all measurements and examinations were conducted by a Clinical Research Unit, minimizing day-to-day variation in the GFR measurements, which was lower than reported in most other studies. Additionally, the use of automated pipetting during the Luminex analysis reduced the risk of variation due to human error.

However, there are certain limitations to acknowledge. While the participation rate in RENIS was high compared to other population studies, there is a possibility of internal selection bias, as healthier individuals may have been more likely to continue participating, while sicker individuals may have been excluded during follow-up. The generalizability of the findings to other age groups and ethnicities is limited since the study only included North European participants aged 50-64 years without baseline CVD, diabetes, or kidney disease. Furthermore, due to the observational design of the study, no casual assumptions can be made.

Misclassification in the incident CKD analysis could have occurred as it was based on a single GFR measurement or estimation at each study visit. The use of the same adjustment variables for all proteins may have led to the misclassification of mediators as confounders, and residual confounding cannot be ruled out. Additionally, the Luminex method used in the analysis had limitations, as it could not analyze unknown proteins, and the discovery of new biomarkers was not feasible. There is a possibility that prolonged storage and freeze-thaw cycles of baseline serum samples prior to protein analysis could have impacted protein

stability and concentration. Furthermore, the manual steps involved in protein analysis are subject to intra-individual variation due to differences in the lab technician’s technique.

7 CONCLUSION

We conclude that the pro-fibrotic biomarker MMP7 is independently associated with GFR decline in individuals without pre-existing diabetes, CVD, or CKD. This association remained consistent and independent regardless of whether GFR was estimated or measured, the duration of follow-up, adjustment variables used, or the definition of GFR decline as an outcome. Furthermore, this association appeared to be stronger among individuals with hypertension or lower baseline GFR. MMP7 also demonstrated improved predictive ability of incident CKD and accelerated GFR decline after a follow-up of 5.6 years. These results suggests that MMP7 may serve as a promising biomarker for early GFR loss and CKD development, potentially representing a target for therapeutic interventions aimed at halting fibrosis development. However, further validation in other cohorts and additional research is necessary to confirm these findings.

Our results also indicate that the dimethylarginines (ADMA and SDMA) are not risk factors for early GFR decline. However, citrulline and ornithine, which represents precursors of NO and are involved in NO metabolism, may play a role in the pathogenesis of age-related GFR decline and CKD development.

In general, we observed that certain serum protein biomarkers showed different associations with GFR decline outcomes when using mGFR compared to eGFR. This indicates the presence of non-GFR related factors that influence the relationship between protein biomarkers and eGFR. Although most of these differences were attenuated by adjustment for covariates, some disparities between the methods persisted. Thus, for some biomarkers, associations with eGFR may not be reproducible with mGFR, or other eGFR methods. Therefore, caution should be exercised when interpreting associations with biomarker studies aimed at identifying GFR decline, and it is advisable to validate such findings preferably using mGFR.

8 PERSPECTIVES

This study underlines the importance of considering non-GFR-related factors that influence the relationship between biomarkers and GFR endpoints when using estimated GFR.

Several of the biomarkers investigated in this thesis have shown promise in previous studies involving different populations using eGFR. However, in this healthy general population, they did not exhibit significant associations with eGFR and/or mGFR decline. Thus, future validation studies using mGFR in populations with underlying diseases are necessary. Particularly, our promising results regarding MMP7 should be validated in other populations.

Given the increasing global burden of CKD, there is a pressing need for improved risk prediction and prevention strategies. Even a moderate reduction in GFR poses a risk for CVD and all-cause mortality. Hence, studies focusing on enhanced prediction and elucidating the underlying mechanisms of early CKD development are highly valuable.

Currently, plans are underway for a third follow-up in the RENIS study (RENIS-4), scheduled to commence in September 2023. In RENIS-4, more participants have likely developed reduced GFR < 60 mL/min/1.73 m^2, thus improving our ability to investigate risk factors for, and the clinical importance of having CKD in old age. In addition, longitudinal studies examining changes in biomarker concentrations could be conducted.

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Papers

Paper 1

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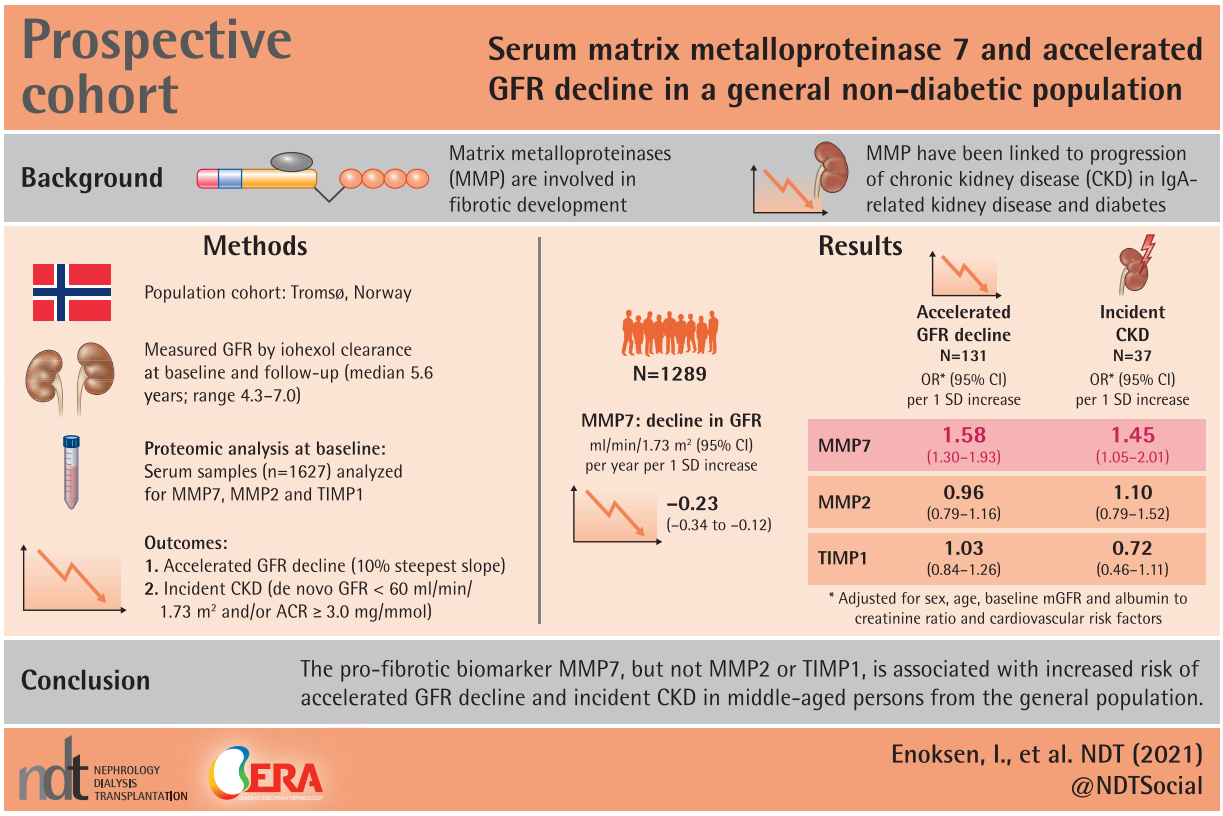
Serum matrix metalloproteinase 7 and accelerated glomerular filtration rate decline in a general non-diabetic population

Inger T. Enoksen [1], Dmitri Svistounov[1], Jon V. Norvik[1,2], Vidar T. N. Stefansson[1], Marit D. Solbu[1,2], Bjørn O. Eriksen[1,2] and Toralf Melsom[1,2]

[1]Metabolic and Renal Research Group, UiT, The Arctic University of Norway, Tromsø, Norway and [2]Section of Nephrology, Clinic of Internal Medicine, University Hospital of North Norway, Tromsø, Norway

Correspondence to: Inger T. Enoksen; E-mail: inger.t.enoksen@uit.no

GRAPHICAL ABSTRACT



ABSTRACT

Background. Age-related reduction of glomerular filtration rate (GFR) is a major contributor to the global chronic kidney disease (CKD) epidemic. We investigated whether baseline serum levels of the pro-fibrotic matrix metalloproteinase 2 (MMP2), MMP7 and their inhibitor, tissue inhibitor of metalloproteinase 1 (TIMP1), which mediates fibrosis development in aging animals, were associated with GFR decline in a general non-diabetic population.

Methods. In the Renal Iohexol Clearance Survey, we measured GFR using iohexol clearance in 1627 subjects aged 50–64 years without self-reported diabetes, kidney or cardiovascular disease.

KEY LEARNING POINTS

What is already known about this subject?

- Matrix metalloproteinases (MMPs) and their inhibitors are involved in fibrotic development and have been linked to progression of chronic kidney disease (CKD) in populations with immunoglobulin A-related kidney disease and diabetes.

What impact this may have on practice or policy?

- It is unknown whether blood levels of MMP7, MMP2 and tissue inhibitor of metalloproteinase 1 (TIMP1) are linked to loss of kidney function in a general population.

What this study adds?

- In middle-aged persons from the general non-diabetic population, we investigated whether serum levels of MMP7, MMP2 and TIMP1 were related to future loss of kidney function assessed by accurate measurements of the glomerular filtration rate (GFR).
- MMP7 was associated with a more rapid loss of the kidney function (GFR) and new-onset CKD, particularly in subjects with hypertension.
- This study suggests that MMP7 may be a promising biomarker for early kidney function loss and CKD development.
- More research is needed to determine if MMP7 may be a useful biomarker for detection of persons at high risk of kidney function loss and new-onset CKD.
- This study may inspire more research on MMP7 as a potential treatment target for halting fibrosis development in the kidney and could lead to new insight into the pathophysiology behind CKD development and kidney function loss in the ageing kidney

After a median of 5.6 years, 1324 had follow-up GFR measurements. Using linear mixed models and logistic regression analyses, we evaluated the association of MMP7, MMP2 and TIMP1 with the mean GFR decline rate, risk of accelerated GFR decline (defined as subjects with the 10% steepest GFR slopes: $\geq$1.8 mL/min/1.73 m^2/year) and incident CKD [GFR $<$60 mL/min/1.73 m^2 and/or urinary albumin to creatinine ratio (ACR) $\geq$3.0 mg/mmol].

Results. Higher MMP7 levels (per standard deviation increase of MMP7) were associated with steeper GFR decline rates [$-$0.23 mL/min/1.73 m^2/year (95% confidence interval $-$0.34 to $-$0.12)] and increased risk of accelerated GFR decline and incident CKD [odds ratios 1.58 (1.30–1.93) and 1.45 (1.05–2.01), respectively, in a model adjusted for age, sex, baseline GFR, ACR and cardiovascular risk factors]. MMP2 and TIMP1 showed no association with GFR decline or incident CKD.

Conclusions. The pro-fibrotic biomarker MMP7, but not MMP2 or TIMP1, is associated with increased risk of accelerated GFR decline and incident CKD in middle-aged persons from the general population.

Keywords: accelerated GFR decline, epidemiology, fibrosis, incident CKD, MMP7

INTRODUCTION

Chronic kidney disease (CKD) affects 30–40% of elderly persons and is associated with increased morbidity and mortality [1]. The death rate by CKD has increased by ~40% worldwide since 1990 [2], and the need for renal replacement therapy in Norway has doubled [3], mainly due to an aging population [2]. Age-related glomerular filtration rate (GFR) decline is a major driving force behind the increasing prevalence of CKD [1]. GFR declines with age even in healthy persons [4], and the decline rate differs significantly between people regardless of risk factors, such as diabetes [5], indicating the potential for preventive measures.

The underlying mechanisms leading to accelerated age-related GFR decline in some persons are unknown. Although interstitial fibrosis (IF) is a key component of the pathophysiology leading to CKD and CKD progression, it is unknown whether increased IF also contributes to accelerated age-related GFR decline. The proportion of healthy kidney donors with IF (defined as $>$5% IF on renal biopsy) has been shown to increase with age, from 1.4% at the age of 18–29 years to 27% at 70–77 years, but there was no effect modification between nephrosclerosis score (including degree of IF) and the decline in GFR with age in this cross-sectional study [6]. However, in another study, the percentage of IF predicted progression of CKD in patients who underwent a radical nephrectomy for a tumour [7]. Only a few longitudinal studies of the general population have investigated the association of fibrotic biomarkers with GFR loss, with inconsistent results [8–10].

Matrix metalloproteinases (MMPs) play a central role in interstitial remodelling. MMP2 and MMP7 contribute to the development of renal fibrosis through conversion of the epithelium to a fibroblastic or myofibroblastic phenotype [epithelial–mesenchymal transition (EMT)] [11–13]. It has been suggested that MMP7 may serve as a non-invasive biomarker for renal fibrosis and that MMP7 could be a potential treatment target to reduce fibrotic development [14]. However, whether increased

levels of circulating MMPs are associated with accelerated GFR decline in the general population has not been investigated.

In the population-based Renal Iohexol Clearance Survey (RENIS), we investigated whether serum MMP2, MMP7 and one of their inhibitors, tissue inhibitor of metalloproteinase 1 (TIMP1), were associated with accelerated decline in measured GFR (mGFR) or incident CKD in middle-aged persons without diabetes or pre-existing CKD.

MATERIALS AND METHODS

Study population

Participants were recruited from the general population as part of The Tromsø Study: Tromsø 6 (T6) [15], a population survey in the municipality of Tromsø, Northern Norway. In total, 2825 persons between 50 and 64 years of age without self-reported kidney disease, cardiovascular disease (CVD) or diabetes were invited to the RENIS-T6 (Figure 1). Of these, 2107 (74%) persons responded positively, and we randomly included 1627 participants according to a predetermined target study size. After a median of 5.6 years (range 4.3–7.0), 1324 (81%) persons participated in the RENIS follow-up (FU), and 88 were randomly selected to have a second FU GFR measurement within 2–8 weeks. Repeated measurements in this subsample enabled us to calculate both the intra-individual variation in GFR measurements and the GFR slope for each individual using a linear mixed model with random intercept and slope [16].

Of the 1324 participants at FU (Figure 1), 25 had baseline GFR <60 mL/min/1.73 m^2, and 30 had urinary albumin to creatinine ratio (ACR) $\geq$3.0 mg/mmol and were excluded in incident CKD analysis. Furthermore, baseline data on smoking and/or ACR were missing for four individuals and C-reactive protein (CRP) was missing for 18 individuals. After calculating the annual GFR slope for each individual who had a FU measurement of GFR, data were available for 1302 individuals in the accelerated GFR decline analysis.

All participants provided written informed consent. The Regional Committee for Medical and Health Research Ethics approved the study, which was conducted in accordance with the Helsinki Declaration. The design and methods of the Tromsø study [15] and RENIS study [17] can be found elsewhere.

Iohexol clearance

GFR was measured at baseline and FU using single sample plasma clearance of iohexol (Omnipaque, 300 mgI/mL; Amersham Health, London, UK) in both surveys [18]. Details are given in Supplementary material. Briefly, 5 mL of iohexol from a syringe was injected into a Teflon catheter in an antecubital vein and flushed with 30 mL isotonic saline. The time of blood sampling after injection of iohexol for each person was determined using Jacobsson's method [19]. The method has been validated and shows substantial agreement with plasma iohexol clearance using multiple sampling methods [20, 21]. The intra-individual day-to-day variation of the GFR measurements in RENIS was 4.2% [95% confidence interval (CI) 3.4–4.9%] [16].

Proteomic analysis

Baseline serum samples ($n = 1627$) were analysed for MMP7, MMP2 and TIMP1 using the Luminex xMap multiplex technology (Bio-Plex 200 systems, Bio-Rad). All samples were analysed in duplicate as described in Supplementary method. Two internal controls were made to measure the coefficient of variation (CV) for the proteins. Intra-assay (variation between duplicates on the same plate) and inter-assay CV (plate-to-plate variation) for the controls for MMP7, MMP2 and TIMP1 were 3.1%, 5.0% and 3.3%, and 19.3%, 6.2% and 5.8%, respectively (Supplementary data, Table S1).

Other measurements

Blood samples were drawn between 08:00 and 10:00 h in the morning after an overnight fast and abstinence from tobacco. Three samples of first void morning spot urine were collected on consecutive days for calculation of ACR (mg/mmol) in unfrozen urine [15]. Data on medication use were collected. Blood pressure (BP) was measured three times with 1 min in between the measurements after the participants had rested for 5–10 min using an automated device (model UA 799; A&D, Tokyo, Japan). The mean value of the last two measurements was used in the analyses. Hypertension was defined as systolic BP (sBP) $\geq$140 mmHg, diastolic BP (dBP) $\geq$90 mmHg and/or the use of antihypertensive medication. Ever smoker was categorized as current or previous daily smoker and never smoker.

Estimated GFR (eGFR) used in sensitivity analyses, was determined using the CKD Epidemiology Collaboration (CKD-EPI) equations based on serum creatinine (eGFRcre) [22], cystatin C (eGFRcys) and the combined creatinine–cystatin equation (eGFRcrecys) [23].

Statistical analysis

Descriptive statistics are given as the means [standard deviations (SDs)] for normally distributed data, medians [interquartile range (IQR)] for skewed data or numbers with percentages.

The association of MMP7 with annual change in mGFR was calculated using mixed model linear regression analyses using random intercept and slopes for time.

Accelerated GFR decline between baseline and FU was defined as the 10% of subjects with the steepest GFR slope. The GFR slope for each subject was calculated using a linear mixed model as described above and adjusted for age, sex and CKD risk factors at baseline [body mass index (BMI), sBP, antihypertensive medication, fasting glucose, high-density lipoprotein (HDL) cholesterol, triglycerides, smoking and CRP]. This method was used to increase the precision of the slope estimates and to minimize confounding in the longitudinal analyses due to baseline associations between the protein biomarkers, CKD risk factors and GFR [24, 25].

In sensitivity analyses, we defined accelerated GFR decline as the 25% fastest decliners based on the GFR slope calculated as described above, and as an annual GFR loss of >3 mL/min/1.73 m^2 or as twice the unadjusted cohort mean (calculated by the

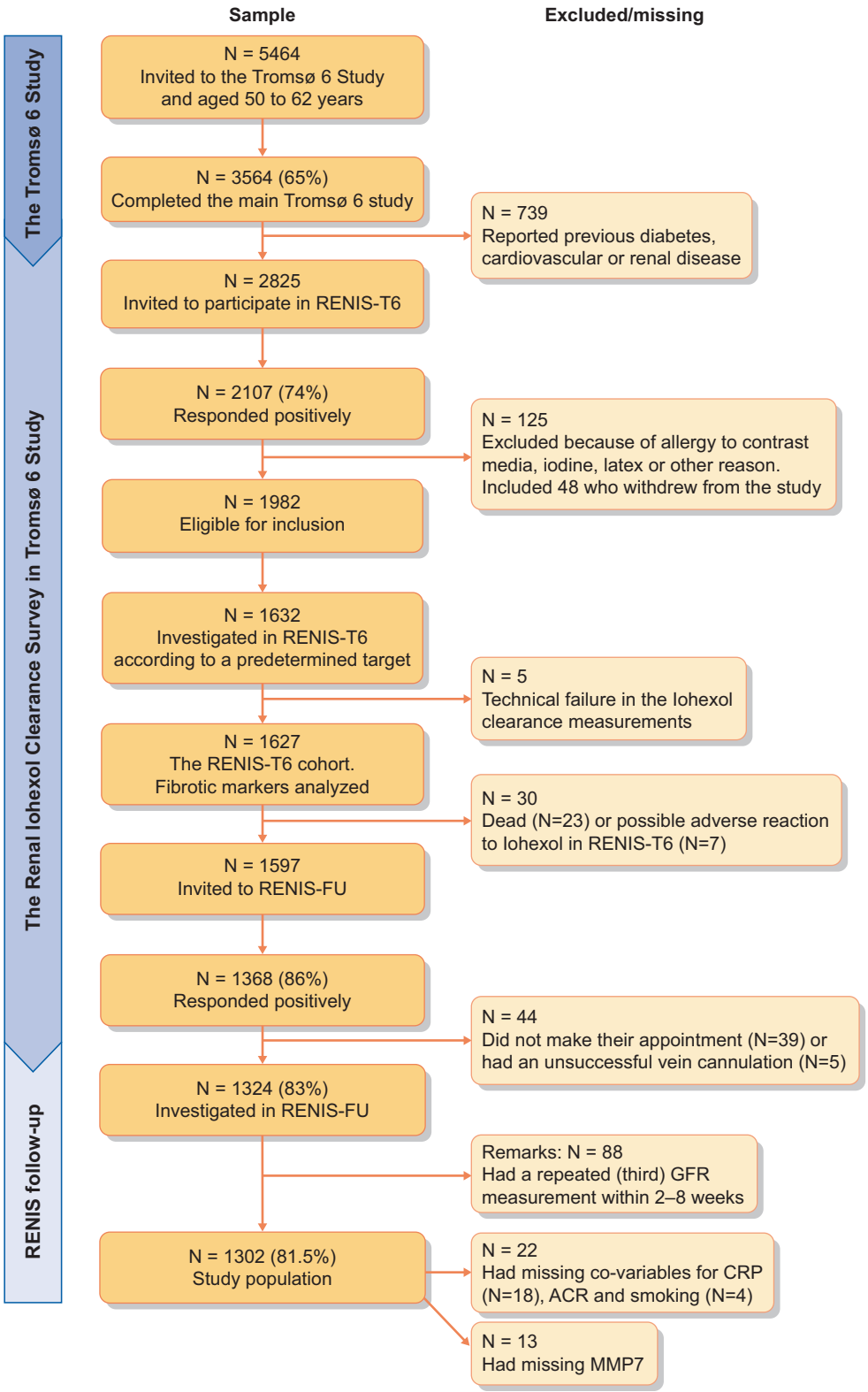


FIGURE 1: Flowchart: an overview of the RENIS cohort. Number (*N*) of invited participants, response rate (%) and number of participants excluded/missing due to different reasons.

difference in GFR from baseline to FU divided by the observation time), methods that have been used in previous studies [8, 26].

The risk for accelerated GFR decline and incident CKD was evaluated using multivariable logistic regression analyses. Fully adjusted models were tested for effect modification by the following subgroups: baseline GFR, sex, smoking, age, BMI, ACR and hypertension, using two-way interaction terms.

Incident CKD was defined as new-onset GFR <60 mL/min/1.73 m^2 and/or ACR ≥ 3.0 mg/mmol at FU [5]. Three alternative

definitions were used in sensitivity analysis (Supplementary data, Table S2).

We adjusted for the following covariates in three separate models. Model 1: sex, age, baseline GFR and ACR; Model 2: Model 1 + BMI, sBP and fasting glucose; Model 3: as Model 2 + ever smoker and the use of antihypertensive medication. Non-linear associations between the fibrotic markers and accelerated GFR decline were investigated in generalized additive models with the mgcv package in R [27].

To evaluate the improvement in risk prediction in models with and without the fibrotic markers, we compared the area under the receiver-operating characteristics curve (AUC) for the nested logistic regression model using the likelihood ratio test [28]. We also assessed the net reclassification index (NRI) and relative integrated discrimination improvement (rIDI) [29], the continuous NRI was used because it overcomes the problem of using categories that do not naturally exist [30].

To evaluate the improvement in prediction of incident CKD we included the newly developed incident CKD risk prediction equation by Nelson *et al.* [31] as the base model.

Finally, internal validation was performed using bootstrapping ($N = 1000$) to all analyses.

All statistical analyses were performed with STATA version 16.1 (StataCorp, College Station, TX, USA) and R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria; www.r-project.org). A P-value of <0.05 was considered statistically significant. For more details on methods see Supplementary material.

RESULTS

Participant and MMP7 characteristics

The study population characteristics at baseline grouped by quartiles of MMP7 are presented in Table 1, and by quartiles of MMP2 and TIMP1 (Supplementary data, Tables S3 and S4). Participants with baseline MMP7 in the upper quartile were older, had higher BP and ACR, had lower GFR, and were more likely to be current or ever smokers and users of antihypertensive medication compared with the three lower quartiles.

Almost all characteristics had a statistically significant change from baseline to FU, except for weight, triglycerides, BMI and ever smokers (Supplementary data, Table S5). These changes were not necessarily clinically significant.

MMP7 concentration showed a statistically significant weak correlation with baseline GFR (r −0.14, $P < 0.001$, Supplementary data, Figure S1), age (r 0.15, $P < 0.001$) and ACR (r 0.13, $P < 0.001$). MMP7 and MMP2 were not correlated, while TIMP1 had a weak correlation with both MMP7 (r 0.15, $P < 0.001$) and MMP2 (r 0.08, $P = 0.001$).

Table 1. Baseline study population[a] characteristics according to quartile of MMP7

Characteristics	Quartile of MMP7, pg/mL (IQR)				P-value linear trend
	Quartile 1 ($n = 322$) (49–1436)	Quartile 2 ($n = 322$) (1437–1900)	Quartile 3 ($n = 322$) (1901–2347)	Quartile 4 ($n = 323$) (2348–7463)	
Sex (men) [*n* (%)]	161 (50)	168 (52)	148 (46)	165 (51)	0.43
Age (years)	57.2 (53.5–61.0)	58.0 (54.3–61.1)	58.6 (55.0–61.3)	60.5 (56.0–61.9)	<0.001
Height (cm)	171.0 (8.9)	171.2 (8.5)	170.4 (8.7)	170.6 (8.3)	0.22
Weight (kg)	78.3 (12.6)	81.1 (13.9)	79.0 (14.4)	79.8 (14.5)	0.84
BMI (kg/m^2)	26.7 (3.4)	27.6 (4.0)	27.1 (4.0)	27.3 (4.0)	0.29
mGFRiohexol (mL/min/1.73 m^2)	95.7 (13.2)	95.7 (13.6)	92.8 (14.2)	91.1 (15.6)	<0.001
eGFRcre	97.1 (90.5–101.6)	97.4 (92.0–101.4)	96.9 (90.0–100.6)	95.6 (87.1–100.5)	<0.001
eGFRcys	108.6 (10.5)	107.3 (11.7)	105.0 (11.3)	102.0 (13.5)	<0.001
eGFRcrecys	105.4 (10.3)	104.6 (10.5)	102.6 (10.3)	100.0 (12.6)	<0.001
Urinary ACR (mg/mmol)	0.20 (0.1–0.46)	0.1 (0.1–0.37)	0.27 (0.1–0.62)	0.31 (0.1–0.66)	<0.001
sBP (mmHg)	125 (114–138)	126 (116–138)	129 (118–143)	131 (119–142)	<0.001
dBP (mmHg)	82 (9)	83 (9)	83 (10)	85 (10)	<0.001
Antihypertensive medication [*n* (%)]	38 (12)	37 (11)	48 (15)	105 (33)	<0.001
RAS inhibitors	16 (5)	25 (8)	30 (9)	61 (19)	<0.001
Fasting blood glucose (mmol/L)	5.3 (0.5)	5.4 (0.5)	5.4 (0.6)	5.3 (0.5)	0.83
LDL cholesterol (mmol/L)	3.6 (0.8)	3.7 (0.9)	3.7 (0.9)	3.7 (0.8)	0.08
HDL cholesterol (mmol/L)	1.6 (0.4)	1.5 (0.4)	1.5 (0.4)	1.5 (0.4)	0.39
Triglycerides (mmol/L)	1.0 (0.7–1.4)	1.0 (0.7–1.4)	1.0 (0.8–1.5)	1.1 (0.8–1.5)	0.03
CRP (mg/L)	1.02 (0.6–1.8)	1.21 (0.66–2.13)	1.17 (0.63–2.28)	1.3 (0.72–2.8)	<0.001
Daily smoker [*n* (%)]	203 (63)	216 (67)	216 (67)	236 (73)	<0.001
Yes, currently	45 (14)	51 (16)	59 (18)	95 (29)	<0.001
Yes, previously	158 (49)	165 (51)	157 (49)	141 (44)	0.12
Never	119 (37)	106 (33)	106 (33)	87 (27)	<0.001
MMP7 (pg/mL)	1192 (918–1345)	1684 (1561–1805)	2113 (1992–2233)	2773 (2519–3186)	<0.001
MMP2 (ng/mL)	311.7 (49.8)	309.8 (52.8)	316.5 (51.7)	323.7 (76.9)	0.017
TIMP1 (ng/mL)	156.8 (24.0)	160.7 (37.7)	163.3 (25.4)	166.3 (26.0)	<0.001

The data are presented as the means (SD) and median (IQR) for continuous variables, and proportions (%) for dichotomous variables.
[a]Study population $N = 1302$, with 13 missing for MMP7 ($N = 1289$ for MMP7 analyses).
mGFRiohexol, mGFR with iohexol; RAS, renin–angiotensin system; LDL, low-density lipoprotein.

Accelerated GFR decline and incident CKD

The median annual change in GFR was −0.87 mL/min/1.73 m^2/year (IQR −1.33 to −0.41). A negative rate of change signifies a decline in GFR. Higher baseline levels of MMP7 were associated with steeper GFR change rates calculated using linear mixed model [−0.23 mL/min/1.73 m^2/year per SD increase in MMP7 in the fully adjusted model, P-value < 0.001 (Supplementary data, Table S6)]. The threshold to define accelerated GFR decline, using the 10th percentile of the distribution of the GFR slope, was −1.78 mL/min/1.73 m^2/year ($n = 131$). Of the 1269 persons with GFR >60 mL/min/1.73 m^2 and ACR <3.0 mg/mmol at baseline, 37 individuals had developed CKD at FU.

Higher levels of MMP7 were associated with an increased risk of accelerated GFR decline ($n = 131$) and incident CKD ($n = 37$) in crude and adjusted analyses (Table 2). Odds ratios (ORs) in the fully adjusted model were 1.58 (95% CI 1.30–1.93) and 1.62 (95% CI 1.19–2.19), respectively (only four variables were included in the fully adjusted model for incident CKD due to few endpoints). All the models were well-calibrated, judged by calibration plots (Supplementary data, Figures S2 and S3). There was a non-linear association between MMP7 and accelerated GFR decline (P = 0.010) in Model 3 of Table 2 as illustrated in Figure 2.

Prediction of accelerated GFR decline improved after addition of MMP7 to a model with age, sex, baseline GFR and ACR and in the fully adjusted model. In the fully adjusted model, the AUC increased from 0.74 (95% CI 0.70–0.79) to 0.77 (95% CI 0.73–0.81) (P < 0.001) (Table 3). The improvement in rIDI was 0.25 (95% CI 0.08–0.41, P = 0.003) and the total NRI was 0.25 (95% CI 0.07–0.43) (Supplementary data, Table S7). The event NRI was −2% while the non-event NRI was 26%, indicating that the total NRI improvement is driven by a positive value for non-events, meaning that adding MMP7 to the model mostly down-classifies individuals correctly to the non-accelerated decline category (to a lower risk). For incident CKD based on

Table 2. Multiple logistic regression analyses with ORs for accelerated GFR decline and incident CKD

Outcome definition:	Accelerated decline[a]			Incident CKD[b]		
Model	OR	95% CI	P	OR	95% CI	P
Crude: Protein only.						
MMP7, per SD increase	1.52	1.28–1.80	<0.001	1.91	1.43–2.55	<0.001
MMP7, first quartile	Ref.			–	–	–
MMP7, second quartile	0.59	0.33–1.07	0.08	–	–	–
MMP7, third quartile	0.90	0.52–1.53	0.69	–	–	–
MMP7, fourth quartile	1.77	1.10–2.84	0.019	–	–	–
MMP7, first to third quartiles	Ref.			Ref.		
MMP7, fourth quartile	2.11	1.45–3.07	<0.001	3.00	1.55–5.78	0.001
Model 1						
MMP7, per SD increase	1.68	1.39–2.04	<0.001*	1.62	1.19–2.19	0.002**
MMP7, first quartile	Ref.			–	–	–
MMP7, second quartile	0.56	0.30–1.02	0.06	–	–	–
MMP7, third quartile	0.84	0.48–1.47	0.54	–	–	–
MMP7, fourth quartile	1.74	1.05–2.87	0.031	–	–	–
MMP7, first to third quartiles	Ref.			Ref.		
MMP7, fourth quartile	2.18	1.46–3.25	<0.001	2.35	1.19–4.64	0.01
Model 2				–	–	–
MMP7, per SD increase	1.66	1.37–2.01	<0.001*	–	–	–
MMP7, first quartile	Ref.			–	–	–
MMP7, second quartile	0.56	0.30–1.03	0.06	–	–	–
MMP7, third quartile	0.82	0.47–1.45	0.54	–	–	–
MMP7, fourth quartile	1.72	1.04–2.85	0.031	–	–	–
MMP7, first to third quartiles	Ref.			–	–	–
MMP7, fourth quartile	2.17	1.45–3.24	<0.001	–	–	–
Model 3				–	–	–
MMP7, per SD increase	1.58	1.30–1.93	<0.001*	–	–	–
MMP7, first quartile	Ref.			–	–	–
MMP7, second quartile	0.56	030–1.04	0.07	–	–	–
MMP7, third quartile	0.81	0.46–1.42	0.46	–	–	–
MMP7, fourth quartile	1.52	0.90–2.56	0.11	–	–	–
MMP7, first to third quartiles	Ref.			–	–	–
MMP7, fourth quartile	1.93	1.27–2.92	0.002	–	–	–

Model 1: adjusted for: sex, age, baseline mGFR and ACR (mg/mmol).
Model 2: adjusted for: Model 1 + BMI, sBP and fasting glucose.
Model 3: adjusted for: Model 2 + ever smoker and BP medication.
[a]Defined as 10% steepest decline (annual GFR decline rate >1.78 mL/min/1.73 m^2 calculated using linear mixed model regression). $n = 1289$ in total and $n = 131$ with accelerated GFR decline.
[b]Incident CKD, defined as new-onset GFR <60 mL/min/1.73 m^2 and/or ACR ≥3.0 at FU ($n = 1256$ in total and $n = 37$ with incident CKD). Only four variables were included in the fully adjusted model (Model 1) due to few outcomes.
*P = 0.008, P = 0.011 and P = 0.009 for the quadratic term of MMP7 in Models 1–3.
**P = 0.23 for the quadratic term of MMP7 in Model 1.

mGFR, MMP7 improved the prediction beyond the recent CKD prediction model proposed by Nelson *et al.* [31], as AUC increased from 0.74 (95% CI 0.64–0.83) to 0.77 (95% CI 0.68–0.86) (P = 0.001) (Supplementary data, Table S8).

The associations with accelerated GFR decline were robust across subgroups (Figure 3). However, a statistically significant effect modification was found for baseline GFR and hypertension. The association of MMP7 with GFR decline was stronger for participants with baseline GFR below the median level (94 mL/min/1.73 m^2, $n = 641$) and for those with hypertension ($n = 542$) (Figure 3).

Internal validation using bootstrapping ($N = 1000$) gave identical ORs but slightly wider 95% CIs and prediction analysis remained similar (Supplementary data, Table S9).

Neither MMP2 nor TIMP1 was associated with accelerated decline or incident CKD (Supplementary data, Table S10).

Sensitivity analyses

Analyses using three alternative definitions for accelerated decline [a GFR decline rate >3 mL/min/1.73 m^2/year ($n = 138$), twice the cohort mean (>1.68 mL/min/1.73 m^2/year, $n = 409$)], and the 25% steepest GFR slope ($n = 325$), revealed similar results (Supplementary data, Table S11). Similar results were also obtained after adjustment for current smoking instead of ever smoking (Supplementary data, Table S12), with three alternative definitions of incident CKD (Supplementary data, Table S2), and after additional adjustment for variables included in Models 2–3 (Table 2) in the incident CKD analyses.

Due to a high inter-assay CV of MMP7, we excluded 30 participants on one Luminex assay where all six standards in the standard curve deviated from expected concentrations by 7.5–28%. Accordingly, the MMP7 inter-assay CV for the total study population was reduced from 19.3% to 13.7%. The ORs for accelerated GFR decline and incident CKD increased slightly for the main analyses and remained similar in the sensitivity analysis (Supplementary data, Tables S13 and S14).

Analysis with eGFR based on the three CKD-EPI equations instead of mGFR revealed similar and statistically significant results in all analyses given in Table 2, although with lower ORs in most models (Supplementary data, Table S15).

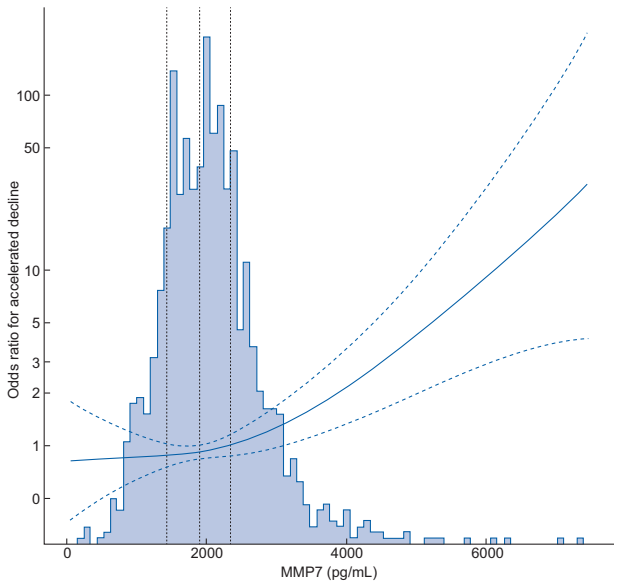


FIGURE 2: The non-linear association between MMP7 concentration and risk of accelerated GFR decline based on Model 3 in Table 2. Baseline MMP7 concentration is on the *x*-axis, the ORs for accelerated decline are on the y-axis and dotted lines indicating the change point for each quartile of MMP7 concentration. The dashed lines indicate 95% CIs. MMP7 concentrations are shown as picogram per millilitre.

DISCUSSION

In this middle-aged cohort from the general population, without self-reported kidney disease, diabetes or CVD, we found that higher baseline serum MMP7 concentrations were independently associated with an increased risk of accelerated GFR decline and incident CKD. MMP2 and TIMP1 showed no association with GFR decline or incident CKD.

We are not aware of previous studies of MMP7 and kidney outcomes in the general population. In patients with diabetes and kidney disease, higher levels of urinary MMP7 (uMMP7) have been linked to increased risk of end-stage renal disease and mortality [32]. In immunoglobulin A nephropathy, higher serum and uMMP7 levels predicted risk of renal failure and disease progression [33, 34]. Among patients with Type 2 diabetes with and without albuminuria, the risk of accelerated GFR decline (defined as ≥5 mL/min/1.73 m^2/year) increased with increasing baseline levels of MMP7 [35]. Our study extends these findings by showing that serum MMP7 is also associated with accelerated GFR decline and incident CKD in a non-diabetic general population without pre-existing CKD or CVD.

We observed a baseline association between higher MMP7 and lower GFR, but it remains unclear whether elevated levels of serum MMP7 originate from a general fibrotic process in the body, from renal fibrosis as part of CKD development, from

Table 3. Prediction of accelerated GFR decline[a] before and after addition of MMP7 to the models

Prediction model (AUC, IDI and NRI)	Model 1	P	Model 2	P	Model 3	P
C-statistics without MMP7 (95% CI)	0.717 (0.672–0.763)	–	0.717 (0.672–0.763)	–	0.743 (0.697–0.789)	–
C-statistics with MMP7 (95% CI)	0.758 (0.717–0.799)	<0.001	0.757 (0.716–0.798)	<0.001	0.769 (0.726–0.812)	<0.001

Model 1: adjusted for age, sex and baseline GFR.
Model 2: adjusted for age, sex, baseline GFR and ACR (mg/mmol).
Model 3: as Model 2 and further adjusted for BMI, ever smoker, sBP, BP medication and fasting glucose. All the analysis includes 1285 participants.
[a]Accelerated mGFR decline is defined as 10% steepest decline (RENIS mean annual decline rate >1.78 mL/min/1.73 m^2, $n = 131$).

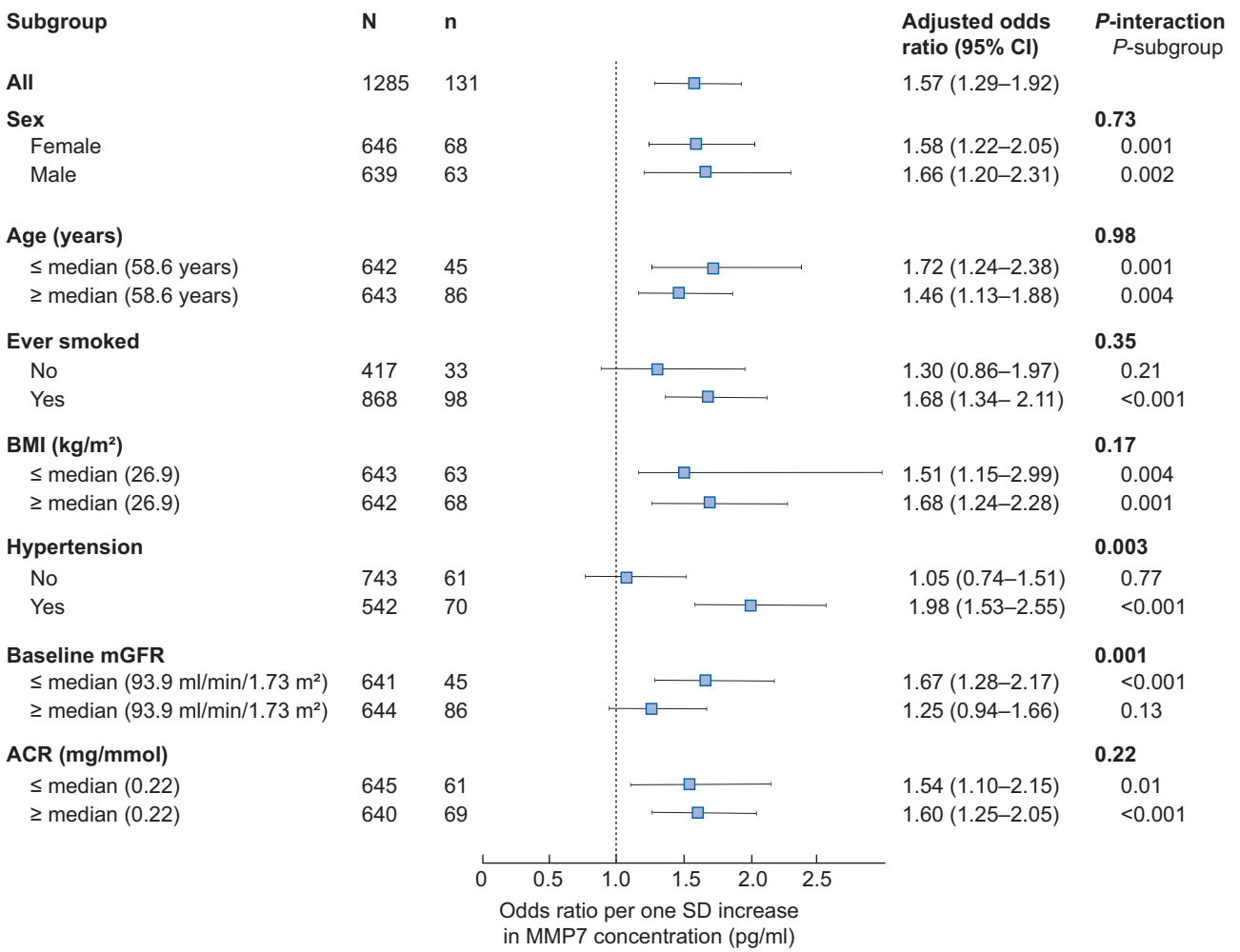


FIGURE 3: Interaction analysis (forest plot) of the adjusted ORs for accelerated decline by various groups per SD increase in MMP7 concentration. Adjusted for sex, age, baseline GFR, BMI, ever smoker, sBP, BP medication, ACR and fasting glucose. P-values for the interaction between MMP7 and the given variables are shown in addition to the P-values for the different subgroups.

other conditions upregulating MMP7, or due to reduced renal elimination [14, 36, 37].

However, an increase in MMP7 expression was reported in renal biopsies of CKD patients compared with healthy controls, which was accompanied by increased uMMP7 and slightly increased serum MMP7. In addition, uMMP7 correlated closely with the fibrosis score of the biopsies [14]. Furthermore, healthy human and mouse kidneys do not express MMP7 [12, 38], but expression increases with CKD severity and is upregulated in renal fibrosis [12, 14, 38]. In addition to being a direct target of the pro-fibrotic Wnt/β-catenin pathway [13, 38], MMP7 is a causal mediator of renal fibrosis by activating β-catenin via a Wnt-independent pathway [14].

MMP7 has been proposed as a therapeutic target for halting CKD progression [14, 39, 40]. A direct role for MMP7 in renal fibrosis development was demonstrated in unilateral ureteral obstruction (UUO) animal models of renal fibrosis. Drug inhibition of MMP7 *in vivo* in these models using MMP inhibitor II showed reduced fibrotic development in MMP7-knockout mice compared with their wild-type counterparts [14]. Studies of the renal protective abilities of resveratrol using human kidney cell lines and UUO models found a reduction in EMT, kidney damage and fibrosis due to MMP7 inhibition [39]. Furthermore, sodium–glucose cotransporter 2 (SGLT2) inhibitors, which delay diabetic kidney disease, were recently studied using *in silico* modelling and plasma samples from a 2-year clinical trial. The results indicated that a reduction in MMP7 levels contributed to the renal protective abilities of SGLT2 inhibitors [40].

The mean GFR decline observed in the RENIS study population was mainly due to age-related GFR decline rather than specific causes of CKD, since these individuals were relatively healthy at inclusion. In rodent models, MMP7 kidney gene expression has been found to increase with age [12, 41]. One study found a 500-fold increase in MMP7 gene expression from young to old animals [12], and another study found an >8-fold increase in gene expression, which also correlated highly to fibrosis grade [41]. However, the increase could not be explained by ageing itself since these associations only were found in kidneys with fibrosis and dysfunction, and not in those without kidney impairment, suggesting that MMP7 plays a role in the pathogenesis of fibrosis and may represent a fibrotic marker.

In our study, those with hypertension or GFR <94 mL/min/1.73 m^2 had a stronger association between MMP7 and

accelerated GFR decline. Although these subgroup effects should be interpreted with caution, we speculate that MMP7 may improve the prediction of subjects with early kidney damage and increased risk of CKD. Similar to our results, another study of patients with CKD also found a stronger association between uMMP7 and GFR loss in those with eGFR <90 mL/min/1.73 m^2 [14].

There is no consensus on the definition of accelerated GFR decline in population-based studies, and some have used an absolute or percentage change in GFR based on the baseline to FU assessment [8, 9, 26]. This method provides an imprecise estimate of GFR change that is more prone to misclassification compared with using the rate of GFR change. Therefore, in this study, accelerated GFR decline was defined as the top 10% of subjects with the steepest GFR slopes calculated by linear mixed models. A slope obtained by a linear mixed model was recently found to be more precise and closely associated with risk of end-stage renal disease compared with a slope based on linear regression, particularly in subjects with baseline GFR >60 mL/min/1.73 m^2 [25]. The majority of patients in the RENIS study had only two GFR measurements and a subset had three GFRs, which is sufficient for using linear mixed models. However, for optimal calculation, three GFRs for a majority of participants would be preferable.

Adding MMP7 to conventional CKD risk factors improved the prediction of accelerated GFR decline assessed by AUC. The prediction increment was only modest; however, although several biomarkers for GFR decline have been proposed, very few improved the prediction of GFR decline or incident CKD over a conventional model using age, sex, baseline GFR and proteinuria/ACR [8, 42].

MMP7 also improved the prediction of incident CKD when added to a recently proposed CKD prediction model developed by Nelson *et al.* [31]. Although accelerated GFR decline and CKD have been associated with increased risk of end-stage kidney disease and mortality in high- and low-risk groups of the general population, it is questionable whether additional risk prediction afforded by MMP7 may be of clinical utility or that its use would be cost-effective. Nevertheless, our findings provide information regarding possible mechanisms that may contribute to accelerated GFR decline with age.

Previous studies have indicated that MMP2 and TIMP1 may be involved in renal fibrosis [11, 43, 44], but our findings do not support an important role for these biomarkers in age-related GFR decline in a general population without CKD, diabetes or CVD.

Potential explanations for the lack of associations with MMP2 and TIMP1 in our study may be that we investigated a relatively healthy baseline population without established kidney disease, compared with others who mostly investigated CKD patients, and low statistical power in the incident CKD analyses. TIMP1 has been found to increase later than MMPs during development of kidney failure [44]. In addition, another study indicated that a threshold level of MMP2 could be necessary to initiate fibrosis [45], and this level may not have been reached in our baseline sample. Our results may indicate a similar threshold level for MMP7, as we found a non-linear association with GFR decline, with increased risk primarily in the upper quartile of MMP7.

Although the intra-assay CV was low, the inter-assay CV for MMP7 was 19.3%, which is relatively high. In part, it may be due to low concentrations in one of our internal controls, which fell in the lower part of the 5-PL standard curve. However, any large inter-assay CV would most likely attenuate any true association between MMP7 and GFR decline. Indeed, the association of MMP7 and GFR decline was stronger in the main analysis after we had excluded subjects ($n = 30$) from the Luminex assay with some faults in the standard curve.

The main strengths of our study are that we used accurate GFR measurements in a prospective study of the general population. eGFR from creatinine or cystatin C is imprecise in the near-normal range of GFR [46] and is biased by muscle wasting and inflammation in aging individuals [47, 48]. Our findings persisted after adjustment for several covariates, and sensitivity analyses showed consistently increased risk across different outcome definitions for kidney function decline.

There are also several limitations. First, our sample consisted only of middle-aged persons without self-reported CVD and diabetes mainly of North European ancestry, and the results cannot be generalized to other ethnicities or age groups. Second, prolonged storage of serum samples before analysis and repeated freeze–thaw cycles can affect protein stability and concentration. However, MMP7 has been found to have excellent stability at temperatures below −75°C [49], although studies on TIMP1 and MMP2 are scarce and conflicting [49, 50]. Third, only baseline serum samples of the proteins of interest were analysed. Whether changes in protein concentration over time, or if additional urine samples could add increased predictive value, should be investigated in future studies. Fourth, analyses for incident CKD lack power due to few incident cases. Fifth, misclassification of incident CKD could have occurred because the CKD diagnosis was based on one GFR and urinary ACR measurement only. Finally, we cannot imply causality based on the associations observed since this was an observational study.

To conclude, our results indicate that the pro-fibrotic biomarker MMP7, but not MMP2 or TIMP1, is independently associated with GFR loss in persons without diabetes or pre-existing CKD. MMP7 improved the prediction of accelerated GFR decline and incident CKD in this population. Further research is needed to determine if MMP7 may be a useful biomarker for detection of persons at high risk of accelerated GFR decline and incident CKD.

SUPPLEMENTARY DATA

Supplementary data are available at ndt online.

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AUTHORS' CONTRIBUTIONS

All authors meet the requirements for authorship.

CONFLICT OF INTEREST STATEMENT

None declared.

DATA AVAILABILITY STATEMENT

The data underlying this article cannot be shared publicly since this was not included in the research permission, due to ethical considerations and the privacy of individuals that participated in the study. The data can be shared on request as part of research collaboration.

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Paper 2

Paper 3

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CLINICAL RESEARCH

Nitric Oxide Precursors and Dimethylarginines as Risk Markers for Accelerated Measured GFR Decline in the General Population

Nikoline B. Rinde[1], Inger Therese Enoksen[1], Toralf Melsom[1,2], Ole Martin Fuskevåg[3], Bjørn Odvar Eriksen[1,2] and Jon Viljar Norvik[1,2]

[1]Metabolic and Renal Research Group, UiT The Arctic University of Norway, Tromsø, Norway; [2]Section of Nephrology, University Hospital of North Norway, Tromsø, Norway; and [3]Division of Diagnostic Services, Department of Laboratory Medicine, University Hospital of North Norway, Tromsø, Norway

Introduction: Nitric oxide (NO) deficiency is associated with endothelial dysfunction, hypertension, atherosclerosis, and chronic kidney disease (CKD). Reduced NO bioavailability is hypothesized to play a vital role in kidney function impairment and CKD. We investigated the association of serum levels of endogenous inhibitors of NO, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA), and precursors of NO, arginine, citrulline, and ornithine, with a decline in glomerular filtration rate (GFR) and new-onset CKD.

Methods: In a prospective cohort study of 1407 healthy, middle-aged participants of Northern European origin in the Renal Iohexol Clearance Survey (RENIS), GFR was measured repeatedly with iohexol clearance during a median follow-up time of 11 years. GFR decline rates were analyzed using a linear mixed model, new-onset CKD (GFR $<$ 60 ml/min per 1.73 m^2) was analyzed with interval-censored Cox regression, and accelerated GFR decline (the 10% with the steepest GFR decline) was analyzed with logistic regression.

Results: Higher SDMA was associated with slower annual GFR decline. Higher levels of citrulline and ornithine were associated with accelerated GFR decline (odds ratio [OR], 1.43; 95% confidence interval [CI] 1.16–1.76 per SD higher citrulline and OR 1.23; 95% CI 1.01 to 1.49 per SD higher ornithine). Higher citrulline was associated with new-onset CKD, with a hazard ratio of 1.33 (95% CI 1.07–1.66) per SD higher citrulline.

Conclusions: Associations between NO precursors and the outcomes suggest that NO metabolism plays a significant role in the pathogenesis of age-related GFR decline and the development of CKD in middle-aged people.

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The prevalence of CKD is increasing, and, at present, CKD affects 8% to 10% of the world's population.[1] CKD is a major contributor to morbidity and mortality, and it is among the few noncommunicable diseases with increasing age-standardized mortality rates worldwide.[1,2] In addition, with an aging population, the incidence of CKD will continue to rise[1] because the loss of GFR with age is an important risk factor for CKD.[3] This age-related loss in GFR varies between persons; some individuals have a moderate age-related GFR loss, whereas others exhibit a steeper GFR decline.[4] A more rapid loss of GFR is associated with an increased risk for end-stage kidney disease and death.[5] However, differences in GFR decline rates are only partly explained by conventional CKD risk factors, such as obesity and hypertension.[6] Therefore, investigations of potential biomarkers that may shed light on underlying mechanisms of age-related GFR decline are essential as an early step toward CKD prevention.

NO is an important vascular regulator of vasodilation and antithrombotic processes.[7] NO synthase converts arginine to NO and citrulline (Figure 1). Ornithine and citrulline are the precursors of arginine through the urea cycle.[8] NO deficiency is considered to

Correspondence: Nikoline B. Rinde, Metabolic and Renal Research Group and Department of Clinical Medicine, University in Tromsø (UiT) The Arctic University of Norway. E-mail: nri006@uit.no
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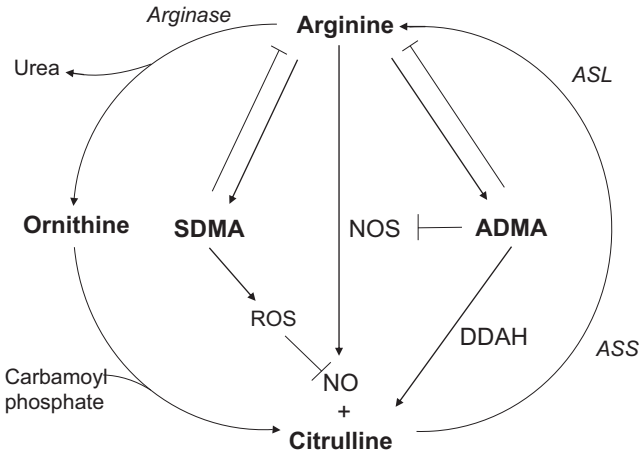

Figure 1. Pathway of NO and arginine metabolism. Arginase converts arginine to ornithine and urea. Arginosuccinate synthase and lyase converts citrulline to arginine, completing the urea cycle. NOS converts arginine to citrulline and NO. ADMA inhibits NOS. SDMA stimulates ROS, which inhibits NO. DDAH converts ADMA to citrulline and dimethylamine. ADMA, asymmetric dimethylarginine; ASL, arginosuccinate lyase; ASS, arginosuccinate synthase; DDAH, dimethylarginine dimethylaminohydrolase; NO, nitric oxide; NOS, nitric oxide synthase; ROS, reactive oxygen species; SDMA, symmetric dimethylarginine.

contribute to endothelial dysfunction, atherosclerosis, as well as pulmonary and systemic hypertension.[9,10] ADMA has a direct inhibiting effect on NO,[10] and SDMA has an indirect inhibiting effect on NO.[11] Both have been identified as independent risk factors for cardiovascular disease (CVD) and death in patients with CKD and the general population.[12–15] Because CKD and CVD share several risk factors, increased levels of ADMA and SDMA could potentially also increase the risk of GFR loss and incident CKD. Although cross-sectional studies have found impaired NO metabolism in persons with decreased GFR,[15,16] it is unknown whether markers of impaired NO metabolism, such as deviations of ADMA, SDMA, arginine, citrulline, and ornithine, are associated with increased risk of accelerated age-related GFR decline and incident CKD.

To the best of our knowledge, no longitudinal studies have investigated the association between ADMA and SDMA and the decline in GFR or new-onset CKD in a general population.[15,17,18] A few have investigated the association between arginine, citrulline, and ornithine and GFR decline. In a longitudinal study, citrulline has been associated with new-onset CKD.[19,20] In addition, a cross-sectional study showed increased levels of arginine and citrulline to be associated with reduced estimated GFR (eGFR) and CKD prevalence. These studies were based on eGFR from serum creatinine, a method that lacks precision in the normal range of GFR and is biased by non–GFR-related factors.[21,22]

The present study aimed to investigate the serum levels of ADMA, SDMA, arginine, citrulline, and ornithine as risk markers for GFR decline using iohexol clearance in a population-based cohort study. We hypothesized that higher levels of dimethylarginines and/or deviations in arginine, citrulline, and ornithine levels were associated with accelerated GFR decline and incident CKD.

METHODS

Study Population

The Tromsø Study is a population-based prospective study with repeated health surveys of representative samples of the inhabitants of the municipality of Tromsø in Northern Norway.[23] The RENIS is a substudy of the Tromsø Study. The baseline investigation of RENIS (RENIS-T6), performed between 2007 and 2009, included 1627 individuals aged 50 to 62 years as described in detail elsewhere.[24] Participants with self-reported kidney disease, CVD, or diabetes were excluded. Of the 1627 participants at baseline, 1324 (81%) were examined in the RENIS follow-up study (RENIS-FU, between 2013 and 2015),[24,25] and 1174 (72%) were examined in RENIS-3 (2018–2020)[26] (Figure 2a). The median follow-up time was 10.86 years (range: 4.39–12.82).

In this study, participants without baseline dimethylarginines and NO precursor measurements were excluded ($n = 3$). In addition, participants had to be present at baseline and have at least 1 follow-up to be included, resulting in a study population of 1407 participants for the analyses (Figure 2b).

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

Baseline Data and Measurements

Measurements were performed in the morning after overnight fasting at the Clinical Research Unit at the University Hospital of Northern Norway. All participants answered a questionnaire about smoking status and current medications. Body height and weight were measured, and body mass index was calculated as weight in kilograms divided by the square of height in meters (kg/m^2). Blood pressure was measured as automated office blood pressure as described previously.[27] Three first-void morning urine samples were collected on separate days before the GFR measurement, the urinary albumin-to-creatinine ratio (ACR) was calculated for each urine specimen, and the mean ACR value was used in the analyses.[28] Fasting plasma samples for glucose and C-reactive protein were drawn for biochemical analyses from a Teflon catheter placed in

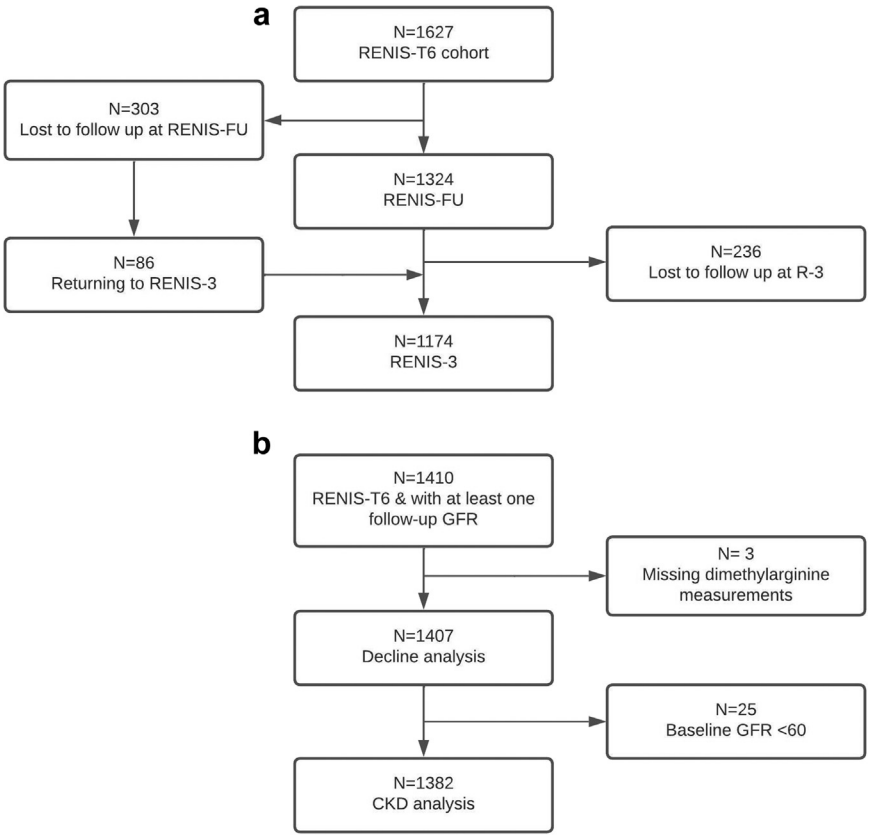

Figure 2. (a) Inclusion of participants in the Renal Iohexol Clearance Survey from Tromsø 6 (RENIS-T6), the first follow-up (RENIS-FU), and third Renal Iohexol Clearance Survey (RENIS-3). (b) Number of baseline participants with at least 1 follow-up GFR measurement and the corresponding number of participants in the different analyses. CKD, chronic kidney disease; GFR, glomerular filtration rate; RENIS-FU, Renal Iohexol Clearance Survey Follow-Up.

an antecubital vein. Serum samples used to measure ADMA, SDMA, arginine, citrulline, and ornithine levels were stored at −80 °C and thawed the day of the analysis.

Iohexol Clearance

GFR was measured at RENIS-T6, RENIS-FU, and RENIS-3 using single-sample plasma iohexol clearance, previously described in detail.[25] Single-sample iohexol clearance has been validated against gold-standard methods.[29] A Teflon venous catheter was used to inject 5 ml of iohexol (Omnipaque, 300 mgI/ml, Amersham Health, London, UK). The optimal time from injection to the sampling of blood for iohexol measurements was calculated using Jacobssons's method.[30] The serum iohexol concentrations were measured by using high-performance liquid chromatography at baseline and RENIS-FU and liquid chromotography–mass spectrometry at RENIS-3.[31] The iohexol measurements in RENIS-T6 and RENIS-3 were calibrated to the RENIS-FU measurements by reanalysis of frozen samples (Supplementary Methods). GFR was calculated using the equations of Jacobsson[30] and standardized to a body surface area of 1.73 m^2.[32] The intra-assay coefficient of variation during the study period was 3.0%, 3.1%, and 2.8% for RENIS-T6, RENIS-FU, and RENIS-R3, respectively.[33,34] The intraindividual variation in the GFR measurements for a random sample of 88 participants who received repeated follow-up measurements within 8 weeks was 4.2%.[35]

Dimethylarginines and NO Precursors

The measurement of ADMA, SDMA, arginine, citrulline, and ornithine in the RENIS cohort has been described in detail previously.[21] Serum levels of dimethylarginines and NO recursors were analyzed by a liquid chromatography–mass spectrometry system consisting of a Waters Acquity UPLC *I*-Class flow through needle system with an autosampler and a binary solvent delivery system (Waters, Milford, MA) interfaced with a Waters Xevo TQ-X benchtop tandem quadrupole mass spectrometer (Waters, Manchester, UK). ADMA, SDMA, arginine, citrulline, and ornithine were obtained from Sigma-Aldrich (St. Louis, MO), and labeled versions were purchased from Toronto Research Chemicals (Ontario, Canada). The precision of the interday coefficient of variation was measured at <8% on 3 different days for ADMA, SDMA, arginine, citrulline, and ornithine.

Statistical Analysis

Continuous variables are given as the means ± SD for normally distributed variables or the median and interquartile range for skewed variables. The baseline data were presented across quartiles of the measured GFR (mGFR).

The association between the dimethylarginines and NO precursors and the annual GFR change rate was analyzed using linear mixed models with random intercept and slope and an unstructured covariance matrix.[5] In these analyses, a negative GFR change rate corresponds to a mean annual reduction in GFR. ADMA, SDMA, arginine, citrulline, and ornithine were studied as independent variables in separate models adjusted for known CKD risk factors (age, sex, body mass index, systolic blood pressure, use of blood pressure–lowering drugs [use of angiotensin-converting enzyme inhibitors, angiotensin receptor II blockers, diuretics, calcium blockers, beta-blockers, or other antihypertensive medications (yes/no)], fasting glucose, smoking status, C-reactive protein and ACR).

There were 3 models for multiple linear mixed models. Model 1 was unadjusted. Model 2 was adjusted for baseline age, sex, and body mass index. Model 3 was adjusted as model 2 with the addition of adjustments for baseline office blood systolic pressure, the use of blood pressure–lowering drugs, fasting glucose, current smoking, ACR, and C-reactive protein as a marker of inflammation because of SDMA's effect on reactive oxygen species. Whether there was an interaction between the GFR change rate and sex was assessed by including a 3-way cross product between sex, the time variable, and the dimethylarginines or NO precursors.

The annual GFR change rate (in ml/min per 1.73 m^2 per year) for each participant was calculated using a linear mixed model adjusted for age, sex, and known CKD risk factors, body mass index, systolic blood pressure, and blood pressure–lowering drugs at baseline using a within-person centered time variable.[36] The centered time variable was used to minimize confounding due to baseline associations between dimethylarginines or NO precursors, CKD risk factors, and GFR.[5,36] The associations between accelerated decline and ADMA, SDMA, arginine, citrulline, and ornithine were studied in separate multiple logistic regression models. Accelerated GFR decline was defined as a dichotomous variable (yes/no), indicating the 10% of participants with the steepest annual GFR decline rate calculated using the mixed model described above.[5,36,37]

Incident CKD was defined as new-onset GFR $<$ 60 ml/min per 1.73 m^2.[38] The exact time when the outcome occurred was not observed but was only known to occur between 2 observations. Therefore, associations between baseline levels of dimethylarginines and NO precursors and incident CKD were examined with interval-censored Cox regression. The time interval of the event was defined as the last observation with GFR $\geq$ 60 ml/min per 1.73 m^2 and the first observation with GFR $<$ 60 ml/min per 1.73 m^2. Participants with GFR $<$ 60 ml/min per 1.73 m^2 at baseline ($n = 25$) were excluded from the interval-censored Cox analyses of incident CKD. Because only 50 of the remaining 1594 persons (3%) died before we started the last wave of iohexol clearance measurements, we did not perform an analysis with death as a competing risk

In the multiple logistic models and interval-censored Cox regression, the same 3 models were used but adjusted for baseline mGFR in models 2 and 3. Interval-censored Cox regression was not adjusted for ACR and C-reactive protein in model 3 because of few events. Interactions between accelerated GFR decline or new-onset CKD and sex were assessed by including a 2-way cross product between sex and dimethylarginine or NO precursor in question.

Interval-censored Cox regression analyses for new-onset CKD with/without albuminuria (mGFR $<$ 60 ml/min per 1.73 m^2 and/or new-onset ACR $>$ 3 mg/mmol) were performed and shown in Supplementary Table S2. As a sensitivity analysis, rapid GFR decline, defined as an annual loss $>$ 3 ml/min per 1.73 m^2 per year calculated using the slope described previously in this article, was analyzed as multiple logistic regression models. For comparison, in additional analyses, we used eGFR instead of mGFR as the dependent variable for each outcome in the supplement.

A P value below 0.05 was considered statistically significant. Statistical analyses were performed with STATA/MP software, version 17.0 (StataCorp LP, College Station, TX).

RESULTS

The baseline characteristics of the study population ($N = 1407$) are presented across quartiles of the mGFR in Table 1.

GFR was negatively correlated with ADMA ($r = -0.18$), SDMA ($r = -0.43$), and citrulline ($r = -0.20$) with a P value of <0.001 (Supplementary Table S1). ACR did not correlate with any of the dimethylarginines or the NO precursors at baseline (Supplementary Table S1). The pairwise correlations between the dimethylarginines and the NO precursors were in the range of 0.15 to 0.47.

In the multivariable-adjusted linear mixed model analyses, ornithine was associated with a steeper

Table 1. Baseline characteristics of the Renal Iohexol Clearance Survey participants according to mGFR quartiles ($N = 1407$)

	Quartile of glomerular filtration rate, range (ml/min per 1.73 m^2)			
	Quartile 1 ($n = 352$)	Quartile 2 ($n = 352$)	Quartile 3 ($n = 352$)	Quartile 4 ($n = 351$)
Characteristic	**≤85.3**	**85.4–93.9**	**94–103.5**	**>103.6**
Sex, men *n* (%)	111 (32)	161 (46)	188 (53)	236 (67)
Age, yr	59.1 (3.5)	58.2 (3.8)	57.5 (3.9)	57.1 (4.0)
BMI, kg/m^2	27.3 (4.2)	27.0 (3.9)	27.2 (3.7)	27.4 (3.7)
Systolic BP, mm Hg	129.4 (17.9)	129.5 (17.8)	129.1 (17.5)	129.0 (16.3)
Diastolic BP, mm Hg	83.17 (10.1)	83.89 (10.0)	83.08 (9.9)	83.40 (8.9)
Antihypertensive medication, *n* (%)	72 (20)	57 (16)	62 (18)	58 (17)
Fasting glucose, mmol/l	5.2 (0.5)	5.3 (0.5)	5.4 (0.5)	5.5 (0.6)
Current smoking, *n* (%)	55 (16)	59 (17)	62 (18)	91 (26)
CRP, mg/l (IQR)	1.2 (0.7–2.3)	1.1 (0.6–2.1)	1.2 (0.7–2.2)	1.2 (0.6–2.1)
ACR, mg/mmol (IQR)	0.2 (0.1–0.5)	0.2 (0.1–0.5)	0.2 (0.1–0.5)	0.3 (0.1–0.5)
mGFR ml/min per 1.73 m^2	76.1 (9.1)	89.6 (2.5)	98.6 (2.7)	111.7 (6.9)
ADMA, µmol/l	0.442 (0.05)	0.435 (0.05)	0.423 (0.05)	0.417 (0.05)
SDMA, µmol/l	0.67 (0.1)	0.64 (0.1)	0.61 (0.1)	0.57 (0.1)
Arginine, µmol/l	92.3 (16.3)	93.4 (16.5)	93.3 (16.6)	95.4 (17.5)
Citrulline, µmol/l	23.0 (6.4)	22.0 (6.1)	20.6 (6.0)	19.7 (5.6)
Ornithine, µmol/l	63.5 (14.5)	63.0 (14.3)	62.0 (14.2)	62.3 (14.6)

ACR, albumin-to-creatinine ratio; ADMA, asymmetric dimethylarginine; Arg, arginine; BMI, body mass index; BP, blood pressure; CRP, C-reactive protein; DMA, dimethylarginine; GABR, global arginine bioavailability ratio, GFR, glomerular filtration rate; IQR, interquartile range, mGFR, measured glomerular filtration rate; SDMA, symmetric dimethylarginine.
Data are shown as mean (SD), median (interquartile range), or *n* (%).

annual GFR change rate (per SD increment; −0.07 ml/min per 1.73 m^2 per year, 95% CI, −0.13 to −0.001; $P < 0.05$) (model 3, Table 2). Participants with a higher baseline level of SDMA, however, had a slower annual GFR decline rate (0.07 ml/min per 1.73 m^2 per year; 95% CI, 0.002–0.13; $P = 0.04$). The other biomarkers were not statistically significantly associated with the annual GFR change rate. There was no interaction between sex and the levels of dimethylarginines or the NO precursors (data not shown).

The cutoff for the 10% with steepest mGFR decline was set at >1.62 ml/min per 1.73 m^2 per year.

Higher baseline levels of citrulline and ornithine were associated with higher odds of accelerated GFR decline (Table 3). The odds of an accelerated GFR decline increased by 43% (OR, 1.43; 95% CI, 1.16–1.76) per SD increment citrulline level and 23% (OR, 1.23; 95% CI, 1.01–1.49) per SD higher ornithine level in the multivariable-adjusted logistic regression analyses (model 3, Table 3).

Of the 1382 participants without CKD at baseline, 94 (7%) developed CKD during follow-up. The interval-censored Cox regression analyses showed an association between the development of CKD and higher baseline levels of citrulline (hazard ratio, 1.33; 95% CI, 1.07–1.66 per SD higher citrulline) in the multivariable-adjusted model (model 3, Table 4).

In sensitivity analyses, 48 (3.6%) participants had a rapid GFR decline >3 ml/min per 1.73 m^2 (Supplementary Table S3). Analyses with eGFR instead of mGFR are presented in Supplementary Tables S4, S5, and S6. SDMA and citrulline show associations with

Table 2. Linear mixed model regression analyses of the associations between dimethylarginines, nitric oxide precursors, and their ratios with measured glomerular filtration rate change rates ($N = 1407$)

	Model 1		Model 2		Model 3	
Independent variable	**ml/min per 1.73 m^2 per yr per SD[a] (95% CI)**	***P* value**	**ml/min per 1.73 m^2 per yr per SD[a] (95% CI)**	***P* value**	**ml/min per 1.73 m^2 per yr per SD[a] (95% CI)**	***P* value**
ADMA	−0.00 (−0.07 to 0.06)	0.99	−0.00 (−0.07 to 0.06)	0.97	0.00 (−0.06 to 0.07)	0.93
SDMA	0.07 (0.00–0.13)	0.05	0.06 (−0.00 to 0.13)	0.05	0.07 (0.00–0.13)	0.04
Arginine	−0.05 (−0.11 to 0.02)	0.14	−0.05 (−0.12 to 0.02)	0.13	−0.05 (−0.12 to 0.01)	0.13
Citrulline	−0.05 (−0.11 to 0.02)	0.17	−0.05 (−0.11 to 0.02)	0.15	−0.04 (−0.10 to 0.03)	0.26
Ornithine	−0.07 (−0.14 to −0.01)	0.03	−0.07 (−0.14 to −0.01)	0.03	−0.07 (−0.13 to −0.00)	0.04

ADMA, asymmetric dimethylarginine; Arg, arginine; CI, confidence interval; OR, odds ratio; SDMA, symmetric dimethylarginine.
[a]A negative coefficient indicates a steeper decline.Model 1: crude.
Model 2: adjusted for age, sex, and BMI.
Model 3: model 2 and adjusted for systolic blood pressure, use of angiotensin-converting enzyme inhibitors, angiotensin receptor II blockers, diuretics, calcium blockers, beta-blockers, or other antihypertensive medications (yes/no), fasting glucose, smoking status (yes/no), C-reactive protein, and albumin-to-creatinine ratio.
Each row represents a separate regression model.

Table 3. Associations of biomarkers with accelerated GFR decline defined as the 10% with the steepest GFR decline rate

	Model 1		Model 2		Model 3	
Independent variable	OR per SD (95% CI)	*P* value	OR per SD (95% CI)	*P* value	OR per SD (95% CI)	*P* value
ADMA	1.16 (0.97–1.37)	0.1	1.13 (0.94–1.35)	0.2	1.11 (0.91–1.36)	0.30
SDMA	1.06 (0.89–1.27)	0.50	1.08 (0.88–1.33)	0.47	1.14 (0.91–1.41)	0.26
Arginine	1.28 (1.08–1.52)	0.005	1.19 (0.99–1.42)	0.06	1.07 (0.88–1.30)	0.48
Citrulline	1.39 (1.17–1.64)	<0.001	1.52 (1.26–1.84)	<0.001	1.43 (1.16–1.76)	0.001
Ornithine	1.39 (1.18–1.63)	<0.001	1.36 (1.15–1.62)	<0.001	1.23 (1.01–1.49)	0.04

ADMA, asymmetric dimethylarginine; CI, confidence interval; GFR, glomerular filtration rate; OR, odds ratio; SDMA, symmetric dimethylarginine.
Model 1: crude.
Model 2: adjusted for age, sex, BMI, and baseline GFR.
Model 3: model 2 and adjusted for systolic blood pressure, use of angiotensin-converting enzyme inhibitors, angiotensin receptor II blockers, diuretics, calcium blockers, beta-blockers, or other antihypertensive medications (yes/no), fasting glucose, smoking status (yes/no), C-reactive protein, and albumin-to-creatinine ratio.
Each row represents a separate regression model.

the 10% with accelerated eGFR decline, OR 1.41 (CI, 1.16–1.73) and 1.24 (CI, 1.03–1.49), respectively. With eGFR, 58 (4.3%) participants fulfilled the criteria for CKD. In the analysis with Cox interval-censored regression, citrulline (HR, 1.34; 95% CI, 1.03–1.73) was associated with new-onset CKD. There were no statistically significant interactions between sex and the levels of dimethylarginines or NO precursors for accelerated GFR decline or incident CKD (data not shown).

DISCUSSION

Although NO deficiency has been proposed as a common pathway for progressive kidney disease, its role in early CKD development has been unclear. In the present prospective, population-based cohort study, there were no associations between higher levels of ADMA or lower arginine with accelerated GFR decline or new-onset CKD. However, participants with higher baseline levels of serum citrulline had a higher risk of accelerated GFR decline and new-onset CKD, whereas higher baseline levels of serum ornithine were associated with a higher risk of accelerated GFR decline.

To our knowledge, this is the first study assessing the association between ADMA and SDMA with the decline of GFR or new-onset CKD in a general population.[15,17,18] Consistent with previous cross-sectional studies, we found a correlation between higher SDMA and reduced GFR.[39,40] This is not surprising because 90% of SDMA is eliminated by glomerular filtration in the kidneys.[17] In contrast, ADMA was poorly associated with mGFR. This may partially be due to ADMA clearance being more dependent on the metabolism of dimethylarginine dimethylaminohydrolase than the GFR.[12,16,17]

NO deficiency has been of interest when investigating the underlying mechanism behind endothelial dysfunction in CKD.[41] Animal studies performed *in vitro* and *in vivo* have investigated the association between dimethylarginines and NO precursors.[7,9,11,16,41] Higher levels of ADMA and lower levels of arginine have been proposed as the underlying mechanisms leading to GFR decline and CKD progression.[7] SDMA limits the arginine supply to NO metabolism[11] and has also been suggested as a marker of kidney function.[42] Therefore, our findings that higher SDMA was associated with slower annual GFR decline may be contraintuitive relative to previous studies. However, the effect on the GFR change rate was small, may not be clinically relevant, and should be interpreted with caution. Most previous studies investigating ADMA and SDMA as biomarkers for diseases have assessed the risk of CVD and mortality[12–15,17,43–45] and not kidney outcomes. A small number of studies have observed an association between

Table 4. Cox interval–censored regression analyses for new-onset chronic kidney disease with measured glomerular filtration rate < 60 ml/min per 1.73 m^2 ($n = 94$)

	Model 1		Model 2		Model 3	
Independent variable	HR per SD (95% CI)	*P* value	HR per SD (95% CI)	*P* value	HR per SD (95% CI)	*P* value
ADMA	1.29 (1.06–1.57)	0.01	1.03 (0.85–1.26)	0.75	1.03 (0.84–1.28)	0.76
SDMA	1.84 (1.52–2.22)	<0.001	1.25 (1.00–1.56)	0.05	1.23 (0.98–1.54)	0.07
Arginine	1.05 (0.85–1.29)	0.68	1.06 (0.87–1.30)	0.56	1.07 (0.87–1.31)	0.51
Citrulline	1.59 (1.31–1.93)	<0.001	1.30 (1.06–1.58)	0.01	1.33 (1.07–1.66)	0.01
Ornithine	1.17 (0.95–1.43)	0.13	1.19 (0.97–1.47)	0.10	1.17 (0.93–1.47)	0.19

ADMA, asymmetric dimethylarginine; CI, confidence interval; HR, hazard ratio; SDMA, symmetric dimethylarginine.
Model 1: crude.
Model 2: adjusted for age, sex, BMI, and baseline GFR.
Model 3: model 2 and adjusted for systolic blood pressure, use of angiotensin-converting enzyme inhibitors, angiotensin receptor II blockers, diuretics, calcium blockers, beta-blockers, or other antihypertensive medications (yes/no), fasting glucose, and smoking status (yes/no).
Each row represents a separate regression model.

increased serum concentrations of ADMA and SDMA and the progression of CKD.[12,13,17,41,43] In comparison to our study, however, these studies included participants with preexisting CKD. In addition, they assessed kidney function with eGFR, which may be influenced by non–GFR-related factors[46] such as CVD risk factors, inflammation, and levels of dimethylarginines,[21,22,47] possibly leading to spurious associations with GFR change rates during follow-up. Indeed, SDMA was associated with accelerated GFR decline using eGFR (Supplementary Table S4), but not with mGFR, indicating confounding due to non–GFR-related factors. Another explanation for the unexpected association of SDMA with a slower mean mGFR decline rate could be an association of SDMA (an inflammatory marker) with hyperfiltration, leading to a phase of increasing GFR in participants with, for example, prediabetes or metabolic syndrome. A phase of hyperfiltration may persist for several years in relatively healthy persons, for example, with those with prediabetes.[33] In line with this hypothesis, SDMA was borderline associated with an increased risk of incident CKD at the end of follow-up.

We found associations between serum citrulline and accelerated GFR decline and new-onset CKD, as well as an association between serum ornithine and accelerated GFR decline. Ornithine competes for the same transport system as arginine.[48] An increase in ornithine may result in a reduction in intracellular arginine availability. Only a few longitudinal studies investigated the association of citrulline with new-onset CKD in the general population.[19,20] Consistent with our observations, the Framingham Heart Study observed a 50% increased risk of CKD development per SD increase in citrulline levels.[19] Another cohort study found a strong association between high levels of citrulline and new-onset CKD.[20] Citrulline is produced from ornithine and further to arginine in the urea cycle,[8] and all are necessary precursors in the biological pathway of NO (Figure 1). An early elevated level of citrulline for persons developing CKD may be explained by reduced citrulline to arginine conversion[49] and/or overproduction of citrulline trying to compensate for the NO shortage.[50] Although no association between arginine and new-onset CKD or accelerated GFR decline was found in our study, disorders of arginine metabolism may be associated with kidney function decline. The citrulline and ornithine association suggests a disorder in endothelial function not captured by measuring serum arginine levels.

The main strength of the present study is the prospective design and the repeated measurements by iohexol clearance in a cohort recruited from the general population, an accurate method to establish GFR without the influence of non–GFR-related factors.[21,22] Previous studies have used eGFR with creatinine or cystatin C to examine the association. Another strength is the relatively long observation period from 2007 to 2020, where only a few participants (27.8%) were lost to follow-up.

Our study has limitations. The enrollment of only middle-aged participants of Northern European origin limits the generalizability of the study. In addition, individuals participating in cohorts over time tend to be healthier,[51] and the study had few (94 [7%]) CKD events. The concentration of the dimethylarginines and NO precursors was analyzed at one time point only. The concentration of the dimethylarginines and NO precursors may vary over the years.

In conclusion, higher levels of citrulline and ornithine, but not ADMA, SDMA, and arginine, were associated with accelerated GFR decline in a general population without diabetes, CKD, or CVD. This suggests that NO metabolism plays a role in age-related GFR decline and early CKD. Our findings warrant further investigation into the pathogenesis of NO metabolism and the possible therapeutic targets and preventive measures.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods. Calibration of the HPLC and LC/MS analyses of serum iohexol.

Table S1. Spearman correlation coefficients between dimethylarginines and the nitric oxide precursors and the baseline characteristics.

Table S2. Interval-censored Cox regression analyses for chronic kidney disease (defined as new-onset measured glomerular filtration rate < 60 ml/min per 1.73 m^2 and/or new-onset albumin-to-creatinine ratio > 3 mg/mmol).

Table S3. Associations of biomarkers with rapid GFR decline defined as an annual loss > 3 ml/min per 1.73 m^2 ($n = 48$).

Table S4. Associations of biomarkers with accelerated GFR decline defined as the 10% with the steepest estimated

GFR decline rate (cutoff 10-percentile −1.48 ml/min per 1.73 m^2 annually).

Table S5. Cox interval–censored regression analyses for new-onset chronic kidney disease with estimated glomerular filtration rate < 60 ml/min per 1.73 m^2 ($n = 58$).

Table S6. Linear mixed model regression analyses of the associations between dimethylarginines, nitric oxide precursors, and their ratios with estimated glomerular filtration rate change rates ($N = 1407$).

Strobe Checklist.

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