UiT The Arctic University of Norway

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Novel biomarkers in hypertension

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Abbreviations

ABP	Ambulatory blood pressure
ABPM	Ambulatory blood pressure monitoring
ACC/AHA	American College of Cardiology/American Heart Association
ACEI	Angiotensin-converting enzyme inhibitor
ARB	Angiotensin receptor blocker
CVD	Cardiovascular disease
CKD	Chronic kidney disease
CV	Coefficient of variability
EGF	Epidermal growth factor
EGF-Cr	Epidermal growth factor creatinine ratio
eGFR	Estimated glomerular filtration rate
GFR	Glomerular filtration rate
HBPM	Home blood pressure monitoring
HMOD	Hypertension mediated organ damage
IL-1RA	Interleukin 1 receptor antagonist
IL-18	Interleukin-18
KDIGO	Kidney Disease: Improving Global Outcomes
mGFR	Measured GFR
MCP-1	Monocyte Chemoattractant Protein-1
NAG	N-acetyl-β-D-glucosaminidase
NAG-Cr	N-acetyl-β-D-glucosaminidase-creatinine ratio
NGAL	Neutrophil Gelatinase-Associated Lipocalin
OCR	Orosomucoid-creatinine ratio
OPN	Osteopontin
RANTES	Regulated upon Activation Normal T-cell Expressed and Secreted
SD	Standard deviation
AASK	The African American Study of Kidney Disease and Hypertension
CENS	The cardiovascular remodelling in living kidney donors with reduced glomerular filtration rate study
DASH	The Dietary Approaches to Stop Hypertension Study
ESC	The European Society of Cardiology
ESH	The European Society of Hypertension
IDA	The individualized blood pressure treatment: a multidisciplinary approach to uncontrolled hypertension in order to reduce morbidity and mortality study
ISH	The International Society of Hypertension
IDNT	The Irbesartan Diabetic Nephropathy Trial
LoB	The limit of blank
LoD	The limit of detection
LoQ	The limit of quantification
MDRD	The Modification of Diet in Renal Disease
REIN-2	The Ramipril Efficacy in Nephropathy-2 trail
RENAAL	The Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan Trial
RENIS	The Renal Iohexol Clearance Survey in Tromsø study
RENIS-T6	The Renal Iohexol Clearance Survey in Tromsø6
SPRINT	The Systolic Blood Pressure Intervention Trial
TNF	Tumour Necrosis Factor
UAE	Urinary albumin excretion
ACR	Urine albumin-to-creatinine ratio
Na/K-ratio	Urine sodium-potassium ratio
vWF-A2	Von Willebrand Factor A2

List of presented papers

Paper 1: The association between urinary sodium-potassium ratio, kidney function, and blood pressure in a cohort from the general population

Paper 2: Novel biomarkers in patients with uncontrolled hypertension with and without kidney damage.

Paper 3: Associations of urinary orosomucoid, N-acetyl-β-D-glucosaminidase, and albumin with blood pressure and hypertension after 7 years. The Tromsø Study.

Summary

Hypertension is the foremost global risk factor for mortality and stems from an intricate interplay of genetic and environmental factors that cumulatively impact cardiovascular and renal function.

A diet high in sodium contributes to hypertension. A low potassium intake and subclinical kidney dysfunction have been implicated as a cause of salt sensitivity. We studied the urinary sodium-potassium ratio in middle-aged North Europeans without specific health conditions and found a significant association with blood pressure, irrespective of other cardiovascular risk factors. This association was not mediated by kidney function, suggesting that a diet high in sodium and low in potassium can lead to higher blood pressure regardless of kidney function. These findings indicate that the effect of the urinary sodium/potassium ratio may extend to a healthier population than previously studied.

Hypertension-mediated organ damage serves as a link between high blood pressure and the development of advanced cardiovascular and chronic kidney diseases. Current blood and urine tests lack the sensitivity to detect kidney damage caused by hypertension at an early stage, when prevention could be most effective. We evaluated plasma levels of biomarkers related to endothelial and kidney cell pathology, inflammation and fibrosis in healthy individuals and patients with hypertension. The examined biomarkers lacked consistent discrimination across hypertension severity levels. However, plasma osteopontin may have the potential to identify those at risk for hypertensive kidney damage. Furthermore, in a population-based study, we explored and compared the associations between urine orosomucoid, N-acetyl-β-D-glucosaminidase, albumin, blood pressure, and the development of hypertension. Among the three biomarkers, orosomucoid had the strongest relationship with blood pressure and hypertension development. There were only varying and weak

relationships between NAG, blood pressure and hypertension. Further investigation is warranted to assess the potential utility of the examined biomarkers in the treatment of hypertension.

1 Introduction

According to the World Health Organization, hypertension is the largest single contributor to overall morbidity and mortality[1, 2]. Inadequate control of blood pressure leads to damage in vital organs, which, if identified early, can be intervened upon to prevent the development of severe diseases[3]. Although hypertension can be effectively managed at relatively low cost, almost half of the individuals with hypertension are unaware of their condition and half of the patients receiving treatment fail to achieve optimal blood pressure control, thus remaining at a heightened risk of complications[2, 4].

Other cardiovascular risk factors, such as dyslipidemia, diabetes and obesity, frequently accompany hypertension. This clustering has a cumulative impact on cardiovascular risk, emphasizing the significance of assessing the overall cardiovascular risk[5]. The quantification of total cardiovascular risk, which refers to the likelihood of experiencing a cardiovascular event within a specific timeframe, plays a critical role in the risk stratification process for patients with hypertension[5]. However, the conventional risk factors only explain a part of the association between hypertension and cardiovascular disease (CVD)[6].

Hypertension is a risk factor for the initiation and progression of chronic kidney disease (CKD), and CKD is known to exacerbate hypertension[7, 8]. Even in cases of mild CKD, there is an increase in the risk of developing CVD and mortality[9]. Observational studies have consistently demonstrated that standard clinical measures of kidney disease, a decrease in estimated glomerular filtration rate (eGFR) and the presence of albuminuria are independent and synergistic risk factors for cardiovascular events[9]. However, it should be noted that a significant decline in kidney function is required to detect a decrease in eGFR, making it an insensitive marker for early detection of kidney damage[5, 10]. Furthermore, urinary albumin excretion (UAE) lacks sensitivity and specificity as a marker of kidney

damage in hypertension [3, 11-13]. Consequently, both eGFR and UAE are limited in identifying early kidney damage in hypertension, a critical stage where preventive interventions could be most effective[5].

Unexplored and unidentified risk factors likely play a mediating role in the heightened risk of CVD among individuals with even mild CKD[14]. Discovering biomarkers associated with hypertension and early kidney damage represents a stride in unravelling the underlying mechanisms of hypertension and facilitating targeted treatments.

2 Background

2.1. Blood pressure and blood pressure measurement

Systolic blood pressure is the pressure in the systemic blood vessels during ventricular contraction when the heart pumps oxygenated blood into the circulation. Diastolic blood pressure represents the pressure on the systemic blood vessels during ventricular relaxation. Blood pressure is measured in units of millimetres of mercury (mmHg). The readings are given in pairs, with the systolic value first, followed by the diastolic value. Office blood pressure refers to the measurement of blood pressure taken in a healthcare setting, typically in a doctor's office or clinic, with attention to the standardized conditions recommended for a valid measurement[5]. Out-of-office blood pressure measurements refer to measuring blood pressure outside of a healthcare setting[5]. This can be done using one of two methods. With home blood pressure monitoring (HBPM), individuals measure their own blood pressure, with two readings in the morning and the evening for at least 3 days following the recommended guidelines from their healthcare provider[3]. Ambulatory blood pressure monitoring (ABPM) involves wearing a portable device that automatically measures blood pressure at regular intervals throughout a 24-hour period. ABPM provides monitoring of blood pressure during daily activities and sleep[3]. Out-of-office blood pressure measurements offer several advantages over office measurements. They are more reproducible, providing more consistent

and reliable data over time, and they are more closely associated with hypertension mediated organ damage (HMOD) and risk of cardiovascular events[15-17]. However, conventional office blood pressure has guided treatment in clinical outcome trials, and it is not clear whether using out-of-office blood pressure measurements to guide treatment will reduce illness or death[18].

2.2 Hypertension

2.2.1 Definition of hypertension

The relationship between blood pressure and cardiovascular and kidney events is continuous, and observational research indicates that the risk may start at the low normal systolic blood pressure level of 115 mmHg[19, 20]. The term hypertension is used to identify the blood pressure levels at which the benefits of treatment outweigh the potential risks[3, 5].

The American College of Cardiology/American Heart Association (ACC/AHA) defines hypertension as office systolic blood pressure values ≥130 mmHg and/or diastolic blood pressure values ≥80 mmHg[21]. The European Society of Cardiology (ESC), the European Society of Hypertension (ESH), and the International Society of Hypertension (ISH) define hypertension as office systolic blood pressure values ≥140 mmHg and/or diastolic values ≥90 mmHg[3, 5, 22]. The American guidelines recommend pharmacological therapy for individuals with blood pressure levels of 130-139/80-89 with established CVD or a 10-year absolute cardiovascular risk over 10%. Those with lower cardiovascular risk are advised to make lifestyle changes and undergo periodic monitoring[21]. In contrast, the ESC/ESH 2018 and the ISH 2020 guidelines classify blood pressure levels of 130-139/85-89 as high normal and recommend adopting healthy lifestyles[3, 5, 22]. All guidelines reference the same randomized controlled trials, but the American guidelines rely more on observational data and the Systolic Blood Pressure Intervention Trial (SPRINT), which showed that targeting systolic blood pressure below 120 mmHg reduced mortality[5, 21-23]. However, the

inclusion of patients already on antihypertensive treatment in SPRINT raises concerns about the generalizability of the results to untreated individuals. The European and international guidelines' perspective is to withhold treatment until the benefits are clearly proven[5, 22]. Despite the various definitions, overall, there are only minor distinctions in the recommendations for when to initiate treatment. For this thesis, the European 2018 hypertension guidelines were used to define hypertension[5]. The ESH released updated guidelines in autumn 2023, where the definition of hypertension remains unchanged[3]. However, the recently released ESH guideline now suggests pharmacological treatment for individuals with high-normal blood pressure and a history of established cardiovascular disease, relying on recent meta-analyses derived from randomized controlled trials[3].

Hypertension is grouped into primary and secondary hypertension. Primary hypertension accounts for 85-95% of cases, and the causes are unknown. Secondary hypertension is caused by identifiable conditions such as renal artery stenosis, adrenal adenoma, or single-gene mutations[24].

Using office and out-of-office measurements, hypertension is classified into three different phenotypes known as white coat hypertension, masked hypertension and sustained hypertension. White coat hypertension refers to the condition when blood pressure is high in the doctor's office but normal during out-of-office measurements[5]. Masked hypertension is the opposite, where blood pressure is normal in the doctor's office but high under out-of-office measurements[5]. Patients with masked hypertension tend to have a high normal office blood pressure, and the condition must be suspected when there are signs of HMOD or high cardiovascular risk[3, 25, 26]. Sustained hypertension is when blood pressure is high in both at-office and out-of-office measurements[5]. The impact of white coat hypertension on cardiovascular outcomes is debated, and there is insufficient evidence from randomized controlled trials to determine if the condition requires drug treatment[5, 26-28]. The risk of

cardiovascular events is significantly higher in masked hypertension compared to normal blood pressure, approaching or surpassing that of sustained hypertension[26]. No studies have investigated how antihypertensive drugs affect cardiovascular outcomes in people with masked hypertension. However, it is recommended to consider treatment with blood pressure-lowering medication for these patients[5].

Controlled hypertension describes the situation in which patients with hypertension has their blood pressure successfully managed within the recommended guidelines. Conversely, when patients with hypertension fail to achieve blood pressure levels within these guidelines, they are categorized as having uncontrolled hypertension[2].

2.2.2 Prevalence and health burden of hypertension

In 2019, over 1.3 billion people worldwide had hypertension, which has doubled since 1990[29]. Among adults aged 30-79, the age-standardised prevalence of hypertension was 32% in women and 34 % in men[2, 29]. The prevalence of hypertension remains high globally, irrespective of the income status of countries, encompassing lower, middle- and higher-income nations (Table 1)[2, 29, 30].

Table 1: Age-standardized prevalence of hypertension among adults aged 30–79 years, and among those with hypertension, diagnosis, treatment and effective treatment coverage in 2019, by WHO region.

Region	Hypertension (%)	Diagnosis coverage (%)	Treatment coverage (%)	Effective treatment coverage[a] (%)
African	36 (38, 33)	43 (46, 39)	27 (30, 24)	12 (14, 9)
The Americas	35 (38, 33)	70 (73, 67)	60 (64, 57)	36 (41, 32)
South-East Asia	32 (36, 29)	39 (44, 34)	30 (34, 25)	14 (18, 10)
European	37 (39, 35)	66 (69, 63)	53 (56, 50)	26 (29, 23)
Eastern Mediterranean	38 (41, 35)	49 (53, 45)	39 (43, 34)	15 (19, 13)
Western Pacific	28 (32, 25)	54 (59, 48)	41 (47, 35)	18 (23, 14)
Global	33 (35, 32)	54 (56, 51)	42 (45, 40)	21 (23, 19)

a. Controlled hypertension among all hypertension. Controlled hypertension is defined as blood pressure <140 mmHg systolic and <90 mmHg diastolic and taking medication for hypertension.

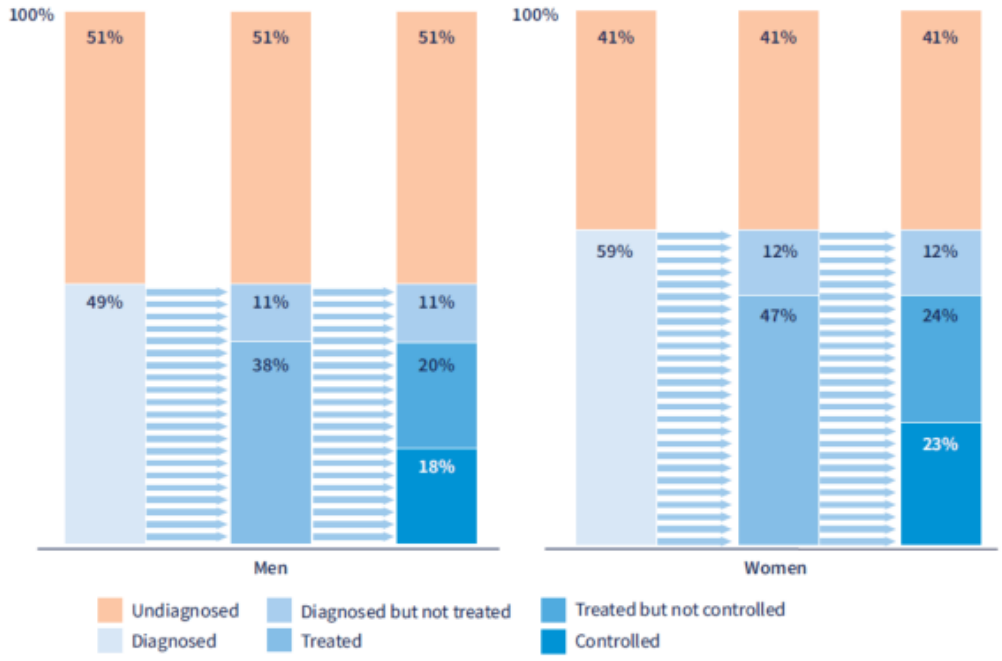
Note: Data in parentheses are 95% uncertainty intervals.

Source: Global Health Observatory (GHO). Noncommunicable diseases: risk factors [online database] *(4)*.

Global report on hypertension: the race against a silent killer. Geneva: World Health Organization; 2023. Licence: CC BY-NC-SA 3.0 IGO

In 2019, the treatment rates were around 38% for men and 47 % for women, with control rates at approximately 20 % for both genders (Figure 1)[2, 29].

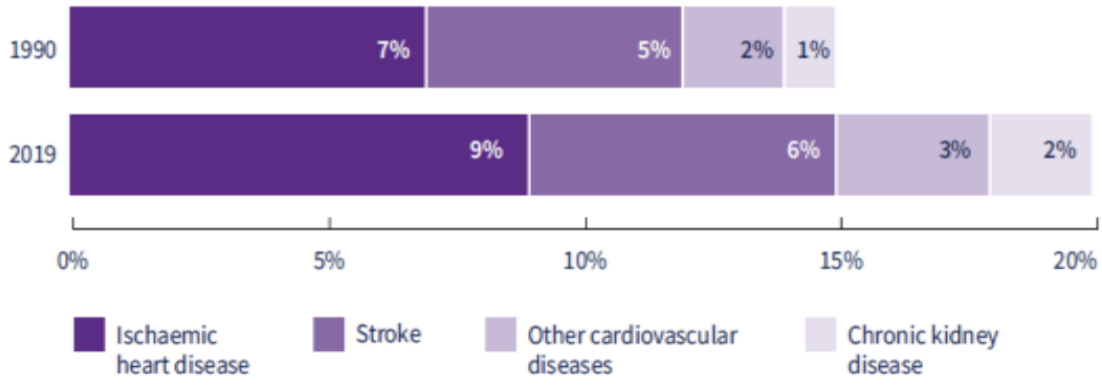
Figure 1: Hypertension treatment cascade in 2019, for adults 30–79 years of age globally, by sex. Age-standardized rates.



Global report on hypertension: the race against a silent killer. Geneva: World Health Organization; 2023. Licence: CC BY-NC-SA 3.0 IGO

Treatment and control rates were slightly improved compared with previous global reports[2, 31, 32]. Hypertension is still the leading preventable risk factor for CVD and overall mortality globally (Figure 2) [1, 2].

Figure 2: Percentage of global deaths attributable to high systolic blood pressure (1990 and 2019), by cause of death.



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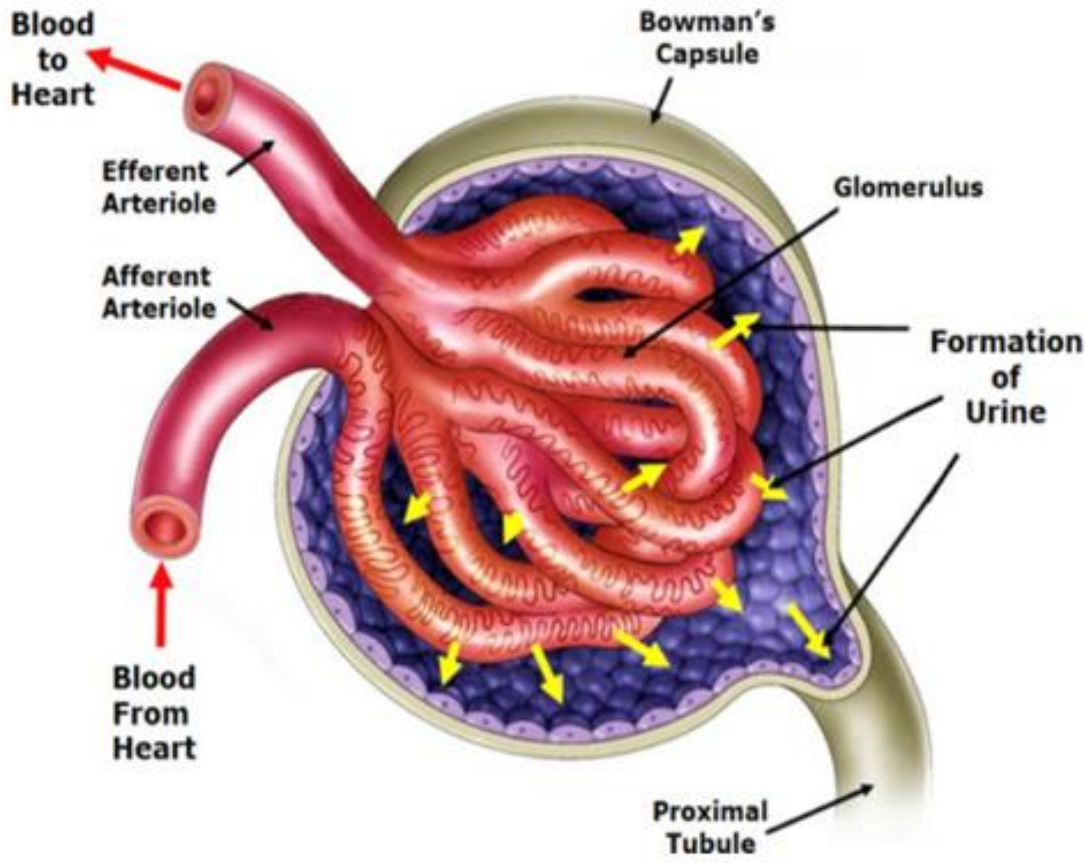
2.3 Kidney function

The kidneys have many functions including excretion and metabolism of substances, regulation of volume, blood pressure, blood osmolality, acid-base, bone and mineral homoeostasis, and erythropoietin production. Assessing overall kidney function is complex[33].

2.3.1 Glomerular filtration rate (GFR)

Glomerular filtration rate (GFR) is a critical measure of kidney function, representing the volume of plasma that flows from the glomerulus into Bowman's space over a specific time period (Figure 3)[34].

Figure 3: The glomerulus.



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It's important to note that GFR cannot be directly measured in humans, making it impossible to determine the 'true' GFR with absolute certainty[33]. Notably, substantial interindividual variability persists even within cohorts of healthy individuals[35, 36]. Furthermore, GFR varies due to diet, time of the day, exercise, pregnancy, drugs and hemodynamic factors. Even in stable conditions, within-person variability of GFR is common and contributes to day-to-day variation[33]. GFR is assessed through the clearance of a filtration marker, a measurable substance excreted via glomerular filtration. An ideal filtration marker should be excreted by the kidneys fully and freely filtered through the glomerular filtration membrane, not be eliminated by other organs, not be protein-bound, and not be metabolized, secreted or reabsorbed in the tubules[33]. Filtration markers can either be introduced externally or

produced endogenously. The normal range for GFR depends on factors such as age, gender, and body size, spanning a range of approximately 90 to 120 millilitres per minute per 1.73 m^2[37]. GFR is considered the best indicator of kidney function, as it often aligns with the loss of other functions[33]. GFR is used as an overall assessment to diagnose and stage CKD, and to determine medication doses[33, 38]

2.3.2 Measured GFR (mGFR)

The exogenous filtration marker inulin has served as a reference filtration marker for determining GFR. However, its application necessitates a labour-intensive procedure characterized by continuous inulin infusion, catheterization, and meticulously timed urine and blood sampling[33]. Due to its complexity, this method is not commonly used today[33]. Instead, two common alternatives are used: urinary clearance of iothalamate and plasma clearance of iohexol. These markers meet the criteria for reliable exogenous filtration markers and correlate well with inulin clearance. In the case of plasma clearance, a known amount of iohexol is injected into the bloodstream, and blood samples are taken over time to calculate the clearance based on the substance's disappearance from the blood[33]. Regardless of the chosen method, utilizing exogenous markers for clearance measurement is intricate, costly, and impractical for routine clinical use, but these methods provide the most accurate measures of GFR[37].

2.3.1 Estimated GFR (eGFR)

Endogenous markers like creatinine can be employed for GFR estimation through timed urine collections and concurrent blood sampling, albeit this approach is laborious and error-prone[37, 39]. In a stable physiological state, the blood level of an endogenous marker like creatinine or cystatin C inversely correlates with GFR, offering an estimation without the need for urine collection[33]. As of today, optimal endogenous filtration markers do not exist. Other factors than GFR, such as tubular processes, filtration maker generation, and non-renal

elimination, impact the serum levels of these markers and the eGFR[33]. Estimating equations for GFR include demographic and clinical variables as surrogates for the impact of the non-GFR determinants, making the estimates more accurate than the blood concentrations alone[33]. Nevertheless, eGFR is still relatively insensitive in detecting and monitoring early kidney disease[33]. The 2012 Kidney Disease: Improving Global Outcomes (KDIGO) guidelines recommend the 2009 CKD-EPI creatinine equation for adults[38, 40]. In 2012, the CKD-EPI Creatinine-Cystatin C equation was developed to estimate GFR from serum creatinine and cystatin C[41]. The equation had a similar bias to the creatinine or cystatin C equations but was more precise and therefore had greater overall accuracy[41]. These two equations include a term for race that, for any given creatinine value, results in a higher eGFR for African Americans than other individuals[42]. The inclusion of the term was based on the observation that individuals in the United States who self-identified as African Americans had higher serum creatinine levels for the same level of GFR compared with non-African Americans[43]. Studies have shown that this coefficient was not applicable to women and other black populations[33, 44, 45]. In 2021, the CKD-EPI group developed a CKD-EPI equation for estimating GFR from serum creatinine without a term for race, as the term has no biological justification[46]. Compared with the 2009 CKD-EPI creatinine equation, the 2021 equation is slightly less accurate, particularly in the European population[44, 46, 47].

2.3.2 Urine albumin excretion

Albuminuria is the abnormal loss of albumin, a plasma protein, in the urine. Albumin can be found in the urine in normal subjects, but often appears in larger quantities in patients with kidney disease[13]. Albuminuria is a common but not uniform finding in CKD. It can be the earliest marker of glomerular diseases, including diabetic glomerulosclerosis, where it can appear before the reduction in GFR. It can be a marker of hypertensive kidney disease, but may not appear until after the reduction in GFR[13]. The pathophysiology of albuminuria is

likely heterogeneous, with different cell types in the glomerulus and possibly the tubule contributing to leakage of albumin[48]. A 24-hour urine collection is the gold standard for detecting increased albuminuria[13, 49]. However, 24-hour timed collection is cumbersome and prone to measurement errors[50]. The impact of changes in urine volume can be avoided by calculating the urine albumin-to-creatinine ratio (ACR) in an untimed urinary sample. ACR is the preferred screening strategy for increased albuminuria [13, 51-53]. An elevated UAE, or moderately increased albuminuria in today's terminology, is defined as urine albumin >30 mg/24 hours or as ACR >3.0 mg/mmol)[38]. Albuminuria is associated with cardiovascular risk factors such as hypertension, obesity, and diabetes, but it is not known if albuminuria is causally related to CVD and CKD[54]. Epidemiologic data demonstrates a strong graded relationship between UAE and kidney and CVD risk, even with ACR levels below the moderately increased albuminuria cut-off and normal eGFR [9, 55-57]. However, todays reference intervals for ACR fail to consider differences in creatinine excretion related to age and sex, as well as the continuous increase in risk tied to albumin excretion[12]. Furthermore, considerable differences exist between methods for measuring albumin and creatinine, and the accuracy is uncertain due to the absence of reference measurement procedures and materials for albumin and creatinine in urine[12]

2.3.3 Definition of chronic kidney disease

The 2012 KIDIGO guidelines define CKD as kidney damage or reduced kidney function lasting three months or more, regardless of the cause, with health implications[13]. Reduced kidney function refers to a GFR, measured or estimated, below 60 mL/min per 1.73 m^2. Kidney damage encompasses an elevated UAE, defined as >30 mg/24 hours or ACR >30 mg/g (>3 mg/mmol). Both GFR and ACR are used to stage CKD (Figure 4)[13]. Pathological abnormalities identified through biopsy, imaging, electrolyte issues due to tubular disorders,

urinary sediment abnormalities and kidney transplantation are also included in the concept of kidney damage[13, 38].

Figure 4: Prognosis of CKD by GFR and albuminuria categoris: KDIGO 2012.

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.

Prognosis of CKD by GFR and albuminuria categories: KDIGO 2012

GFR categories (mL/min/1.73m²) 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			

Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group. KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. Kidney Inter., Suppl. 2013; 3: 1–150.

2.3.4 Prevalence and health burden of chronic kidney disease.

CKD is a common disorder with a global prevalence of approximately 10%. The overall global prevalence of CKD increased by 29.3% across all age groups between 1990 and 2017. However, the age-standardized prevalence of CKD remained stable[58-60]. The global mortality rate for CKD increased by 41.5% for all age groups, but the age-standardized

mortality rate remained steady. The rates of years of life lost and years lived with disability due to CKD decreased by 9.6% and 4.3% respectively. This indicates that CKD-related deaths are occurring at an older age and that non-fatal cases of CKD are less severe on average[61]. The increasing prevalence of CKD can be attributed to several factors, including aging populations, a rising prevalence of type 2 diabetes and hypertension, and a low rate of early-stage CKD detection along with therapeutic inertia in its early management[5, 62-64]. The principal risk factors for CKD are impaired fasting plasma glucose, elevated blood pressure, a high body mass index, and a diet with excessive sodium consumption[61]. Despite the high global burden of CKD, only 0.1% of the world`s population has end-stage kidney disease with the need for kidney replacement therapy[65]. The risk for death and CVD with decreasing kidney function exceeds the risk of progressing to end-stage kidney disease, even after adjustment for other established risk factors[64, 66, 67]. CKD is, therefore, considered one of the strongest risk factors for CVD[68].

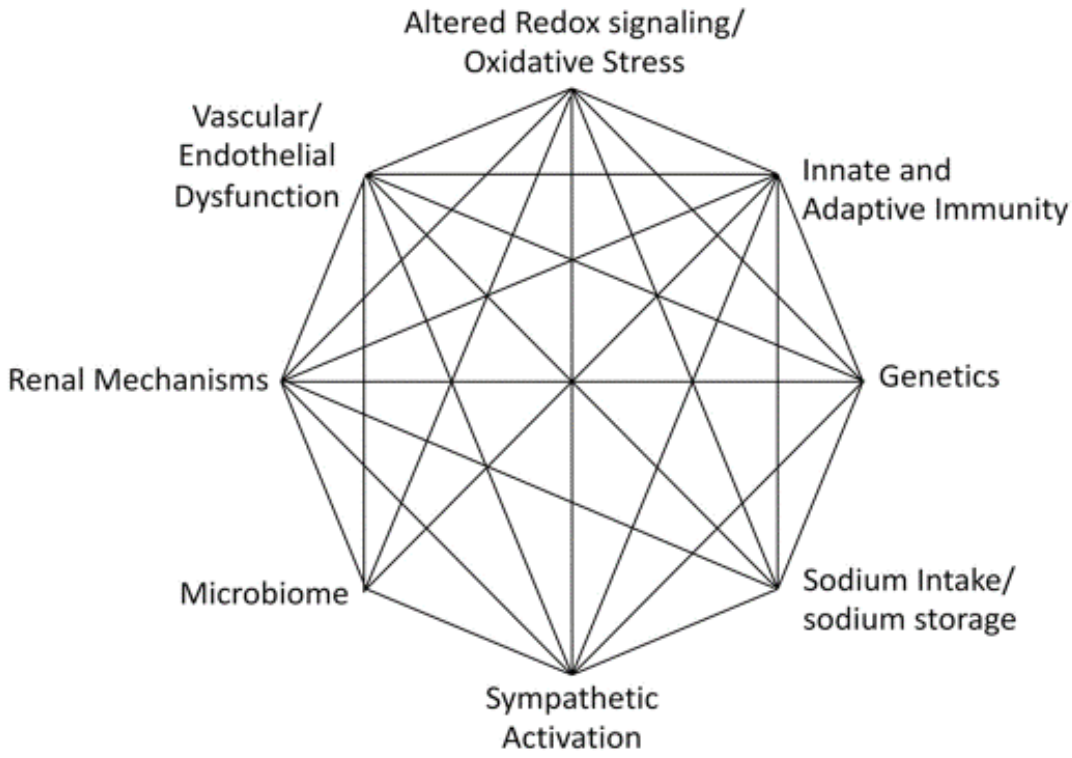
2.4 Hypertension and chronic kidney disease

The aetiology of primary hypertension remains inadequately elucidated. However, it is widely believed to arise from a complex interplay of various genetic and environmental factors, which exert cumulative influences on cardiovascular and kidney structures and function[3, 24, 69, 70].

The kidney has four major roles in hypertension; (1) it regulates blood pressure by adjusting urine volume and sodium excretion, (2) it produces renin, which is crucial for activating the renin-angiotensin system and generating angiotensin-II, which regulates blood volume, electrolyte balance and vascular resistance, (3) it plays a role in modulating the sympathetic tone, and (4) the kidney can contribute to immune activation in hypertension[24].

Kidney disease is associated with developing hypertension in the general population, and both eGFR and UAE are limited in their ability to identify early kidney damage in hypertensive individuals[5, 8, 10, 12]. Subclinical kidney dysfunction may influence changes in blood pressure through altered sodium intake. However, the specific renal abnormalities responsible for this connection have yet to be completely understood[24, 71, 72]. A high sodium intake in the general population is associated with hypertension, CKD, and cardiovascular disease[73-76]. Restricting sodium intake has a blood pressure-lowering effect[75-77]. When the blood pressure rises, the kidney increases urine and sodium excretion to return the blood pressure back to normal. This process is known as pressure natriuresis[78]. Guyton suggested that for hypertension to persist, there needs to be a shift in the pressure natriuresis curve to sustain higher blood pressure levels[78]. The mechanisms for a change in the pressure natriuresis curve are multifactorial and involve alterations in sodium transporters, the tubulointerstitial compartment and the kidney vasculature[8, 24]. These changes are intricately interrelated to actions of the renin-angiotensin-aldosterone system, adrenergic stimulation, inflammation, and actions involving oxidative stress[24, 79-81]. Furthermore, studies have shown that the relationship between dietary sodium intake and blood pressure also is influenced by the skin interstitium and involves immune activation, and possibly gut microbiota (Figure 5) [24, 82-85].

Figure 5 Pathophysiology of hypertension: A revised Mosaic Theory incorporating new understanding of cellular, environmental and genetic mechanisms.



A revised Mosaic Theory incorporating new understanding of cellular, environmental and genetic mechanisms.
From Circ Res. 2021 Apr 2; 128(7): 847–863. Doi: 10.1161/CIRCRESAHA.121.318082. (License Number 562408045311

Further, a higher potassium intake is also associated with lower blood pressure and reduced risk of cardiovascular disease[76, 86-90]. A diet high in sodium and low in potassium can stimulate salt reabsorption along the distal nephron[83]. A low potassium intake may also catalyst renal vasoconstriction, leading to persistent preglomerular arteriolopathy, tubular ischemia, and interstitial inflammation, generating deviant renal sodium handling and increasing blood pressure[91]. High sodium and low potassium consumption may also disrupt the endothelial glycocalyx barrier, induce endothelial stiffening, and trigger endothelial dysfunction with increasing blood pressure[92-94].

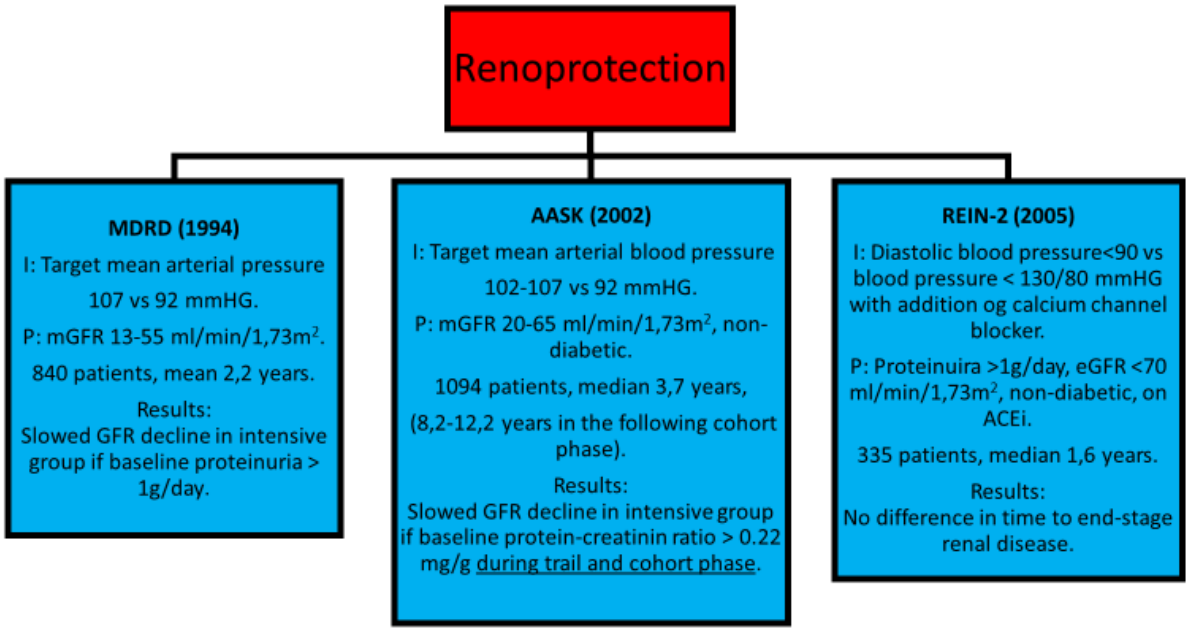
Although the cause of hypertension is complex and involves many mechanisms, hypertension represents a prominent risk factor for initiation and progression of CKD, and CKD is known to exacerbate hypertension[7, 8, 95]. It is important to note that even subtle renal injury not

reflected in changes in standard clinical measures of kidney function can initiate a vicious cycle with increasing blood pressure and kidney damage[8, 24, 78].

2.4.1 The current evidence on hypertension and kidney disease.

Current evidence on the protective effect of blood pressure treatment on the progression of CKD originates mainly from three trials: The Modification of Diet in Renal Disease (MDRD), the African American Study of Kidney Disease and Hypertension (AASK), and the Ramipril Efficacy in Nephropathy-2 trail (REIN-2)[96-100] (Figure 6). The main objective of these studies was to investigate whether more intensive blood pressure reduction could reduce the decline in kidney function. These trials exclusively enrolled CKD patients and examined how different blood pressure levels affected kidney endpoints. In patients with baseline proteinuria, a more intensive blood pressure control slowed the rate of GFR decline compared with standard blood pressure control in the Modification of Diet in Renal Disease study and the extended African American Study of Kidney Disease and Hypertension cohort study[97, 99]. However, no such benefit was seen in those without proteinuria. In both trials, participants were randomized to different mean blood pressure levels, which cannot be readily extrapolated to systolic and diastolic blood pressure values used in everyday clinical practice[3]. Further, these studies did not consider the potential benefits of intensive blood pressure control on cardiovascular endpoints, and there were relatively few non-kidney events during the trials[96-100].

Figure 6: Current evidence on the protective effect of blood pressure treatment on the progression of CKD.



The Modification of Diet in Renal Disease (MDRD), the African American Study of Kidney Disease and Hypertension (AASK), and the Ramipril Efficacy in Nephropathy-2 trail (REIN-2), intervention (I), participants (P), angiotensin-converting enzyme inhibitors (ACEi), measured GFR (mGFR), estimated glomerular filtration rate (eGFR).

In contrast, a large trial that did not exclusively enroll CKD patients, the Systolic Blood Pressure Intervention Trial (SPRINT), used cardiovascular events as the primary outcome. The study included a significant number of CKD patients and aimed to assess the impact of intensive blood pressure control on cardiovascular outcomes. The trial showed a lower risk of the composite primary outcome in the intensive blood pressure group, but inconclusive results on CKD progression[23, 101]. There was an increased risk of acute kidney injury and incident CKD in the intensive group[23, 101]. However, the decline in kidney function may be reversible, and the study's follow-up time might be insufficient to fully understand long-term effects[23, 101, 102]. The study excluded patients with proteinuria >1 g/day or diabetes. It utilized a non-standard office blood pressure measurement, which is prone to yielding a lower value than a standard office blood pressure measurement[103].

Other cardiovascular risk factors often accompany hypertension, and globally, diabetes stands out as the primary risk factor for CKD[61]. The RENAAL (Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan) trial and the IDNT (Irbesartan Diabetic

Nephropathy trial) both demonstrated enhanced protection against kidney disease progression through the use of blood pressure lowering angiotensin receptor blockers (ARB) in albuminuric CKD associated with type 2 diabetes[104, 105].

Furthermore, in patients with CKD, higher levels of UAE are associated with a more rapid decline in GFR, irrespective of the underlying cause of kidney disease and the initial GFR[13, 106, 107]. For individuals with hypertension and severely increased albuminuria, the recommended first-choice antihypertensive agents are renin–angiotensin system inhibitors, angiotensin-converting enzyme inhibitors (ACEI) or angiotensin receptor blockers (ARB)[3, 96, 104, 105, 108-111].

Moreover, several new drugs, which also have a blood pressure lowering effect, have also shown benefits in preventing a decline in kidney function in diabetic and non-diabetic kidney disease[112-119]. However, it's important to note that these effects are not solely attributable to the blood pressure-lowering effect[120, 121].

Observational studies and analyses of achieved blood pressure in clinical trials often show a continuous and graded association between blood pressure and cardiovascular and renal outcomes[20, 122]. However, variables associated with lower blood pressure levels may include more favourable health characteristics at baseline and during follow-up, and the apparent benefit of lower blood pressure may result, in whole or in part, from confounding[122]. In a recent study with participants aged 50 to 62 from northern Norway without baseline cardiovascular disease, diabetes, or kidney disease, higher daytime ambulatory blood pressure (ABP) was associated with a shift in measured GFR distribution toward lower values over an 11-year follow-up. The study also found cross-sectional associations between ABP and wider GFR distribution (higher and lower GFRs) at baseline, indicating an early onset of these associations. It's also worth noting that results using eGFRs

differed from mGFR. Additionally, office blood pressure did not reveal time-dependent associations with the GFR distribution[123].

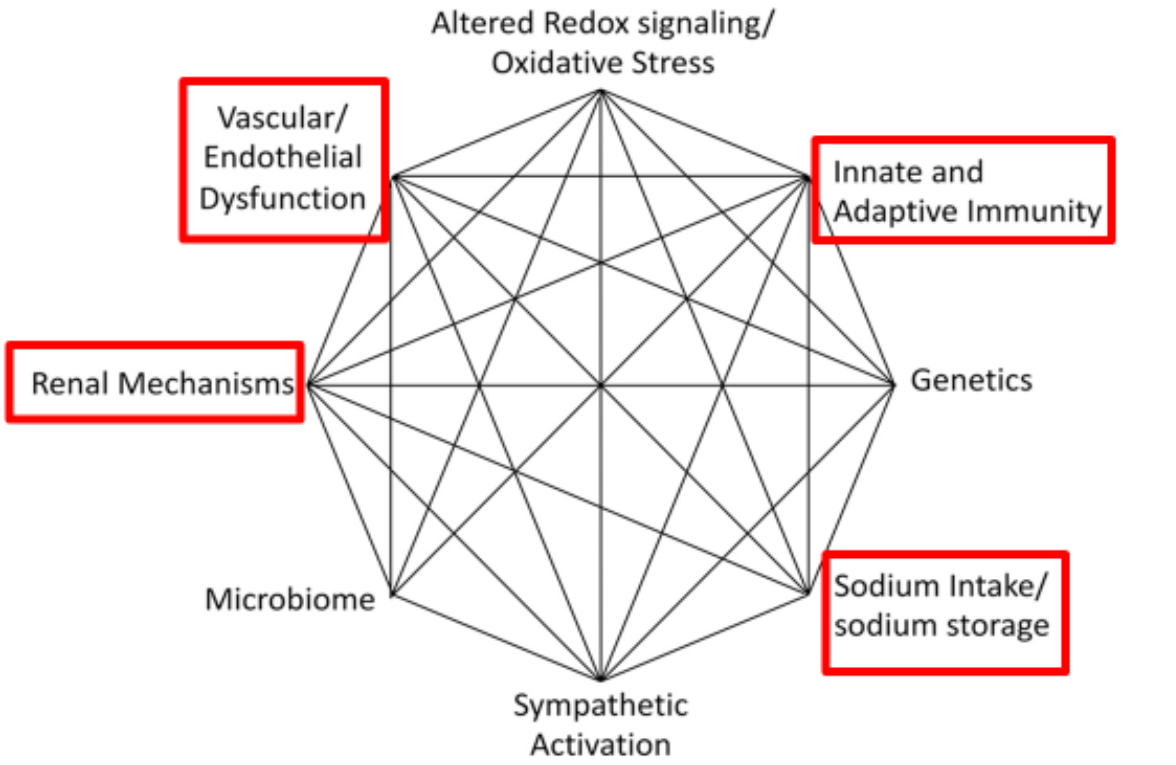
Overall, lowering blood pressure can delay the progression of CKD, and reduce the incidence of CVD and all-cause mortality[23, 96, 99, 100, 124, 125]. However, randomized controlled trials have not proven a beneficial long-term effect of blood pressure on kidney function assessed as the GFR in persons without CKD or individuals with CKD without severely increased albuminuria[3, 96-100, 126]. Further, the available evidence does not provide a clear consensus regarding the optimal blood pressure target in CKD[3, 126].

2.5 Novel biomarkers in hypertension.

Hypertension is frequently accompanied by other cardiovascular risk factors, such as dyslipidemia, diabetes and obesity, exerting a cumulative impact on cardiovascular risk. While risk scores are critical in the risk stratification of hypertensive patients, a significant gap exists between predicted and actual event rates[3, 127, 128]. Biomarkers may aid in further stratifying individual patient risk[128]. HMOD acts as a crucial intermediate stage between cardiovascular risk factors and advanced CVD or CKD[3]. The relationship between hypertension and CKD forms a harmful cycle, where each condition worsens the other[7, 8, 95]. ACR and eGFR are insensitive biomarkers for early detection of kidney HMOD[3, 10-13]. Biomarkers that can detect HMOD early and are readily available for clinical use may improve risk stratification of patients.

In this thesis, we have investigated potential biomarkers within 4 key mechanisms of the mosaic theory of hypertension as advocated by Irvine Page ~60 years ago, which have been revised after the addition of new knowledge (Figure 7)[24, 129, 130].

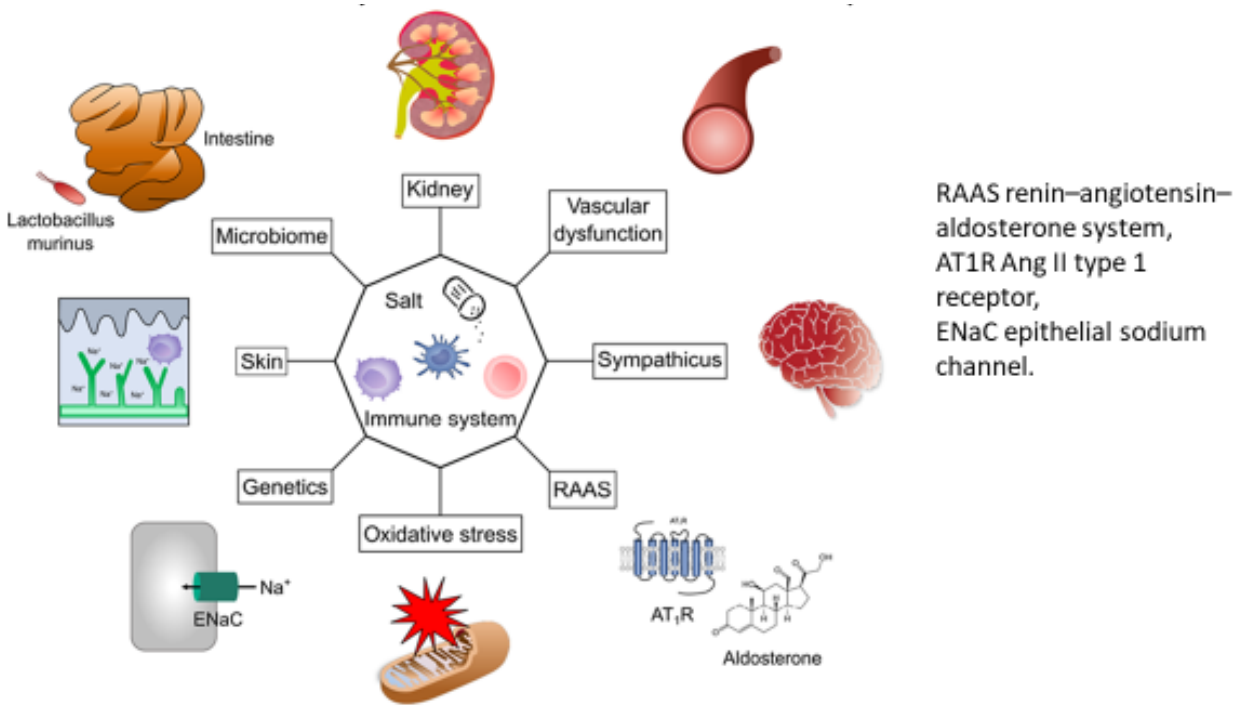
Figure 7: Pathophysiology of hypertension: A revised Mosaic Theory incorporating new understanding of cellular, environmental and genetic mechanisms.



A revised Mosaic Theory incorporating new understanding of cellular, environmental and genetic mechanisms.
From Circ Res. 2021 Apr 2; 128(7): 847–863. Doi: 10.1161/CIRCRESAHA.121.318082. (License Number 562408045311

The urinary sodium-potassium ratio (Na/K-ratio) is a biomarker for sodium and potassium intake. It has shown a stronger association with blood pressure, hypertension and cardiovascular disease than urinary sodium or potassium alone[87, 131, 132]. Further, in animal studies, salt-induced renal and vascular injury has an inflammatory component (Figure 4)[130, 133-135]. Hypertension is associated with chronic inflammation in the kidneys and blood vessels, which might induce further organ damage and fibrosis, and further increased blood pressure (Figure 8)[136-139].

Figure 8: Revised version of the mosaic theory of hypertension. Salt and immune cells are placed in the center because they influence all other mosaic pieces.



Hengel FE, Benitah JP, Wenzel UO: Mosaic theory revised: inflammation and salt play central roles in arterial hypertension. Cell Mol Immunol 2022, 19(5):561-576.

Hypertension induces arterial changes with augmented collagen, arterial wall thickening, and kidney changes including tubulointerstitial injury and fibrosis fibrosis[138, 140]. Kidney tubulo-interstitial damage and fibrosis are prognostic for subsequent kidney failure in biopsy studies but cannot be reliably detected by standard clinical measures[141]. Vascular HMOD can be examined by pulse wave velocity[3]. Two meta-analyses have found that pulse wave velocity improved cardiovascular risk stratification, especially for younger or middle-aged patients with low or moderate risk levels[142, 143]. However, the reproducibility of these measurements is low and depends on the operator, and they are only available at specialized centers[3]. Biomarkers readily available for clinical use that can reflect early inflammation, vascular and kidney tubule and interstitial disease may provide additional information on the risk of CKD and associated adverse clinical endpoints, above and beyond eGFR and

albuminuria. In this thesis, we investigated potential biomarkers for sodium and potassium balance, inflammation, kidney and vascular damage. We have investigated the association between biomarkers, blood pressure and hypertension.

2.5.1 Biomarkers included in the thesis

Table 2 provides an overview of the biomarkers examined in this thesis. All biomarkers have previously shown associations with either hypertension, CKD or CVD. However, knowledge about the causal effect of these associations is limited for most of the biomarkers. Our studies must thus be regarded as validation studies and an extension of previous work. Additional information, including references, can be found in the appendix.

Table 2: Overview of the biomarkers

Biomarker name	Function	Associated with blood pressure/ hypertension.	Associated with CKD	Associated with CVD
Novel biomarkers of kidney damage in hypertension.				
N-acetyl-β-D-glucosaminidase (NAG)	Marker of kidney tubular cell injury.	N/A	NO	YES
Neutrophil Gelatinase-Associated Lipocalin (NGAL)	Marker of kidney tubular cell injury.	YES	YES	YES
Uromodulin (Tamm-Horsfall protein)	Marker of intact tubular cells/renal tissue	YES	YES	YES
Epidermal growth factor (EGF)	Marker of intact tubular cells/renal tissue	YES	YES	N/A
Novel biomarkers of vascular damage in hypertension.				
Orosomucoid (α_1-Acid glycoprotein)	Marker of general endothelial dysfunction and a damaged glomerular filtration barrier	YES	YES	YES
von Willebrand Factor A2 (vWF-A2)	Marker for endothelial injury and activation.	YES	YES	Conflicting results
Novel biomarkers of inflammation in hypertension.				
Interleukin 1 receptor antagonist (IL-1RA)	Indicates immune activation.	YES	YES	YES
Interleukin-18 (IL-18)	A pro-inflammatory cytokine.	YES	YES	YES

Tumour Necrosis Factor (TNF)	Regulator of the inflammatory response	YES	YES	YES
Monocyte Chemoattractant Protein-1 (MCP-1)	A chemotactic protein for monocytes.	YES	YES	YES
Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES)	A chemotactic molecule for monocytes and T cells	YES	Conflicting results	Conflicting results
Osteopontin (OPN)	A chemotactic molecule for monocytes and T cells	YES	YES	YES
Biomarker for sodium and potassium balance				
Sodium-potassium ratio (Na/K-ratio)	A proxy for sodium and potassium intake.	YES	YES	YES

3 Aims

3.1 Primary aim

The primary objective of this project was to investigate how biomarkers of early kidney and endothelial dysfunction are related to blood pressure and hypertension. Additionally, we intend to examine whether markers of kidney function mediate the association between sodium and potassium intake and blood pressure.

3.1.1 Aims in paper 1

In a cross-sectional study, we aimed to assess the associations between urinary Na/K-ratio, blood pressure and hypertension in a cohort of healthy individuals, and to examine if this association is mediated through the kidney function variables measured GFR, ACR and epidermal growth factor-to-creatinine ratio **(**EGF-Cr). We hypothesised that the Na/K-ratio is associated with blood pressure and different hypertension phenotypes independently of traditional cardiovascular risk factors, and that measured GFR, ACR and EGF-Cr mediate these associations.

3.1.2 Aims in paper 2

In a cross-sectional study, we aimed to assess the plasma levels of biomarkers representing inflammation, endothelial and kidney dysfunction in healthy controls and hypertensive patients, and to assess the associations between biomarkers, severity of hypertension and kidney damage. We hypothesised that plasma levels of biomarkers: (1) are different between healthy controls and patients with hypertension, (2) can classify patients with hypertension according to the degree of hypertension severity.

3.1.3 Aims in paper 3

In a cross-sectional and prospective observational study, we aimed to compare UAE, orosomucoid and N-acetyl-β-D-glucosaminidase (NAG) in relation to blood pressure and the development and progression of hypertension. We hypothesized that urinary orosomucoid and NAG have stronger associations cross-sectionally to blood pressure and longitudinally to hypertension than UAE.

4 Methods

4.1 Subjects

This thesis includes subjects from the sixth and seventh surveys of the Tromsø study, the Renal Iohexol Clearance Survey in Tromsø6 (RENIS-T6), the cardiovascular remodelling in living kidney donors with reduced glomerular filtration rate study (CENS) (identifier: NCT03729557), and the Individualized blood pressure treatment: a multidisciplinary approach to uncontrolled hypertension in order to reduce morbidity and mortality study (IDA) (identifier: NCT03209154). The Regional Committee for Medical and Health Research Ethics approved the studies, and all participants gave written consent[144-148].

The Tromsø Study is a population-based, prospective study of inhabitants of the municipality of Tromsø, Northern Norway. Since 1974, seven surveys have been conducted[144, 145]. The

Tromsø study cohort represents a general population. The main goal of the Tromsø study was to investigate and prevent cardiovascular mortality. Later, the focus expanded to include various chronic conditions such as atrial fibrillation, venous thromboembolism, diabetes, osteoporosis, and CKD[144].

The Renal Iohexol Clearance Survey in Tromsø (RENIS) study stems from the Tromsø study. Originally, RENIS was designed to investigate the longitudinal associations between baseline GFR and incident CVD using mGFR. In RENIS-T6, 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[148].

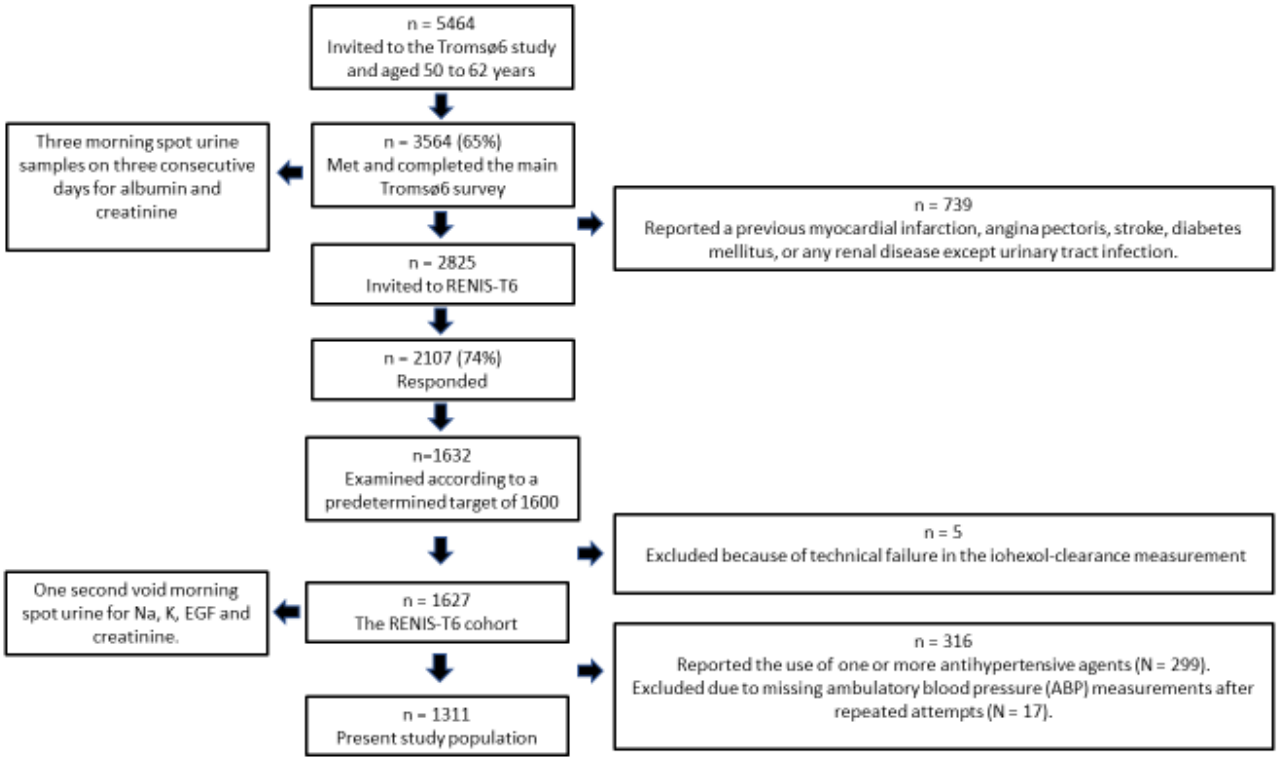
The CENS study is a prospective observational cohort study on living kidney donors. The ongoing study aims to uncover long-term risks for kidney donors, provide insights into CKD prognosis, and identify new targets for CKD-focused cardiovascular therapy[147]. Thorough health screenings for living kidney donors ensure their overall health, and baseline data before a potential nephrectomy, make them an ideal healthy control group with minimal comorbidities.

The IDA study investigated a population of patients with suspected uncontrolled hypertension in Norway. The study examined non-adherence to prescribed antihypertensive drugs and had several aims. Cross-sectionally, it was designed to investigate the prevalence as well as psycho-social and treatment factors associated with non-adherence[146]. Further, in a randomized controlled trial, the study aimed to elucidate the effects of therapeutic monitoring of antihypertensive drugs as a tool to improve blood pressure control. The study gave us access to a very well described population with established hypertension.

4.1.1 Paper 1:

Among the 3564 participants aged 50-62 in the sixth wave of the Tromsø study, 2825 reported no previous myocardial infarction, angina pectoris, stroke, diabetes mellitus, or renal disease. These individuals were invited to RENIS-T6. A total of 2107 agreed, and 1632 subjects were examined according to a predetermined target of 1600. Five subjects were excluded due to technical failure of the iohexol clearance measurement, leaving 1627 participants in the cohort. In paper 1, we excluded 17 participants due to missing ABPM after repeated attempts. Further, 299 participants reporting using one or more antihypertensive agents were excluded, leaving 1311 participants for our study (Figure 9).

Figure 9: Inclusion of participants in the Renal Iohexol-clearance Survey (RENIS-T6) from the main part of the sixth Tromsø survey (Tromsø6).



4.1.2 Paper 2:

For paper 2 we selected individuals with hypertension from a large nationwide multicentre study on hypertension, the IDA study, and healthy controls from an ongoing study of living kidney donors, the CENS study[146, 147]. The inclusion and exclusion criteria for the two respective studies are shown in Table 3.

Table 3: The inclusion and exclusion criteria of the IDA and CENS studies

The Individualized blood pressure treatment: a multidisciplinary approach to uncontrolled hypertension in order to reduce morbidity and mortality (IDA) study	
Inclusion criteria: A stable medication regimen for at least 4 weeks of ≥ 2 antihypertensive agents. No planned changes in antihypertensive drugs. Age > 18 years.	**Exclusion criteria** Inadequate Norwegian language skills. Positive pregnancy test. Known alcohol or drug abuse. Known serious disorders which may limit the ability to evaluate the efficacy or safety of the protocol, i.e. cerebrovascular, cardiovascular, renal or psychiatric diseases. eGFR <30 mL/min/1.73m^2 (2009 creatinine CKD-EPI formula). ACR >300 mg/mmol. Any reason why, in the opinion of the investigator, the patient should not participate.
The cardiovascular remodelling in living kidney donors with reduced glomerular filtration rate (CENS) study.	
Inclusion criteria Age > 18 years. **Donor group:** Accepted as living kidney donor at Oslo University Hospital, Rikshospitalet. **Control group:** Individuals evaluated for donation, but not found eligible due to non-medical causes. Family members related to donors or recipients and blood donors evaluated and fulfilling the Norwegian transplantation protocol for living kidney donors.	**Exclusion criteria** Previous CVD or malignant disease (except carcinoma in situ). **Blood pressure:** (1) Office blood pressure ≥ 140/90 mmHg or 24 hour BP ≥ 130/80 mmHg. (2) Age > 60:24-hour blood pressure < 130/80 mmHg) with one antihypertensive drug. **BMI:** (1) Age <30: BMI≥ 30 kg/m^2 . (2) Age >30 years: Men ≥31 kg/m^2. Women≥32 kg/m^2. **mGFR:** (1) Age <50 years: <90 ml/min/1.73 m^2. (2) Age 50-60 years: <(130 minus age) ml/min/1.73m^2. (3) ≥ 70 years: <70 mL/min/1.73m^2. **(Proteinuria) Albuminuria:** (1) ACR >30 mg/mmol. (2) Age <60 years with ACR > 3 mg/mmol. **Blood glucose:** (1) Diabetes. (2) Age < 60 with impaired oral glucose tolerance test. **Pathological spirometry.** **Pathological stress test ECG at age > 40.** **Pathological chest X-ray.** **Positive serological tests for HIV, TB, HBV, HCV, syphilis and toxoplasmosis.**
Cardiovascular disease (CVD), body mass index (BMI), measured glomerular filtration rate (mGFR), urine albumin-to-creatinine ratio (ACR), electrocardiogram (ECG), human immunodeficiency virus (HIV), tuberculosis (TB), hepatitis B (HBV), hepatitis C (HBC).	

The study on hypertension was an investigator-initiated, prospective, open, multi-center study with four research units located at Oslo University Hospital, Ullevål, Haukeland University Hospital, St. Olav´s University Hospital and the University Hospital of North Norway.

Patients were self-referred, recruited from the nephrology and cardiology outpatient clinics, from private specialists and general practitioners in all four regional health authorities in Norway. We selected participants with controlled hypertension, uncontrolled hypertension and uncontrolled hypertension with kidney damage based on UAE and eGFR. Uncontrolled hypertension was defined as systolic daytime ambulatory blood pressure ≥135 mmHg and kidney HMOD as ACR >3.0 mg/mmol or eGFR<60 mL/min per 1.73 m^2.

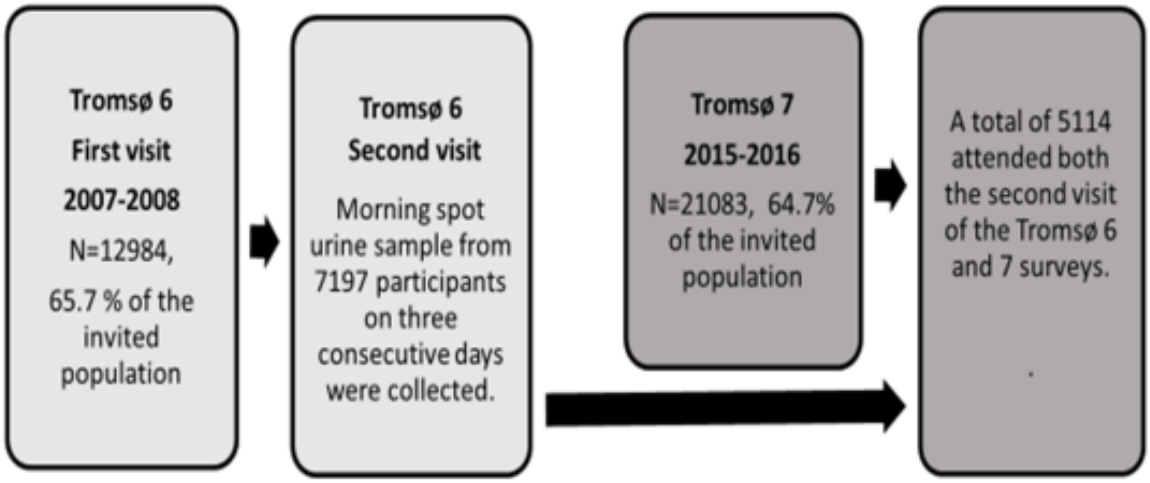
In the ongoing study on living kidney donors, established cooperation with all hospitals performing kidney donation evaluation in Norway, makes it possible for all potential kidney donors in Norway to participate. Participants are either accepted as living kidney donors, or evaluated for donation and not found eligible due to causes other than medical conditions related to the person itself, i.e. immunological incompatibility with the recipient. Family members related to donors or recipients and blood donors evaluated and fulfilling the Norwegian transplantation protocol for living kidney donors are also included. Investigations according to the protocol are performed within three months before unilateral nephrectomy for accepted living kidney donors[147].

The healthy controls and patients with hypertension were age- and sex-matched to the greatest extent possible. A primary objective of our study encompassed the analysis of plasma inflammation biomarkers. Accordingly, individuals receiving systemic immunosuppressive medications were excluded (n=7). We used data collected at the first visit from both studies. A total of 215 individuals, 176 patients with hypertension and 39 healthy controls, were included. Among patients with hypertension, 55 patients had controlled hypertension, 59 had uncontrolled hypertension without kidney damage, and 62 had uncontrolled hypertension with kidney HMOD.

4.1.3 Paper 3:

For paper 3, we included all participants of the sixth wave of the Tromsø Study (Tromsø6 2007-2008), the second visit, in a cross-sectional design. In a longitudinal design, we also included all participants of Tromsø7 (2015-2016) who had attended the second visit of Tromsø6 and did not have hypertension or had controlled hypertension at the time of the examination in Tromsø6 (Figure 10).

Figure 10: Selection of participants from the Tromsø study, 6th and 7th wave.



Brobak KM, Andreassen RM, Melsom T, Hoieggen A, Norvik JV, Solbu MD: Associations of urinary orosomucoid, N-acetyl-beta-D-glucosaminidase, and albumin with blood pressure and hypertension after 7 years. The Tromso Study. *Blood Press* 2022, 31(1):270-283.

DOI: 10.1080/08037051.2022.2128043

The population in Tromsø6 (2007-2008) consisted of four invited groups; those who took part in the second visit of Tromsø4 (1994-1995), a random 10% sample of residents of the municipality of Tromsø aged 30-39, everyone aged 40-42 or 60-87 and a random 40 % sample of residents aged 43-59 years. A total of 12984 attended the Tromsø6, 65,7 % of the invited population[144]. Common cardiovascular risk factors were mapped in the first visit. Further, 7955 were invited to undergo an extensive examination, and the attendance rate for this second visit was 91,8 % (n=7307)[144, 149]. The Tromsø6 second visit included; (1) all

eligible subjects aged 50–62 and 75–84 years from the first visit; (2) A random 20% sample of men and women aged 63–74 from the first visit; and (3) Individuals not previously included in the first two groups who attended the second visit of Tromsø 4[144].

The invited population in the Tromsø7 (2015-2016) consisted of all individuals ages 40 and older. A total of 21083 attended, 64,7% of the invited population[145].

We defined the group without hypertension as those not using blood pressure-lowering drugs and an office blood pressure <140/90 mmHg, and controlled hypertension as using one or more blood pressure lowering drugs and a blood pressure <140/90 mmHg at baseline (Tromsø6). Among those without hypertension from Tromsø6, we defined incident hypertension as using one or more blood pressure-lowering drugs or having a blood pressure ≥140/90 mmHg at follow-up (Tromsø7). Among those with controlled hypertension in Tromsø6, we defined progressive hypertension as an increase in the number of blood pressure-lowering drugs or a blood pressure ≥140/90 mmHg with the same number of blood pressure-lowering drugs in Tromsø7. A total of 7197 participants were included in the cross-sectional study. A total of 5114 attended the second visit of Tromsø6 and Tromsø7 (Figure 10), and 3097 individuals were available for our longitudinal analyses on incident and progressive hypertension.

4.2 Procedures and Methods

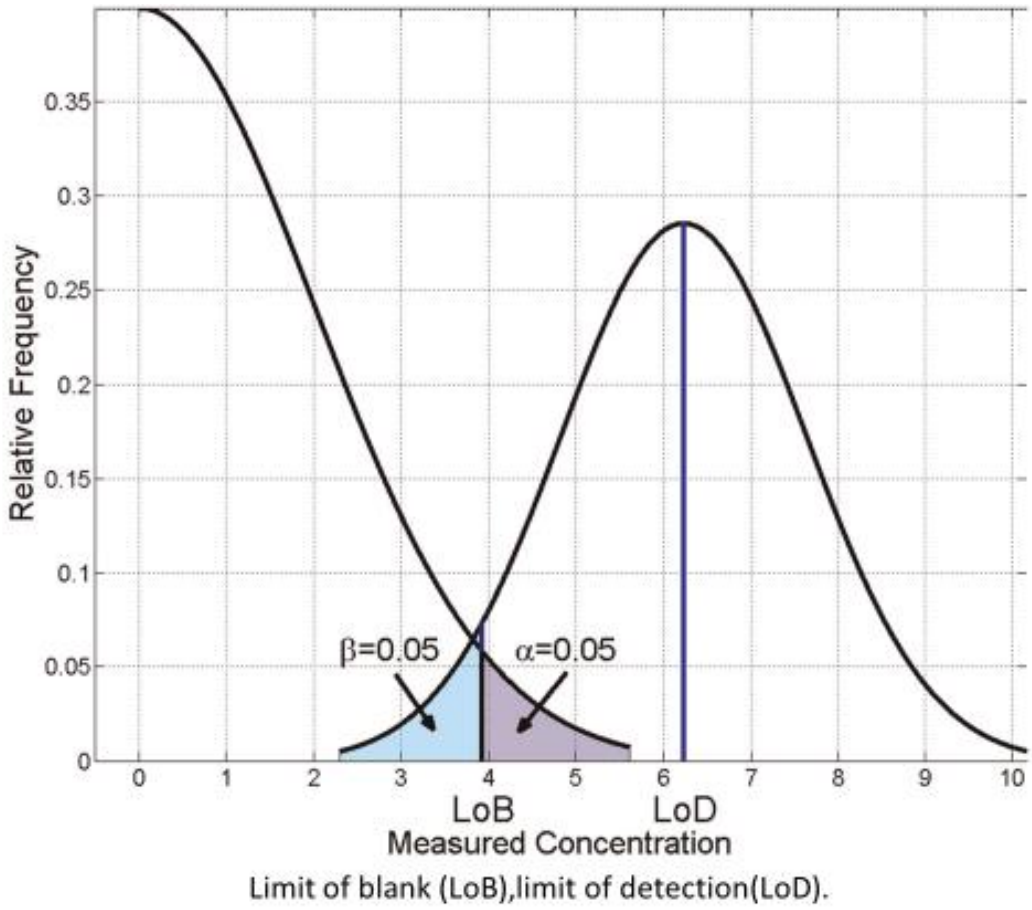
4.2.1 Biomarker measurement and analytic performance

In this thesis, different biomarkers were measured in plasma or one or three spot-urine samples using different measurement techniques (Table 4). Further, some biomarkers were measured in fresh samples, whereas others were measured in frozen, stored and thawed samples (Table 4). All the relevant biomarkers appear to be stable after prolonged frozen storage and after freeze–thaw analysis[150-162].

Biomarker assays can be evaluated for analytical performance, that is, the ability of an assay to measure the biomarker quantity[163]. There will be a certain margin of error or noise in all measurements. In a biochemical analysis, noise can be measured by the analytic device by examining a blank sample, as a buffer solution without an analyte. The limit of blank (LoB) is the highest apparent analyte concentration expected to be found when replicates of a blank sample are tested. By measuring several blank samples, one can find the average and standard deviation (SD) of the noise signal. If LoB is set as the mean blank sample value plus 1.65 times the SD of the blank samples, with a normally distributed blank sample, 95% of the blank sample values will fall within this range and are true negative. The last 5% of the noise signal represents a signal that could come from a tiny amount of the substance analyzed. This false positive signal is called a Type I error in statistics and is marked with α in Figure 11[164, 165]. Specificity = (true negative)/(true negative + false positive), and LoB can be seen as the specificity of an analysis [164, 165].

The limit of detection (LoD) is the lowest amount of an analyte you can detect, but not precisely quantify. The LoD is determined by the measured LoB and test replicates of a sample known to contain a low analyte concentration. If LoD is set as the LoB plus 1.65 times the SD for a low-concentration sample, with a normally distributed low concentration sample, 95% of the measured values will exceed the noise signal. These are the true positives. However, 5% of low concentration values will be lower than the noise signal and appear to contain no analyte, a false negative, which is called a Type II error and is marked with β in Figure 9 [164, 165]. Sensitivity = (true positive)/(true positive + false negative), LoD can be seen as the sensitivity of an analysis[164, 165].

Figure 11: Illustration of the Limit of Detection (LoD) in relation to the Limit of Blank (LoB), depicting a type 1 error (α) where an assay result exceeds the LoB in a blank sample, and a type II error (β) where an assay result falls below the LoB in a sample with an analyte level equal to the LoD.



Pennello GA: Analytical and clinical evaluation of biomarkers assays: when are biomarkers ready for prime time? *Clin Trials* 2013, 10(5):666-676. doi.org/10.1177/1740774513497541

The limit of quantification (LoQ), also called functional sensitivity, is the lowest amount of analyte that can reliably and reproducible be detected and represents the distribution of results for an analyte of low concentration meeting a predefined coefficient of variability (CV)[166]. The CV is a dimensionless number defined as the SD of a set of measurements divided by the mean of the set. The LoQ may be equivalent to the LoD or at a much higher concentration[167].

Most studies measure each sample in duplicate for each analyte. The degree to which the duplicate results differ can be expressed by calculating the mean and SD of the duplicate

results and converting them to the intra-assay CV. The score reflects the repeatability precision[163]. In studies with many samples to be tested, it is necessary to run samples on multiple assay plates. Each plate is run with controls with known concentrations of the analyte. The inter-assay CV is an expression of plate-to-plate consistency calculated from the SD and mean values of controls on each plate. The scores reflect the within-laboratory or within-device performance of the assay[163].

We do not know the analytical performance of the measurements for all biomarkers in this thesis. Table 4 provides an overview of LoD, LoQ, samples in range, and intra- and inter-CV for the performed analyses. The utility of the ACR measurements is compromised due to the absence of recognized standard methods for sample collection, ACR measurement and evaluation of analytical performance in clinical and research settings[12, 163]. We determined the LoD for urine albumin and orosomucoid concentrations based on the values provided in the manufacturer's manual, without calculating the limits through in-house analysis[12]. The manuals did not provide the LoQ, and when reporting samples within the analytical range, it was assumed that no samples fell below the quantification limit (i.e., LoD equals LoQ). These measurements are marked with a red background in Table 4. We do not know whether the limits specified by the eassay producers apply to our surveyed populations. We do not have information on which data the specified limits are calculated from and whether they are based on a validation data set of subjects independent of the data set used in developing the essays[12].

Table 4: Overview of LoD, LoQ, samples in range, and intra- and inter-CV.

Biomarker name	Sample material	Methode (year analyzed)	Under LOD	Under LOQ	In range	Intra CV%	Inter CV%
Biomarkers of kidney damage in hypertension.							
Albumin (paper 1)	Urine, median of 3 morning spot urine samples	Immunoturbidimetric assay (2007-2008)	845 (64)	N/A	466 (36)	N/A	N/A

Albumin (paper 3)	Urine, median of 3 morning spot urine samples	Immunoturbidimetric assay (2007-2008)	4644 (64.5)	N/A	2553 (35.5)	N/A	N/A
N-acetyl-β-D-glucosaminidase (NAG)	Urine, median of 3 morning spot urine samples	Colorimetric analysis (2007-2008)	N/A	N/A	N/A	N/A	N/A
Neutrophil Gelatinase-Associated Lipocalin (NGAL)	Plasma, stored at -80°C	Luminex Multiplex (2022)	1 (0.5)	0	214 (99.5)	1.2-5.6	5.6
Uromodulin (Tamm-Horsfall protein)	Plasma, stored at -80°C	Luminex Multiplex (2022)	0	1 (0.5)	214 (99.5)	0.8-4.6	3.7
Epidermal growth factor (EGF)	Urine, second void morning spot urine, stored at -80°C	Elisa (2016)	0	N/A	1311 (100)	N/A	4.6
Biomarkers of vascular damage in hypertension.							
Orosomucoid (α_1-Acid glycoprotein)	Urine, median of 3 morning spot urine samples, stored at -20°C	Fluorometric immunoassay (DELFIA) (2008)	0	N/A	7197 (100)	N/A	N/A
von Willebrand Factor A2 (vWF-A2)	Plasma, stored at -80°C	Luminex Multiplex (2022)	0	0	215 (100)	1.3-4.4	3.5
Biomarkers of inflammation in hypertension.							
Interleukin 1 receptor antagonist (IL-1RA)	Plasma, stored at -80°C	Luminex Multiplex (2022)	0	0	215 (100)	1.9-5.2	19.5
Interleukin-18 (IL-18)	Plasma, stored at -80°C	Luminex Multiplex (2022)	0	0	215 (100)	0.8-6.5	6.4
Tumour Necrosis Factor (TNF)	Plasma, stored at -80°C	Luminex Multiplex (2022)	1 (0.5)	2 (0.9)	212 (98.6)	1.7-4.3	2.3
Monocyte Chemoattractant Protein-1 (MCP-1)	Plasma, stored at -80°C	Luminex Multiplex (2022)	0	1 (0.5)	214 (99.5)	2.5-6.1	3.3
Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES)	Plasma, stored at -80°C	Luminex Multiplex (2022)	1 (0.5)	0	214 (99.5)	1.0-6.0	3.2
Osteopontin	Plasma, stored at -80°C	Luminex Multiplex (2022)	2 (0.9)	0	213 (99.1)	1.0-3.6	4.1
Biomarker for sodium and potassium balance							
Sodium-potassium ratio (Na/K-ratio)	Urine, second void morning spot urine, stored at -80°C	Ion-selective electrode method (2007-2008)	0	0	1311 (100)	N/A	N/A

Data are number (%) and range as appropriate. Limit Of Detection (LOD), Limit Of Quantitation (LOQ),

Intera assay Coefficients of Variability (intra CV%), Inter assay Coefficients of Variability (inter CV%), not applicable (N/A).

4.2.2 Blood pressure measurement

4.2.2.1 Office blood pressure measurement

In all studies, office blood pressure was measured using a validated automated oscillometer device, in accordance with the ESC/ESH 2018 guidelines[5]. Measurements were performed after at least 5 minutes of rest, with the participant in a relaxed, seated position, without crossing legs and with the cuff at heart level in a quiet room with an observer present. At least three, a maximum of five blood pressure readings were obtained by the investigator at intervals of at least 1 minute. The blood pressure value was the average of the two last measurements.

4.2.2.2 ABPM

In participants included in papers 1 and 2, ABPM was measured with a validated automated oscillometric device using the appropriate cuff size. The results were not visible to the participants. The persons were instructed to keep their arm still during measurement and avoid strenuous exercise during the measurement period, but otherwise participate in their normal daily activities. The device was set to automatic readings every 20 minutes in the daytime. In paper 1, the device was set to automatic readings every 45 minutes at night. Recordings under 22h, with less than 50% of the expected blood pressure readings between 1000 and 2200 h, and under five readings between midnight and 0600 h were repeated. These criteria were adopted from the International Database on Ambulatory Blood Pressure in Relation to Cardiovascular Outcome study[168]. In paper 2, the device was set to automatic readings every 30 minutes at night (22:00 to 6:00). Recordings with less than 70% of the expected blood pressure readings, or two or more consecutive hours without valid readings during daytime, were repeated[146]. We adjusted the readings to the patient-reported day and night periods. These criteria were adopted from the ESC/ESH 2018 guidelines[5, 146].

4.2.3 GFR measurements

4.2.3.1 Estimated GFR (eGFR)

Serum creatinine levels were assessed using an enzymatic technique[169]. Cystatin C was only available by an assay traceable to the international standard reference material in paper 3[13]. For this thesis, the 2009 CKD-EPI Creatinine and 2012 CKD-EPI Creatinine-Cystatin C equations without the race correction factor were assumed to be most accurate in the populations studied and were used to calculate eGFR[44, 170].

4.2.3.2 Measured GFR (mGFR)

GFR was measured with single sample plasma clearance of iohexol in RENIS-T6 (Paper 1). A Teflon catheter was inserted into the antecubital vein, and a null sample was drawn. The syringe was weighed for accuracy after injecting 5 ml of Iohexol (Omipaque 300 mgI/ml; Amersham Health, London, UK). The catheter was flushed with isotonic saline, and participants had a light breakfast before the iohexol blood sample was drawn. The optimal time for measuring Iohexol concentration was calculated using Jacobsson's method based on the GFR estimated by creatinine measured in Tromsø6 for each individual[171]. Sampling time was recorded using a stopwatch, with a minimum of 180 minutes, to ensure complete Iohexol distribution. Serum Iohexol concentration was measured by high-performance liquid chromatography with an intra-assay CV of 3.0% [172, 173]. GFR was calculated following Jacobsson's method, and the single-sample method has been found unbiased compared to multiple-sample methods[171].

4.3 Statistics

We used SPSS Statistics for Windows, Version 27.0 and 29.0 (IBM SPSS Statistics for Windows, Version 27.0/29 .0. Armonk, NY: IBM Corp). For paper 3, we also used *Hayes*' *PROCESS macro* for SPSS (Hayes, 2022)[174].

In all papers, non-normally distributed biomarkers were transformed before regression analysis. In paper 2, all biomarkers also had outliers and influential cases after transformation that changed the magnitude of regression coefficients and biased our statistical models. We performed a symmetric winsorization by replacing outliers with the next highest score that is not an outlier in a systematic way to reduce bias. The number of adjusted measurements in the upper distribution corresponds to the adjusted measurements in the lower distribution for each biomarker. Extreme measurements above three SD from the mean were adjusted to the nearest measured value below three SD[175].

The LoD for the urine albumin assay in papers 1 and 3 was given at 4 mg/L. However, the instrument used for the analysis reported albumin concentrations as low as 1 mg/L. In paper 1, sixty five percent of the participants had albumin concentration under the LoD, and only a few participants had moderately or severely increased ACR. We first used all values of albumin, including those below the LoD. In supplemental analysis, we substituted all observations of albumin concentration lower than 4 mg/L with 4 mg/L in each urine sample, calculated ACR using this value and used the median ACR value of the three samples in the repeated analysis. In doing so, we preserved a relative standing of persons with values below the limit relative to those above the limit, and a relative standing accounting for different urine volumes. The LoD corresponded almost to the median albumin concentration in Tromsø6. In paper 3, we performed supplemental analysis with left-censored observations using the deletion method of albumin concentration lower than 4 mg/L [176, 177]. The presence of measurement error attenuates correlations. If the independent variables have different degrees of measurement error, the correlations among them and with the dependent variable will be differentially attenuated with respect to the correlations that would be obtained if the measures were free of error. By censoring, we possibly reduced some of the measurement errors for the albumin concentration, which could reduce the standardized beta

coefficient. Beyond this, we excluded participants with missing data only from analyses for which the case had missing data in all papers.

Because our biomarkers, as independent variables, were measured in different units of measurement, the biomarker variables were standardized for the regression analysis, and we report the results as beta coefficients or odds ratios per SD. In multiple regression, standardized coefficients are useful for comparing the relative strength of the influence of independent variables on the dependent variable in the given sample. In logistic regression, the calculation of standardized coefficients is more complicated (a more detailed explanation is provided in the appendix). Paper 3 reports the semi-standardized regression coefficients from the Kaufman formula for the logistic regression analysis. The semi-standardized coefficient given by the Kaufman formula measures the change in predicted probability associated with a one SD change in the predictor and is restricted to the interval -1 to 1[178]. The semi-standardized regression coefficients were calculated at the mean predicted probability for the outcome in the population for the two longitudinal analyses. A range of estimated semi-standardized regression coefficients for different probability reference values was calculated and displayed graphically. This allowed us to compare the strength of the associations within models in the population examined[178, 179].

In all papers, multiple significance tests were used for descriptive purposes, and multiplicity corrections were not performed[180]. A 2-sided P value <0.05 was considered statistically significant.

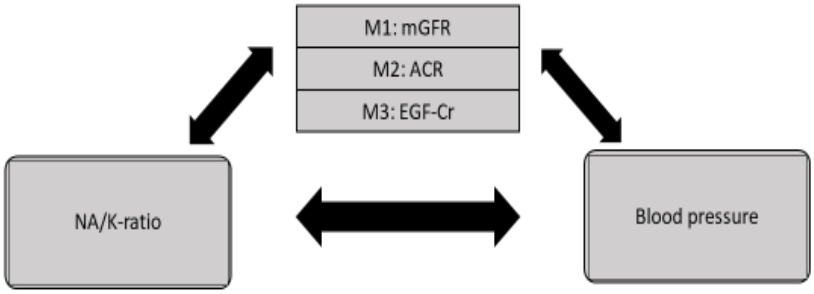
Baseline characteristics were summarized for the cohorts and subgroups with a mean (SD) for normally distributed variables, median (IQR) for skewed variables, and n (percentage) for categorical variables. Characteristics were compared using a t-test, Mann-Whitney test, chi-square test, Wilcoxon-signed-rank test, Fisher´s exact test of independence and Kendall´s

rank correlation (Kendall´s tau), as appropriate. Kendall´s rank correlation was used as a non-parametric test for a correlation in the biomarker's medians in relation to an ordinal scale level variable.

For papers 2 and 3, we used univariable and multivariable linear, logistic and multinomial logistic regression analyses to assess the associations between the plasma biomarkers as independent variables, and blood pressure and the hypertension groups as dependent variables.

For paper 1, we used statistical mediation analysis with bootstrapping as implemented in Model 4 of the PROCESS macro for SPSS[174] to assess the association between blood pressure as a dependent variable, and Na/K-ratio as the independent variable of interest. A mediating variable transmits the effect of an independent variable on a dependent variable[181]. We did a two-components mediation analysis to examine the direct and indirect effects. The direct effect was the effect of urinary Na/K-ratio on blood pressure and hypertension phenotypes, and the indirect effect, the mediation effect of Na/K-ratio on blood pressure and hypertension phenotypes, was through the kidney function variables mGFR, ACR and EGF-Cr (Figure 12).

Figure 12: Two components mediation analysis



M1: mGFR mediates the relationship between Na/K-ratio and blood pressure.
M2: ACR mediates the relationship between Na/K-ratio and blood pressure.
M3: EGF-Cr mediates the relationship between Na/K-ratio and blood pressure.
Glomerular filtration rate as a single-sample plasma clearance of iohexol (mGFR), urine albumin creatinine ratio (ACR), epidermal growth factor to the urine creatinine concentration (EGF-Cr).

5 Main results

5.1 Paper 1

The urinary Na/K-ratio demonstrated a significant association with both systolic office blood pressure and ambulatory blood pressure, even when accounting for cardiovascular risk factors and kidney function markers. A one SD unit rise in the urinary Na/K-ratio was linked to a 1.0 (0.3-1.6) mmHg increase in 24 h mean systolic ambulatory blood pressure, and a 3.6 (2.8-4.5) mmHg increase in systolic office blood pressure. Notably, markers of kidney function, EGF-Cr, ACR and mGFR, did not act as mediators on the association between urinary Na/K-ratio and blood pressure.

5.2 Paper 2

Our healthy controls had all undergone extensive assessment for living kidney donation to ensure their overall health. Our hypertension groups were well characterized, defined by ABPM[182-187], and on stable medication regimes with adherence to the number of reported antihypertensive agents assessed by pharmacological evaluation of serum drug concentrations[188]. The eGFR was not significantly different between those who had

controlled hypertension and those who had uncontrolled hypertension without evidence of kidney HMOD[189]. The latter group had significantly higher urine ACR, although within the normal range (<3 mg/mmol). The group with uncontrolled hypertension and kidney HMOD had, by definition, significantly lower eGFR and higher ACR compared to the other two hypertension groups. As hypertension-related structural abnormalities in the kidney can never completely regress[96, 98, 99, 101], these data suggest that our hypertension groups represent different stages of disease severity.

Plasma concentrations of interleukin-1 receptor antagonist (IL-1RA) and neutrophil gelatinase-associated lipocalin (NGAL) were found to be lower, while uromodulin levels were higher, in healthy controls (n=39) compared to individuals with hypertension (n=176). All biomarkers, except osteopontin (OPN), exhibited significant trends in median biomarker levels across all four study groups, including healthy individuals, those with controlled hypertension, uncontrolled hypertension, and uncontrolled hypertension with kidney HMOD. Moreover, all biomarkers, except regulated upon activation normal T-cell expressed and secreted (RANTES), showed significant trends in median biomarker levels across all three hypertension severity groups. In multinomial logistic regression models including only patients with hypertension and using controlled hypertension as the reference category, none of the biomarkers were associated with both groups with uncontrolled hypertension after adjustment for cardiovascular risk factors. OPN was the only biomarker associated with uncontrolled hypertension without kidney damage in models adjusted for cardiovascular risk factors and eGFR (odds ratio 1.77, 95% confidence interval 1.05-2.98, P=0.03), and RANTES was associated with uncontrolled hypertension with kidney damage (odds ratio 0.57, 95% confidence interval 0.34-0.95, P=0.03).

5.3 Paper 3

In the cross-sectional analysis of 7,197 participants with an average age of 63.5(SD 9.2) years and mean blood pressure of 141/78 mmHg (SD 23.0/10.6), orosomucoid-creatinine ratio (OCR) and ACR, but not N-acetyl-β-D-glucosaminidase-creatinine ratio (NAG-Cr), were significantly associated with systolic and diastolic blood pressure. OCR had a consistently, although marginally, stronger association with blood pressure than ACR. In fully adjusted models with cardiovascular risk factors and all urinary biomarkers included, OCR was the only urine biomarker significantly associated with blood pressure among those with and without treated hypertension and the entire cohort. OCR exhibited the most pronounced significant interactions with sex and age. The age interaction was most prominent for OCR in men, and OCR showed a stronger association with blood pressure in the youngest category (men ≤60 years, p for interaction 0.001).

In the longitudinal assessment involving 2,744 subjects without hypertension during Tromsø6, 681 subjects were identified with incident hypertension in Tromsø7. Here again, OCR and ACR, but not NAG-Cr, demonstrated a significant association with incident hypertension after a seven-year interval, both in unadjusted and adjusted models. The semi-standardized regression coefficients indicated that OCR exhibited a marginally stronger association than ACR with incident hypertension. Further, within the subset of 353 subjects who had controlled hypertension in Tromsø6, 193 individuals experienced progressive hypertension in Tromsø7. Only OCR and ACR, but not the NAG-Cr, were significantly or borderline significantly associated with progressive hypertension in crude and multivariable models. OCR and ACR showed equally strong associations with progressive hypertension in the multivariable models. There were no interactions between the biomarkers-creatinine ratios and age or sex in the longitudinal analysis.

The analyses with ACR left-censored, considering participants with urine albumin concentration ≥4 mg/L only, included 2553 participants in the cross-sectional analysis with blood pressure, and 752 participants in the longitudinal analysis with incident hypertension as the dependent variables. The analysis reinforced the previously described pattern with the strongest associations between OCR, blood pressure and incident hypertension.

The associations of the median concentration of albumin and orosomucoid, without the urine creatinine ratios, did not differ substantially from the corresponding associations with ACR and OCR. Urinary NAG concentration showed a stronger association with blood pressure and incident hypertension than NAG-Cr. However, NAG consistently displayed the weakest association with blood pressure and incident hypertension, regardless of urinary creatinine.

6 General discussion

6.1 Methodological discussion

6.1.1 Assessment of biomarkers

Controlled 24-hour urine collection is the gold standard method for urine biomarker quantification. However, a 24-hour collection is cumbersome for patients and is unreliable due to under- or over-collection and laboratory processing methods[50, 190]. Spot urine albumin, expressed with ratios of urine creatinine to adjust for varying urine concentration and dilution, is a convenient way to estimate UAE, and there is a high correlation between 24-hour urinary albumin excretion and ACR[191, 192]. However, the best estimations for biomarker excretions in urine may vary for glomerular and tubular biomarkers[193, 194]. For instance, plasma uromodulin and EGF are suggested markers of intact tubular cells and remaining functional kidney tissue. Urine creatinine depends on generation, non-renal elimination, glomerular filtration and tubular processes. Adjusting the sample for urine creatinine concentration might bias the associations between the biomarkers and the outcome of interest[190]. To mitigate this issue, a potential solution may involve quantifying

biomarkers excreted in urine over a specified time interval. However, timed urine collections have a known problem for not obtaining precisely timed supplies, especially in outpatient settings[50, 195].

Urine albumin, orosomucoid and NAG were measured in morning urine samples collected on three consecutive days. We reduced the impact of a day-to-day variation using the median values of the three specimens. We do not know the correlations between 24-hour urine collections and single or multiple spot urine measurements of orosomucoid and NAG. Urine EGF was measured in second void morning spot urine, stored at -80°C. There is a moderate correlation between 24-hour urine collections and single spot urine EGF-Cr (r=0.6)[196].

The best way to assess electrolyte intake is through multiple controlled 24-hour urine collections[197-200]. However, this method is prone to errors due to incomplete collections, especially in population studies[195, 197, 199]. Accurate control of these collections is crucial to avoid bias in estimating electrolyte intake[197, 201, 202]. However, it is imperative to note that the current knowledge does not offer a feasible, reliable methodology for evaluating the thoroughness of a 24-hour urine collection[50, 199, 200, 203]. Further, 24-hour samples may pose a high burden on participants, and when strict control measures are applied in larger population studies, there is a chance of unintentionally introducing a systematic bias[195, 197]. The spot urine Na/K-ratio may help mitigate systematic errors from incomplete 24-hour collections in larger studies[199, 201, 202]. Spot urine samples, while less accurate, can still show a moderate correlation with 24-hour collections[202, 204, 205]. Multiple random spot urine examinations are recommended to reduce random error[201, 204]. A limiting factor in our study is the measurement of urine Na/K-ratio in only one second void morning spot urine after an overnight fast[202]. This moderate correlation also implies that a larger sample number of participants would be required, compared to 24-hour urine samples, to identify an association between blood pressure and Na/K-ratio.

In paper 2, the Luminex multiplex method allowed us to analyse multiple biomarkers simultaneously using small volumes, which is preferable when only limited plasma volumes are available. Luminex multiplex kits manufacturers apply their own unique calibration standards, leading to varying concentrations across different kits. Thus, the absolute concentrations obtained using the method cannot be directly compared to measurements from the same method using different kits, or measures obtained using different methods[206]. However, the results remain reliable for a valid comparison between our study groups.

6.1.2 Design

This thesis is primarily based on cross-sectional investigations of data from the Tromsø6, the RENIS-T6, and baseline data from the IDA and CENS studies. The information and data were recorded at a single point in time or within a few months. Thus, no definite conclusion about the cause-and-effect relationship can be drawn in these cross-sectional studies.

This thesis also includes a longitudinal investigation of data from Tromsø6 and Tromsø7 for a prespecified study objective[144, 163, 207]. Essential factors for a reliable longitudinal biomarker study are: (1) a well-designed study with appropriate eligibility criteria, (2) ample available specimens, (3) preplanned analysis, (4) reliable assay performance on stored samples, and (5) masking assay users from clinical data[163, 207, 208]. In our prospective observational study, we assessed urinary biomarkers in relation to hypertension development in 3097 individuals divided into two longitudinal analyses on incident and progressive hypertension. The Tromsø study, with its rigorous conduct, provided a representative study population suitable for the intention of diagnosing hypertension. Further, three baseline spot urine measurements were analysed by personnel blinded to clinical information, aligning with the criteria for unbiased assessment. The analytical performance is further elaborated in section 6.1.1 and Table 4. A limitation is that the longitudinal analysis for progressive hypertension only included 353 participants with 193 outcome events, and the population may

not have provided adequate statistical power to assess the associations between the biomarkers and progressive hypertension.

Furthermore, all the studies in this thesis are observational, and thus, definitive causal conclusions cannot be drawn[163].

6.1.3 Bias

Any systematic error in the design, execution or presentation of a study that results in incorrect estimates of the association between exposure and outcome is referred to as “bias”. Bias causes a lack of internal validity and is generally categorized into two main groups: selection bias and information bias[209].

Systematic errors should be distinguished from random error or lack of precision[209]. Random error could arise from imprecise and inaccurate measurement values due to poorly calibrated instruments with low precision and validity. Measurements of biomarkers have been addressed previously; beyond this, a consistent and detailed research protocol was followed in all studies, and the instruments and equipment utilized were of high quality and calibrated.

6.1.3.1 Selection bias

Randomly sampled subjects are desirable for obtaining a study population representative of the target population to whom a test will be applied in clinical practice[163]. 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[207, 209].

Selection bias in the form of non-responder bias may be present in all studies used in this thesis. These non-responders could differ from the responders regarding the exposure and outcomes of interest. To assess the presence of selection bias in a study, the characteristics of

the responders must be compared with the non-responders. For participants in paper 2, we do not have data to assess this. In the RENIS-T6 and Tromsø surveys, the participants were randomly selected from all eligible individuals. Approximately 35% of the participants invited to Tromsø6 and RENIS-T6 did not participate. We know that the non-attendees in Tromsø6 and RENIS-T6 surveys were more likely to be men across all age groups[144, 210]. Further, 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[144, 210, 211]. This may indicate that participants are healthier than non-participants or that participation influences individuals to adopt healthier lifestyles.

Observational studies on the association between sodium consumption, blood pressure and cardiovascular disease, are prone to reverse causality. Reverse causality in sodium studies occurs when sick individuals reduce their Na intake due to medical advice or illness-related food consumption changes. Studies excluding diseased participants at baseline have been designated as having the lowest risk of bias, while studies recruiting sick participants have been designated as having the highest bias risk[197]. The RENIS study, therefore, comprises a population particularly suited to study the association between sodium and potassium consumption and blood pressure.

Selection bias in the probability of being included or excluded applies to paper 2. Subjects were selected from a large nationwide multicentre study on hypertension and an ongoing study of living kidney donors with the inclusion and exclusion criteria given in Table 3. The individuals participating in the two studies were further age- and sex-matched to the greatest extent possible. This limits how representative the study population is in relation to the population that may be relevant for examination in clinical practice. However, the performance of biomarkers tends to be overstated when only subjects at the extreme ends of the disease spectrum are enrolled. This so-called spectrum effect on test performance can

occur when healthy subjects are studied together with subjects at an advanced disease stage. Leaving out subjects in the middle of the disease spectrum can also make different tests appear more comparable in performance than if the full spectrum of subjects were enrolled[163, 209]. As blood pressure typically rises with age, age-based matching can lead to the "spectrum effect." In our study, the group of healthy controls was younger than the group of patients with hypertension. When we attempt to match them based on age, we face the risk of selecting the oldest and healthiest controls and the youngest and sickest hypertension patients. However, the associations between biomarkers and the severity of hypertension were only tested within the different hypertension groups, and the healthy controls were not included in these analyses. Among the patients with hypertension, the inclusion of an intermediate group, as was done in this study, may limit some of this spectrum effect.

Moreover, in Paper 3, several albumin values were below the LoD, and we conducted additional linear and logistic regression analyses exclusively using participants with urine albumin concentrations above the detection limit. Normal urinary albumin excretion (UAE) is defined as less than 10 mg/day, and our albumin assay had a detection limit of 4 mg/l[13]. The results of spot urine samples will depend on the volume of excreted urine influenced by fluid intake, physical activity and cardiovascular risk factors[212, 213]. Urine albumin was measured in morning urine samples collected on three consecutive days, reducing the impact of a day-to-day variation[214-216]. Individuals with UAE above the LoD may not represent a sample of the general population, indicating a potential for selection bias in this additional analysis.

6.1.3.2 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[209].

In Tromsø6 and RENIS-T6, participants completed a questionnaire before or on the examination day. In the IDA and CENS studies, participants underwent a structured physician-patient interview collecting information about demographic and lifestyle data, socioeconomic factors, and medical and family history[146, 147]. When recalling past lifestyle-related or treatment behaviours, there is a possibility of recall bias, where different subgroups may overestimate or underestimate frequencies (reporting bias) depending on their characteristics (e.g., uncontrolled hypertension) and the specific question being asked[146, 197].

The error arises when the measured overall mean differs from the true overall mean. If the error is systematically related to the exposure or disease status, it can introduce unpredictable effects when estimating relationships between exposures and outcomes of interest[146, 197, 209]. In medical treatment, "adherence" is the ability of the patient to comply with the prescribed treatment[217]. Patients may state that they take their medication every day as prescribed, despite often forgetting to take their medication, or not wanting and refraining from taking their medication regularly[5, 146]. Past studies report widespread drug non-adherence spanning from below 10% to over 80%[4, 146, 218]. This variability is contingent upon the demographic composition of the patient cohorts and evaluation methods for non-adherence[4, 146, 218]. In the IDA study, medication adherence was addressed as self-reported adherence, physician-reported adherence after the structured physician-patient interview and as measured serum concentration of blood pressure lowering drugs. However, the different estimates of medication adherence in the study showed little concurrence[146]. In all three articles in this thesis, we have utilised the self-reported use of blood pressure lowering drugs to define a desired study population or an outcome of interest. Our study groups and results are contingent on participants providing accurate information.

6.1.4 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. External validity relies on internal validity, but having internal validity does not automatically ensure external validity[209].

The population included in the Tromsø Study consists almost exclusively of North European middle-aged and elderly persons, limiting broad generalizations. The population studied in the RENIS-T6 is recruited from the Tromsø study and thus also consists almost exclusively of North European middle-aged persons with generally normal or near normal kidney function. In addition, they were without diabetes, cardiovascular disease or treated hypertension, limiting broad generalizations to other healthy and sick populations. The specific inclusion and exclusion criteria constrain the generalizability of the population with hypertension from the IDA study. The population is additionally constrained by age and gender-matched selection, contingent on the CENS study, limiting the representativeness of the study population, particularly to a hypertension population that could be pertinent in clinical practice. Therefore, caution should be exercised when extrapolating the findings to other populations from other geographical regions with different age distributions or diseases. Ideally, the results presented in this thesis should have been validated in an independent cohort. However, as previously mentioned, all biomarkers examined in this thesis have previously shown associations with either hypertension, CKD or CVD. Our studies must thus be regarded as validation studies and an extension of previous work.

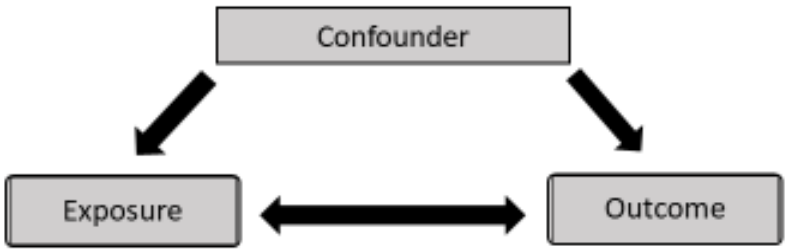
The concentrations of the biomarkers studied may vary between geographical regions or exhibit different concentrations or effects based on sex, age, or underlying diseases. In Tromsø6 and Tromsø7, where we examined a broader population representative of the general population, we observed statistically significant differences in baseline concentrations of urine ACR, OCR and NAG-Cr based on age and sex. We explored interaction effects

between the urinary biomarkers, age, and sex. OCR showed a stronger association with blood pressure among men in the youngest category (men ≤60 years, p for interaction 0.001).

6.1.5 Confounding

A confounder is a variable that should be associated with both the exposure and the outcome and is not on the causal pathway between the exposure and outcome[219]. Confounding occurs when the relationship between an exposure and outcome variable is influenced by a third variable, so that ignoring the third variable leads to incorrect inference about the relation of exposure and outcome (Figure 13)[181, 219].

Figure 13: Confounding



In all papers, we adjusted the analysis for the established cardiovascular risk factors sex, age, body mass index (BMI) or waist-hip-ratio, diabetes or HbA1c. In papers 2 and 3, we also adjusted for prior cardiovascular disease. In paper 2, the healthy controls and patients with hypertension were age- and sex-matched to the greatest extent possible to mitigate the potential confounding effects of age and sex on the biomarker plasma levels. However, we did not adjust for every known or suspected blood pressure and hypertension determinant. For instance, 21% of the RENIS-T6 population reported that they were daily smokers[220]. Smoking tobacco has been associated with higher eGFR, and a steeper rate of mGFR decline[221, 222]. Research utilizing ABPM indicates that individuals with normal office blood pressure who smoke tend to exhibit elevated daily blood pressure compared to non-smokers[25, 223, 224]. In paper 1, we used various dependent variables derived from ABPM

and office blood pressure measurements, and the mediation analysis was not adjusted for the confounding effect of smoking. Furthermore, the causal mechanisms between biomarkers investigated in this thesis and blood pressure have not been fully elucidated. Despite adjustment for every known or potential determinant of blood pressure, residual confounding would remain a concern due to unknown mechanisms involved in the complex pathogenesis of hypertension.

6.1.7 Multiple significance tests

The null hypothesis forms the formal basis for testing statistical significance. The null hypothesis postulates no association between an independent and dependent variable in a population. The probability of making a type 1 error is called α (alpha), or statistical significance. With $\alpha = 0.05$, this represents a 5% probability of erroneously rejecting the null hypothesis. The probability of not rejecting the null hypothesis is 1- α. In studies that test multiple hypotheses or perform multiple comparisons, the likelihood of rejecting the null hypothesis when true in the population increases as the number of comparisons rises. This error rate across multiple statistical tests is called the familywise error rate and can be calculated as $(1- \alpha)^n$, where n is the number of statistical tests conducted[181]. There are various methods to control for multiple testing, which reduces the chance of making a type 1 error, but they also decrease the study's statistical power, the probability of observing an effect in the sample if it exists[180, 181]. In exploratory studies where data is collected with an objective, but without a pre-specified main hypothesis, multiple test adjustments are not strictly necessary[180]. Exploratory studies often require a flexible approach to design and analysis. The choice of tested hypotheses and their quantity may depend on the data, meaning multiple significance tests can only be used for descriptive purposes. Since the number of tests in such studies is often large, appropriate multiple-test adjustment is difficult. Significant results based on exploratory analyses should specify whether multiple testing has been

performed, and these results should be labelled as exploratory[180]. To confirm these results, the corresponding hypotheses must be tested in confirmatory studies, where multiple test adjustments will be strictly necessary[180]. All our studies should be considered exploratory. Further studies are required to clarify whether the examined biomarkers can enhance risk stratification and contribute to a better targeted treatment with a reduction in morbidity and mortality.

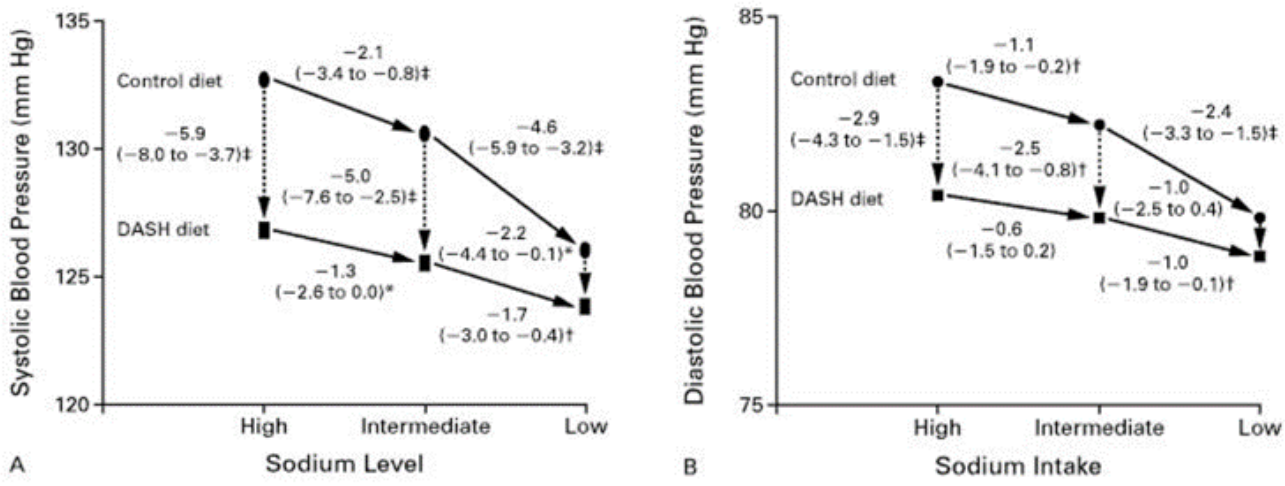
6.2 Discussion of the main results

6.2.1 Paper 1: The association between urinary sodium-potassium ratio, kidney function, and blood pressure in a cohort from the general population

In a middle-aged general population cohort without diabetes, chronic kidney disease, cardiovascular disease or treated hypertension, we found that spot urine Na/K-ratio was significantly associated with systolic office blood pressure and ambulatory blood pressure independently of cardiovascular risk factors and kidney function markers. EGF-Cr, ACR and mGFR did not mediate the relationship between urinary Na/K-ratio and systolic blood pressure, suggesting a relationship between a diet with high sodium and low potassium and higher blood pressure regardless of kidney function. It is important to acknowledge that this study should be considered hypothesis-generating, and additional investigations employing methodologies for a more accurate estimation of urinary sodium and potassium levels are warranted[198, 201, 202, 204]. Spot urine samples will give rise to greater random errors in estimates of sodium and potassium and will bias the relationship between Na and K intake and BP toward the null. The lack of 24-hour urine collections further limited the possibility of examining the dietary contributions of sodium and potassium alone. In the clinical trial Dietary Approaches to Stop Hypertension (DASH), the effects of dietary patterns on blood pressure were assessed in 412 adults randomly assigned to eat either a control diet typical of intake in the United States or the DASH diet, a diet rich in fruits and vegetables and low-fat

dairy products and with reduced saturated and total fat[225]. Sodium intake and body weight were maintained at constant levels. As shown in Figure 14, the sodium intake had approximately a 2x effect on blood pressure in the control diet compared to the DASH diet. Reducing sodium intake from a moderate to a low level had a greater effect than reducing it from a high to a moderate level. The DASH diet resulted in significantly lower systolic blood pressure at all levels of sodium intake and had the greatest effect at a high sodium intake[225].

Figure 14: Effects on blood pressure of reduced dietary sodium and the Dietary Approaches to Stop Hypertension (DASH) diet.



Sacks FM, Svetkey LP, Vollmer WM, Appel LJ, Bray GA, Harsha D, Obarzanek E, Conlin PR, Miller ER, 3rd, Simons-Morton DG *et al*: Effects on blood pressure of reduced dietary sodium and the Dietary Approaches to Stop Hypertension (DASH) diet. DASH-Sodium Collaborative Research Group. *N Engl J Med* 2001, 344(1):3-10. DOI:10.1056/NEJM200101043440101

Furthermore, two studies have shown that replacing salt with a potassium chloride salt substitute has reduced the risk of stroke and cardiovascular disease mortality[88, 89]. The urinary Na/K-ratio does not directly measure of sodium and potassium intake[226]. The importance of a high potassium diet is probably greater with increasing sodium intake, and the association between the Na/K-ratio and blood pressure seems independent of the individual urinary levels of sodium and potassium[227]. In this regard, we emphasize that we

have not looked at the amount of sodium or potassium, but the ratio. Our findings may indicate that the effect of a lower sodium intake and higher potassium intake can be extended to a healthier population relative to previously studied populations[228, 229]. Because estimations of the amount of sodium and potassium intake in a population are difficult and costly to obtain through multiple 24-hour urine collections, a ratio-effect could represent a paradigm that can be broadly and usefully applied in planning and follow-up of public health programs[76, 199, 201, 230, 231]. Conducting a randomized controlled trial would be essential to determine whether dietary interventions, focusing on achieving a recommended Na/K ratio as opposed to recommended sodium and potassium levels measured through 24-hour urine collections, could improve blood pressure and potentially contribute to a further reduction in cardiovascular endpoints.

6.2.2 Paper 2: Novel biomarkers in patients with uncontrolled hypertension with and without kidney damage.

In this study comparing healthy controls and three hypertension groups (controlled, uncontrolled without kidney HMOD, uncontrolled with kidney HMOD), none of the biomarkers examined could consistently differentiate all three hypertension groups when established risk factors were considered. However, our small sample size could account for the biomarkers' inability to differentiate between the hypertension groups. Among the three groups with hypertension, OPN was the only biomarker significantly associated with uncontrolled hypertension without kidney HMOD, independent of cardiovascular risk factors and eGFR. The eGFR was not significantly different between those who had controlled and those who had uncontrolled hypertension without evidence of HMOD. However, the latter group had significantly higher urine ACR, although within the normal range. Subclinical kidney disease is associated with developing hypertension, forming a damaging cycle of worsening kidney damage and elevated blood pressure[7, 8]. Early-stage kidney HMOD biomarkers could enhance risk assessment and guide personalized treatment. Our study

indicates that OPN may be an early biomarker of kidney HMOD in hypertension. OPN has been associated with hypertension, HMOD, CKD progression and all-cause mortality in previous studies[232-237]. In light of this, a study of Chinese patients with uncontrolled hypertension investigated serum OPN levels in individuals with or without CKD, defined as an eGFR < 60 mL/min/1.73 m² or spot urine ACR >2.5 mg/mmol for males and >3.5 mg/mmol for females[233]. Elevated serum OPN was independently associated with reduced GFR and moderately increased ACR, regardless of blood pressure and other cardiovascular risk factors[233]. In our study, which involved a similar population, OPN did not exhibit a similar association with the kidney-related endpoint. It's important to highlight that their study had a larger sample size and excluded patients with diabetes. Furthermore, eGFR is insensitive to early and low-grade kidney injury, leaving a potential for other biomarkers to exhibit an association with kidney injury in regression models that include eGFR in these early stages. As eGFR starts to decline, indicating more substantial kidney damage, it may become increasingly challenging for other biomarkers to demonstrate an independent contribution beyond eGFR for an association with kidney damage.

Nevertheless, further longitudinal studies are required to ascertain whether any of the examined biomarkers can enhance risk assessment in hypertension.

6.2.3 Paper 3: Associations of urinary orosomucoid, N-acetyl-β-D-glucosaminidase, and albumin with blood pressure and hypertension after 7 years. The Tromsø Study.

In a cohort from the general population, it was observed that urinary orosomucoid excretion, compared to ACR, displayed a marginally stronger cross-sectional association with blood pressure, both in individuals receiving treatment and those not under treatment with blood pressure lowering drugs. OCR also showed a marginally stronger association with the development of hypertension after seven years compared to ACR. This may suggest that urinary orosomucoid excretion may serve as an earlier biomarker for kidney and endothelial

damage in hypertension than ACR. It's worth mentioning that this study did not investigate the associations between urine biomarkers and clinically relevant endpoints in hypertension. In previous studies, among individuals with type 2 diabetes and normal UAE, an increase in urinary orosomucoid was found to independently predict cardiovascular mortality[238-240]. In patients with preeclampsia, an increase in urinary orosomucoid was observed before the development of elevated albumin levels and even before the rise in plasma orosomucoid levels[241]. Additionally, a recently published cross-sectional examination of Tromsø6 participants revealed an association between urinary orosomucoid and diastolic dysfunction and carotid arteriopathy. Interestingly, UAE did not exhibit a significant association with diastolic dysfunction, but its relationships with carotid arteriopathy were on par with urinary orosomucoid[242]. However, it is important to note that further research is necessary to elucidate the underlying pathophysiological mechanisms. The observational nature of our study constrains any assertions about causality, and prospective clinical studies are required to determine whether orosomucoid can serve as a clinically valuable biomarker in the context of hypertension.

The role of tubulointerstitial function is pivotal in regulating blood pressure, and tubulointerstitial damage tends to manifest early in CKD[24, 141]. Nevertheless, our findings revealed inconsistent associations between urinary -NAG, blood pressure, and hypertension.

We used a highly sensitive immunoassay to analyze orosomucoid, with a LoD lower than the lowest value in our population[162]. The urine albumin assay in Tromsø6 had a LoD that approximated the median albumin concentration in our study population. Unfortunately, we could not obtain information about the analytical performance of NAG measurements. When independent variables have varying degrees of measurement error, it can affect their correlations with each other and the dependent variable. As numerous albumin values were below the LoD, we conducted additional linear and logistic regression analyses using only

participants with urine albumin concentrations over the detection limit. These analyses confirmed the previously observed patterns, showing the strongest associations between orosomucoid, blood pressure, and incident hypertension. By doing this, we likely reduced some of the measurement errors related to albumin concentration, potentially leading to more accurate standardized beta coefficients for ACR. However, the ensuing smaller sample size results in poorer estimations of the population effect, and whether the results in this study are due to properties of the measured biomarkers, analytical precision, or a combination cannot be determined.

7 Conclusion

We conclude that in a representative sample from the middle-aged North European population without diabetes, CKD, CVD or treated hypertension, there was a significant association between urinary Na/K-ratio and blood pressure independently of cardiovascular risk factors. The association with blood pressure was not mediated through kidney function measures of glomerular and tubular function (mGFR, ACR and EGF-Cr), suggesting a relationship between a diet with high sodium and low potassium and higher blood pressure regardless of kidney function.

Further, healthy controls fulfilling the Norwegian transplantation protocol for living kidney donors, compared to patients with hypertension being prescribed ≥2 antihypertensive agents, had lower IL-1RA, NGAL, and higher uromodulin plasma levels. IL-1RA, IL-18, TNF, MCP-1, OPN, vWF-A2 and NGAL showed significant trends in plasma levels across study groups consisting of controlled hypertension, uncontrolled hypertension without kidney HMOD and uncontrolled hypertension with kidney HMOD. After considering established risk factors, none of the biomarkers could consistently differentiate all three hypertension severity groups. Among patients with hypertension, OPN was significantly associated with

uncontrolled hypertension without kidney HMOD, independent of cardiovascular risk factors and eGFR, suggesting that OPN may be an early biomarker for Kidney damage in hypertension.

Finally, in a North-European general population cohort, urine orosmucoid excretion had a stronger cross-sectional association with blood pressure and a stronger association with incident hypertension after seven years than UAE. Urine orosomucoid excretion may be an earlier biomarker than UAE for kidney and endothelial damage in hypertension. Urinary NAG excretion had varying and weak associations with blood pressure and hypertension.

8 Perspectives

The aetiology of primary hypertension remains inadequately elucidated and probably arises from an interplay between genetic and environmental factors, and their cumulative influences on cardiovascular and kidney structures and function[3, 24, 69, 70]. Elevated systolic blood pressure is the leading global risk factor for mortality[2]. A poor-quality diet, characterized by high sodium intake and low potassium consumption, contributes to hypertension[2, 76, 90]. The World Health Organization recommends reducing sodium and increasing potassium intake[76, 90]. To do this effectively, we need reliable methods for measuring and monitoring sodium and potassium intake studies[195, 197, 199]. Estimating sodium and potassium intake in a population through multiple 24-hour urine collections is challenging and costly[199, 201]. Using a ratio-based approach to assess the population's sodium and potassium intake could be a more manageable and cost-effective method to implement and supervise public health programs to enhance dietary habits[2, 75, 202].

Despite risk scores playing a key role in the stratification of patients with hypertensive, a substantial gap remains between predicted and actual event rates[3, 127, 128]. Biomarkers can enhance individual risk stratification[128]. HMOD acts as a crucial intermediate stage

between cardiovascular risk factors and advanced CVD or CKD[3]. The relationship between hypertension and CKD forms a vicious cycle where each condition worsens the other. This mutual connection highlights the need for early detection of CKD and hypertension, strict blood pressure control, and effective CKD management to prevent disease progression and, in turn, delay cardiovascular events and kidney complications[2]. Biomarkers that can detect kidney HMOD early and are readily available for clinical use may improve risk stratification of patients.

Nevertheless, a novel biomarker must meet various criteria, including prospective validation to demonstrate its accuracy and ability to enhance individual patient risk assessment. Further, it needs to be cost-effective and user-friendly with methodological consensus and reference values[163, 207, 243]. Finally, the clinical value of a biomarker should be assessed by its effect on patient management and outcomes[207]. However, since the studies in this thesis are observational, definitive causal conclusions cannot be drawn. Further research is necessary to determine whether the biomarkers we investigated can genuinely assist the treatment of hypertension[163, 207].

The RENIS-4 study, initiated in September 2023, marks the fourth follow-up in the ongoing research. With the expanded follow-up, RENIS-4 is expected to reveal a higher incidence of reduced GFR and strengthen our capacity to explore risk factors for CKD. Stated to commence in early 2025, spanning through 2026, the Tromsø8 aims to invite individuals aged 40 and above from the Tromsø municipality to participate. Furthermore, the IDA study has initiated long-term data collection. These surveys will be necessary for further longitudinal research, paving the way for investigations into biomarker concentrations in relation to kidney function and blood pressure development.

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Paper 1

The association between urinary sodium-potassium ratio, kidney function, and blood pressure in a cohort from the general population.

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Abstract

Introduction

Subclinical kidney dysfunction may contribute to salt sensitive hypertension. We assessed the association between the urinary sodium-potassium ratio (Na/K-ratio) and blood pressure (BP) in a general population cohort without diabetes, chronic kidney disease, cardiovascular disease or treated hypertension. We investigated whether any such association was mediated by the kidney function markers measured glomerular filtration rate (mGFR), urinary albumin creatinine ratio (ACR) and epidermal growth factor – creatinine ratio (EGF-Cr).

Methods

The Tromsø Study is a population-based study of inhabitants of the municipality of Tromsø, Northern Norway. Participants aged 50-62 years, without diabetes, chronic kidney disease or cardiovascular disease, were invited to the substudy Renal Iohexol Clearance Survey in Tromsø 6 (RENIS-T6; 2007-09). For the present study we excluded participants reporting the use of 1 or more antihypertensive agents, leaving 1311 RENIS-T6 participants for a cross-sectional analysis. We measured office BP, 24-hour ambulatory blood pressure (ABP) and mGFR using iohexol-clearance. Na/K-ratio, ACR, and EGF-Cr were measured in morning urine samples.

Results

Urinary Na/K-ratio was significantly associated with systolic office BP and ABP independently of cardiovascular risk factors and kidney function markers. A one standard deviation unit increase in the Na/K-ratio was associated with increased systolic ABP by 1.0 (0.3-1.6) mmHg. Urinary Na/K-ratio showed a stronger association with office BP than ABP. EGF-Cr, ACR and mGFR did not mediate the relationship between urinary Na/K-ratio and systolic BP.

Conclusions

In a representative sample of the middle-aged North-European population without diabetes, chronic kidney disease, cardiovascular disease or treated hypertension, there was a consistent association between urinary Na/K-ratio and BP. The association with BP was not mediated through kidney function measures, suggesting a relationship between a diet with high sodium and low potassium and higher BP regardless of kidney function.

Introduction

Hypertension is globally the most significant contributor to morbidity and mortality (1). All patients should be empowered to make lifestyle changes that may prevent and improve hypertension and reduce the need for antihypertensive drug therapy (2). An advisable lifestyle modification is sodium restriction (2). The global mean daily sodium intake is estimated to be approximately 4 g/day, equivalent to 10 g/day of salt (3), and about twice the WHO recommended limit (4). A high sodium intake in the general population is associated with hypertension, chronic kidney disease, and cardiovascular disease (5, 6), and sodium restriction has a blood pressure(BP)-lowering effect (7). However, BP response to a change in sodium consumption is heterogeneous and the term salt sensitivity has been applied to persons with the greatest change in BP due to altered sodium intake (8). Subclinical kidney dysfunction with a decrease in whole kidney ultrafiltration and an increase in tubular sodium reabsorption has been implicated as a cause of salt sensitivity (9). The renal abnormalities leading to this increased reabsorption have not yet been fully elucidated. A diet high in sodium and low in potassium can stimulate salt reabsorption along the distal nephron (10). A low potassium intake may also catalyst renal vasoconstriction, leading to persistent preglomerular arteriolopathy, tubular ischemia, and interstitial inflammation, generating deviant renal sodium handling and increasing BP (11). On the other hand, a high potassium intake is associated with lower BP and reduced risk of cardiovascular disease (12, 13). Replacing salt with a potassium chloride salt substitute has reduced the risk of stroke and cardiovascular disease mortality (14, 15). The urinary sodium-potassium ratio (Na/K-ratio) is a proxy for sodium and potassium intake. It is independent of urine volume and body weight and has a higher correlation with 24-h urinary excretion of each electrolyte than the urinary Na or K concentration alone (16). Na/K-ratio has shown a stronger association with BP, hypertension and cardiovascular disease than urinary sodium or potassium alone (17, 18), and the association between Na/K-ratio and BP is independent of the individual urinary levels of Na and K (19).

No population-based studies have addressed the association between Na/K-ratio and BP in the specific context of kidney function. The present cross-sectional study aimed to assess the associations between Na/K-ratio, BP and hypertension in a cohort of individuals representative for the North-European

general population without diabetes, chronic kidney disease or cardiovascular disease (20, 21). Further, we aimed to examine if an association is mediated through the kidney function variables measured glomerular filtration rate (mGFR), urine albumin creatinine ratio (ACR), and Epidermal growth factor (EGF-Cr). We hypothesised that the U-Na/K-ratio is associated with BP and different hypertension phenotypes independently of traditional cardiovascular risk factors, and that mGFR, urine ACR and EGF modify this association.

Materials and Methods

Materials

The Renal Iohexol Clearance Survey in Tromsø6 (RENIS-T6) is a substudy of the population-based Tromsø Study, a prospective study of inhabitants of the municipality of Tromsø, Northern Norway. The sixth survey (Tromsø6) was conducted in 2007–2008. An age-stratified random sample of 19 762 inhabitants between 40 and 87 years of age was invited to participate. A total of 12 984 persons attended (66%). Among the 3564 participants aged 50-62, 2825 reported no previous myocardial infarction, angina pectoris, stroke, diabetes mellitus, or renal disease. These individuals were invited to RENIS-T6. A total of 2107 agreed. One hundred and twenty-five were excluded because of allergy to contrast media, iodine, latex, or for other reasons, including 48 who withdrew. Five subjects were excluded due to technical failure of the iohexol clearance measurement, and 1627 subjects were included according to a predetermined target of 1600. For the present study 17 were excluded due to missing ambulatory blood pressure (ABP) measurements after repeated attempts. Further, 299 participants reporting the use of one or more antihypertensive agents (beta blocking agents, calcium channel blockers, ACE inhibitors, angiotensin II receptor blockers or diuretics) were excluded to limit the possibilities of reverse causality, and because antihypertensive treatments could potentially affect urinary Na or K excretion, leaving 1311 participants for our study, shown in Figure 1.

Measurements

The details of sample procurement in the RENIS-T6 have previously been described (22).

All data were collected during the RENIS -T6 visit, besides urine ACR which was measured in the forerunner Tromsø6 survey. In the RENIS-T6 study participants met between 08:00 and 10:00 a.m. at the Clinical Research Unit at the University Hospital of Northern Norway after an overnight fast. Office BP, urine Na and K were measured simultaneously, and ABP over the following day and night.

Office BP was recorded in triplet by trained personnel using an automatic device (model UA 799; A&D, Tokyo, Japan)). The cuff was chosen according to the measured circumference of the upper right arm. After a 2-min rest, three attended readings were taken in a sitting position, separated by 1-min intervals (23). We used the mean of the second and third readings in the analyses.

ABP was measured from the completion of the iohexol clearance measurement, just before the participant left the Clinical Research Unit, to the next day. A Spacelabs 90207 device was applied (Spacelab Inc., Redmond, Washington, USA), using the appropriate cuff size. The persons were instructed to keep their arm still during measurement and avoid strenuous exercise during the measurement period, but otherwise participate in their normal daily activities. BP was measured at 20-minute intervals from 0800 to 2200 h and at 45-min intervals from 2200 to 0800 h. The registration was valid if it was of at least 22 h duration, at least ten readings between 1000 and 2200 h, and if there were five readings between midnight and 0600 h. These criteria were adopted from the International Database on Ambulatory Blood Pressure in Relation to Cardiovascular Outcome study (24). Persons with invalid registration had their ABP monitoring repeated as soon as possible.

The fall in BP during sleep, the night-time BP dip, was defined as the difference between daytime mean pressure and night-time mean pressure, expressed as a percentage of the daytime value.

The ambulatory white coat effect was defined as the difference between the office BP and daytime mean ABP (office BP minus daytime mean ABP) (2). Hypertension phenotypes were defined as white-coat hypertension, the untreated condition in which office BP was ≥140/90 mmHg, but 24h ABP <130/80 mmHg, masked hypertension, the untreated condition in which office BP was <140/90 mmHg, but 24h ABP≥130/80 mmHg, and sustained hypertension as the untreated condition in which office BP was ≥ 140/90 mmHg, and 24h ABP≥130/80 mmHg (2).

GFR was measured with single-sample plasma clearance of iohexol (mGFR). The serum iohexol concentration was measured by HPLC. The interassay coefficient of variation (CV) for the iohexol analysis with HPLC during the study period was 3.0%. External quality control was provided by Equalis (Equalis AB, Uppsala, Sweden). This method has been validated against gold standard methods and is accurately described in a previous publication (22).

From the RENIS-T6 survey we used a second void morning spot urine, stored at -80°C, to measure urine Na, K, EGF and creatinine. Urine Na and K were measured via the ion-selective electrode method using a COBAS 8000 autoanalyzer (Roche Diagnostic, Indianapolis, IN). EGF was measured using a human EGF immunoassay quantikine enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA). EGF was assessed in duplicate. Urine creatinine was measured using a colorimetric method with a COBAS 8000 autoanalyzer (Roche Diagnostic, Indianapolis, IN). We calculated the ratios of EGF to the urine creatinine concentration (EGF-Cr) to adjust for differences in urine concentration (25).

In Tromsø6, morning spot urine samples were collected on three consecutive days. Albumin and creatinine concentrations were measured at the time of collection in unfrozen samples. Urine albumin concentration was measured by an immunoturbidimetric method with the ABX Pentra Micro-albumin CP (Horiba ABX, Montpelier, France), whereas creatinine was analyzed by a colorimetric method (Jaffes reaction) using an autoanalyser (ABX Pentra, Horiba ABX, Montpellier, France). For each urine sample, we calculated the ACR, and the median of the values assessed on days 1,2, and 3 were used.

The subjects completed a questionnaire regarding medication use. Body mass index (BMI; kg/m^2) was calculated from measured height and weight. Fasting blood samples were drawn to measure lipids, HbA1c, creatinine and cystatin C, as previously described (22). We calculated creatinine-cystatin C based estimated GFR by applying the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (26)

Statistical analyses

Baseline characteristics are summarized for the cohort with a mean (SD) for normally distributed variables, median for skewed variables, and n (percentage) for categorical variables.

We used statistical mediation analysis with bootstrapping as implemented in Model 4 of the PROCESS macro for SPSS (27) to assess the associations between the BP variables and hypertension phenotypes as dependent variables in separate models, and Na/K-ratio as the independent variable of interest. We did a two components mediation analysis to examine the direct and indirect effects, shown in Figure 2. The direct effect was the effect of urinary Na/K-ratio on BP and hypertension phenotypes, and the indirect effect of Na/K-ratio was through the mediators mGFR, ACR and EGF-Cr on BP and hypertension phenotypes. We report the results of all the pathways shown in Figure 2. We adjusted the mediation analysis for the covariates age, sex, and traditional cardiometabolic risk factors (waist-hip-ratio, HbA1c, triglycerides and HDL-cholesterol). We report the mediation analyses direct and indirect effects as beta coefficients or odds ratios for the Na/K-ratio per SD.

The urine albumin assay's limit of detection in Tromsø 6 is given at 4 mg/L, but the PENTRA instrument reports albumin concentrations as low as 1 mg/L. Seventy percent of the participants had albumin concentration under the limit of detection. Due to a large number of observations below the limit and only a few participants with moderately or severely increased ACR we 1) used all values of albumin, including those below limit of detection, 2) substituted all observations of albumin concentration lower than 4 mg/L with 4 mg/L in each urine sample, calculated the ratio of albumin concentrations with the urine creatinine concentration and used the median value of the three samples in the repeated mediation analysis. As so, we preserved the relative standing of persons with values below the limit relative to those above the limit and a relative standing accounting for different urine volumes. The limit of detection for the EGF assay was 0.7 pg/mL. No participants had EGF lower than the limit of detection.

In multivariate linear regression analyses, we tested for collinearity with the variance inflation factor, tolerance statistics, the eigenvalue of the scaled, uncentred cross-products matrix, and variance proportions.

Statistical significance was defined as two-sided P <0.05, and the correspondent *t-statistics* value *1.96* in all analyses.

We used SPSS Statistics for Windows, Version 29.0 (IBM SPSS Statistics for Windows, Version 29 .0. Armonk, NY: IBM Corp) and *Hayes*' *PROCESS macro* for SPSS (Hayes, 2022).

Results

Baseline characteristics

A total of 1311 participants with a mean (SD) age of 58 (3.8) years, BMI of 26.8 (3.8) kg/m2, Na/K-ratio 1.43 (0.76), and mGFR 94.3 (14.2) mL/min/1.73m2 were included in the study (Table 1). Office BP was 128/82 (17.5/9.6) mmHg, 24 h ABP 122/76 (12.3/7.9) mmHg (Table 2). The distribution of mGFR stratified by sex is shown in Figure 3. Among the 360 participants with urine albumin above the assay's limit of detection, 73 participants had high normal ACR (ACR 1.13- <3.40 mg/mmol), 12 participants had moderately increased ACR (ACR 3.40-≤34mg/mmol), and one participant had severely increased ACR (ACR > 34 mg/mmol).

The associations between Na/K-ratio and ABP

We found no sign of multicollinearity in the multivariate linear regression analyses. Results of the regression analysis are presented in the supplementary tables 1-3.

There was a significant direct effect of Na/K-ratio on systolic ABP, and a borderline significant direct effect on 24 h mean and daytime diastolic ABP. A 1 SD increase in Na/K-ratio corresponded to approximately 1 mmHg increase in systolic 24 h mean ABP (Table 3 and Supplementary table 1). We found no significant indirect effects of Na/K-ratio through mGFR, ACR or EGF-Cr (Table 3). Mediation analysis with ACR substituted for values under the limit of detection did not change the results (Supplementary table 4). Hence, none of the three kidney function variables mediated the relationship between Na/K-ratio and ABP.

The Na/K-ratio was not significantly associated with nighttime BP dipping, and we found no significant indirect effects of Na/K-ratio trough mGFR, ACR or EGF-Cr on the dipping pattern

(Supplementary table 2 and 5). Mediation analysis with ACR substituted for values under the limit of detection did not change the results (Supplementary table 6)

The associations between Na/K-ratio, office BP and the ambulatory white coat effect

Na/K-ratio showed a stronger direct effect with office BP than ABP (Table 4). The standardized regression coefficients indicated a 3.6 mmHg increase in systolic office BP per SD unit increase in Na/K-ratio (Table 4 and Supplementary table 3). For the diastolic office BP, the rise was 1.4 mmHg per SD unit Na/K-ratio increase (Table 4 and Supplementary table 3).

We found no significant indirect effects of Na/K-ratio through mGFR, ACR or EGF-Cr (Table 4). Mediation analysis with ACR substituted for values under the limit of detection did not change the results (Supplementary table 7).

There were significant associations between the Na/K-ratio and the ambulatory white coat effect (office BP minus daytime mean ABP) (Table 4 and Supplementary table 3). The estimated increase in the systolic white coat effect was 2.7 mmHg per SD unit increase in Na/K ratio. The corresponding value for the diastolic white coat effect was 1.0 mmHg per SD unit (Table 4 and Supplementary table 3). We found no significant indirect effects of Na/k-ratio through our kidney function variables (Table 4). Mediation analysis with ACR substituted for values under the limit of detection did not change the results (Supplementary table 7).

The associations between Na/K-ratio and hypertension phenotypes.

There was a significant direct effect of Na/K for white coat hypertension and sustained hypertension, but not for masked hypertension (Table 5). We found no significant indirect effects of Na/K-ratio through mGFR, ACR or EGF-Cr for masked or sustained hypertension, but a partial complementary mediation effect through EGF-Cr for white coat hypertension (Table 5). Mediation analysis with ACR substituted for values under the limit of detection did not change the results (Supplementary table 8). Hence, the kidney function variables in our study did not mediate the relationship between Na/K-ratio and sustained hypertension.

Discussion

The present study is, according to our knowledge, the first study to assess the association between urinary Na/K-ratio, BP and hypertension phenotypes, in the context of kidney function markers including GFR assessed with gold standard measurement, ACR and a validated biomarker of functional renal tubular mass (EGF-Cr). In multivariate models, the Na/K-ratio was significantly associated with systolic ABP, office BP and sustained hypertension. Compared to ABP measurements, the Na/K-ratio showed a stronger association with office BP, and the increase in the standardized coefficient for the Na/K-ratio corresponded to the white coat effect. The markers of kidney function did not mediate the relationship between urinary Na/K-ratio, systolic BP or sustained hypertension.

No population-based studies have addressed the association between Na/K-ratio and BP in the specific context of kidney function, but some studies included the estimated glomerular filtration rate as a covariate in the data-analyses (18, 28). In a cross-sectional study by Tabara et al. of a middle-aged population in Japan, the association between Na/K-ratio and office BP was independent of cardiovascular risk factors and estimated GFR (standardized coefficient of urinary Na/K- ratio to systolic office BP 0.12 ($P<0.001$)). Only one previous study has included estimated GFR and ACR as covariates in its analyses (28). This cross-sectional study by Hedayati et al., which also included a large African American population, showed a significant association between Na/K-ratio and office BP after adjustment for cardiovascular risk factors, including estimated GFR and ACR (28). However, these studies included subjects with diabetes and treated hypertension, and the Na/K-ratios was substantially larger than observed in the present study (18, 28). Although in line with our results, the individual or combined effect of adjustments for estimated GFR and ACR was not reported.

However, estimated GFR is an insensitive marker early of renal impairment (29). Estimated GFR is influenced by non-GFR components such as age, sex, muscle mass, dietary protein intake and exercise, resulting in systematic overestimation and underestimation of kidney function, which may influence the effect of kidney function on BP (30). Previous studies (18, 28) may have biased results from non-GFR-related confounders. Accordingly, to investigate the influence of GFR on the Na/K-BP association we measured the GFR using plasma iohexol-clearance. Further, ACR is a marker of

kidney damage and endothelial dysfunction, and thus may be an important mediator for the effect of Na/K-ratio on BP (29, 31). High sodium and low potassium consumption may disrupt the endothelial glycocalyx barrier, induce endothelial stiffening, and trigger endothelial dysfunction with increasing ACR and BP (32-34). A diet high in sodium and low in potassium may also cause kidney glomerular and tubulointerstitial injury, leading to an increased ACR, deviant salt handling and salt-sensitive hypertension (11, 35). In our study, mGFR and ACR did not mediate the association between the Na/k ratio, BP and hypertension.

Furthermore, Epidermal growth factor (EGF), a marker of functional tubular mass, may contribute to BP regulation (36-38). In animal studies, EGF has been shown to regulate ENaC-mediated sodium transport in the distal part of the kidney tubule (38). An increased renal cortical expression of the EGF-receptor has been found in salt-sensitive prehypertensive and hypertensive rats (37). However, it is unknown whether this expression is a cause or a consequence of salt-sensitive hypertension (36). Intrarenal EGF may represent a potential molecular mechanism underlying kidney dysfunction and salt sensitivity (36). We have not found previous studies that have assessed the association between Na/K ratio and BP in the context of EGF. In our study, urinary EGF did not mediate the association between Na/K-ratio, BP, masked- or sustained hypertension. Inconsistent effect modification by EGF-Cr was demonstrated with a small partial mediation effect on white coat hypertension, but no significant mediation effect was shown with the ambulatory white coat effect as a continuous dependent variable. Thus, the mediation effects via EGF on white coat hypertension cannot be ascribed significance until confirmatory findings in other cohorts (39).

The Na/K ratio showed a stronger association with office BP than ABP, and the increase in the standardized coefficients corresponded to the ambulatory white coat effect. The ambulatory white coat effect has been shown to increase with increasing age and BMI (40). The direct effect of urinary Na/K-ratio was highly significant after adjustment for cardiometabolic risk factors and we found no significant indirect effects trough mGFR, ACR or EGF-Cr

Guyton and coworkers proposed that hypertension could not occur with a preserved natriuresis, which would restore plasma volume and normotension (41). Recent studies have shown that subclinical kidney dysfunction may be implicated as a cause of salt sensitivity (9, 42). In our study of a middle-aged population without diabetes, chronic kidney disease, cardiovascular disease or treated hypertension, kidney function variables did not mediate the association between urinary Na/K-ratio, systolic BP or sustained hypertension. Thus, although we cannot make inferences about causality in this cross-sectional study, the findings support that a high sodium and/or low potassium diet alone may stimulate salt reabsorption along the distal nephron, leading to an increased BP (43, 44). However, recent insights have launched new hypotheses as potential explanations for salt sensitivity, suggesting primary vascular dysfunction, primary sympathetic nervous system dysfunction, immune activation, and skin hypertonicity to be involved (10).

A strength of this general population cohort study is the exclusion of participants with cardiovascular disease, kidney disease, diabetes and antihypertensive medication at baseline, limiting the possibility for reverse causality. Previous studies on the association between Na/K-ratio and BP have included subjects with either cardiovascular disease, diabetes or treated hypertension (18, 28, 45-48). Further, the accurate measurement of GFR using iohexol clearance reduces the risk of bias and residual confounding or effect modification compared to using estimated GFR. Also, ABP measurements are more strongly associated with target organ damage than office BP and provide the opportunity to study dipping patterns and the ambulatory white coat effect (2, 49).

However, there are several limitations. Excretion measured in multiple controlled 24-hour urine collections is the gold standard method to assess electrolyte intake (50). Controlled 24-hour urine collection is also the gold standard method for urine biomarker quantification. However, the method is subjected to error by incompleteness of the collection and is demanding to perform adequately in population studies (50). Thorough control of 24-hour urine collections is crucial for accurate and reliable results. Neglecting this process can introduce a systematic bias in the estimation of electrolyte intake (50, 51). However, when strict control measures are applied in larger population studies, there is a chance of unintentionally introducing a similar bias. In this regard, we would like to emphasize

that the utilization of the Na/K-ratio offers advantages in mitigating systemic errors arising from incomplete 24-hour urine collections that can confound results in association studies (51, 52). There is a moderate correlation between 24-h urine collections and single second void morning spot urine Na/K-ratio (r=0.6), and between 24-h urine collections and single spot urine EGF-Cr (r=0.6) (16, 25, 51). Spot urine samples, on the other hand, will give rise to greater random errors in estimates of sodium and potassium intake and will bias the relationship between Na and K intake and BP toward the null. Furthermore, potassium intake is negatively correlated with sodium intake and BP, and modifies the relationship between sodium intake and BP. When potassium and sodium are measured in spot urine samples, the correlation of their errors may complicate the interpretation of coefficients in linear models. It is important to acknowledge that this study should be considered as hypothesis-generating, and additional investigations employing multiple random spot urine samples for a more accurate estimation of the urinary Na/K-ratio are warranted (52). Moreover, the population in the RENIS-T6 consists almost exclusively of North-European middle-aged persons with generally normal or near normal kidney function, limiting broad generalizations to other healthy and sick populations. Further limitations include residual confounding, and we did not exclude potential hypertension patients with ongoing BP follow-up and guided lifestyle interventions.

Conclusion

In this study of a healthy middle-aged population the Na/K-ratio showed a consistent association with systolic BP and sustained hypertension. This effect was not mediated through the kidney function variables mGFR, ACR or EGF-Cr. Our findings do not support the suggestion that kidney function mediates the relationship between sodium and potassium intake and BP. A high sodium and low potassium diet may be sufficient to stimulate salt reabsorption leading to increased BP. Longitudinal and interventional studies should address this and other explanations for salt sensitivity.

Statement of ethics

The present study obtained ethical approval form the Regional Ethical Committee (REK Nord 2017/1970), adhered to the principles outlined in the Declaration of Helsinki, and written informed consent was obtained from all participating individuals. The registration and handling of patient data

were conducted in compliance with national personal laws and received approval form local data safety officers.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Authors contribution

Research idea and study design: KMB, TM and MDS; data acquisition: BOE, TM, MDS; data analysis/interpretation: KMB, MDS, TM, BOE; statistical analysis: KMB; supervision or mentorship: MDS, TM, BOE, AH, JVN; drafting of paper: KMB, MDS; revision of the article: MDS, TM, BOE, AH, JVN; approval of final version: all authors.

Data availability statement

The data underlying this article cannot be shared publicly due to ethical considerations and the privacy of individuals that participated in the study. The data are available from the corresponding author on reasonable request (Karl.Brobak@unn.no).

List of abbreviations

ABP: 24-hour ambulatory blood pressure
ACR: Urinary albumin creatinine ratio
BP: Blood pressure
EGF-Cr: Epidermal growth factor – creatinine ratio
GFR: Glomerular filtration rate
mGFR: Measured glomerular filtration rate (mGFR)
Na/K-ratio: Urinary sodium-potassium ratio

Fig. 1. Inclusion of participants in the Renal Iohexol-clearance Survey (RENIS-T6) from the main part of the sixth Tromsø survey (Tromsø6).

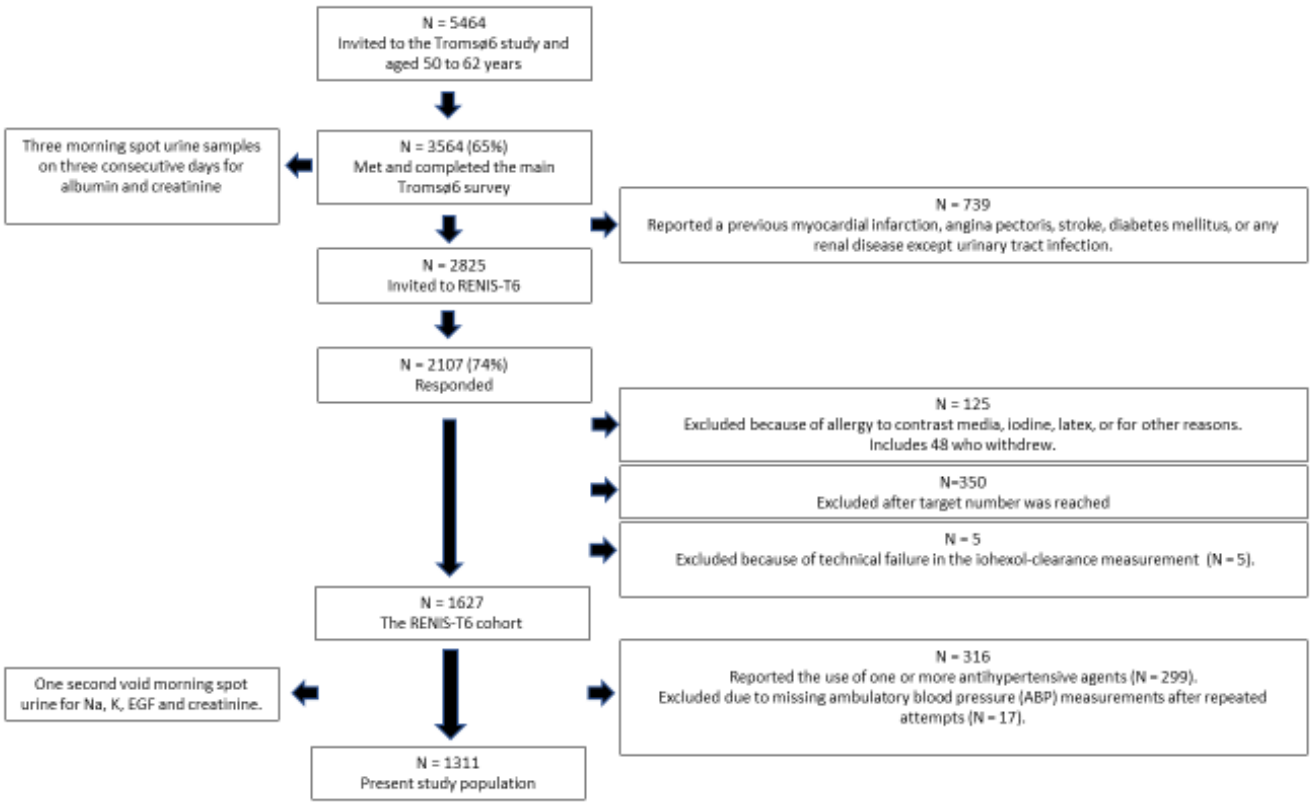
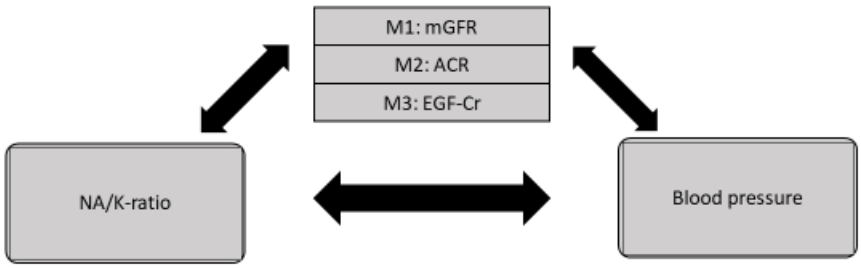
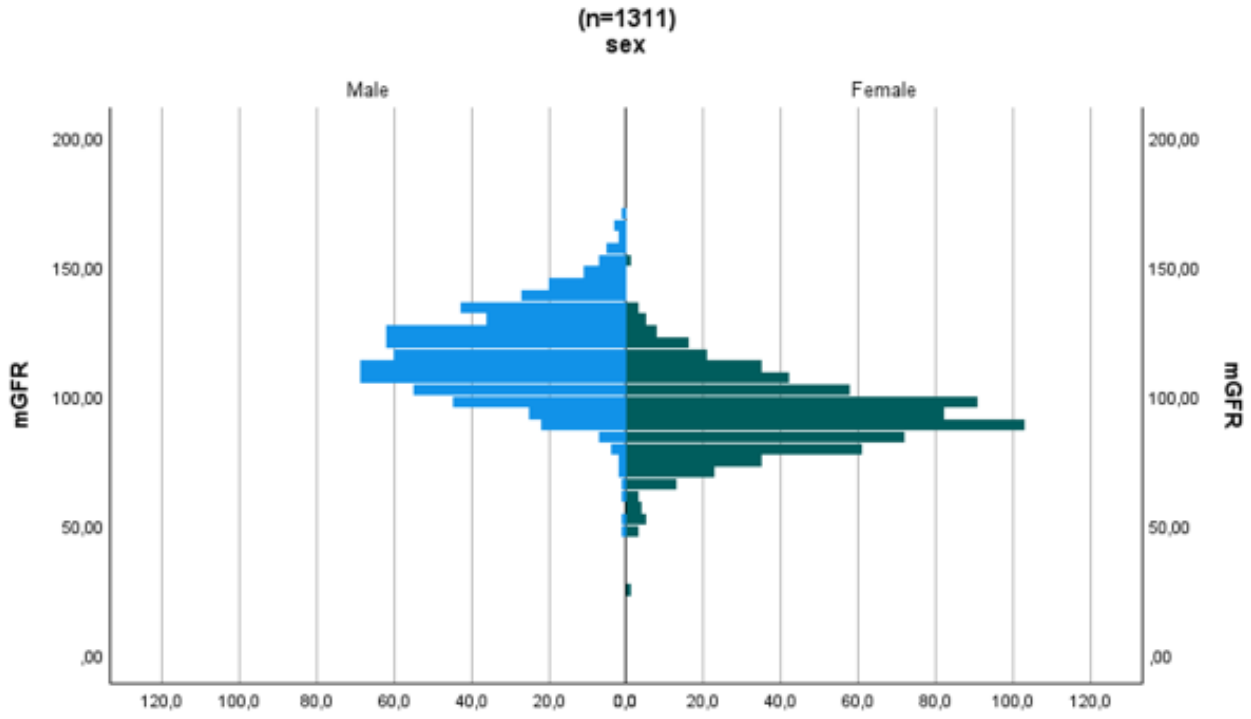


Fig. 2. Two components mediation analysis.



M1: mGFR mediates the relationship between Na/K-ratio and blood pressure.
M2: ACR mediates the relationship between Na/K-ratio and blood pressure.
M3: EGF-Cr mediates the relationship between Na/K-ratio and blood pressure.
Glomerular filtration rate as a single-sample plasma clearance of iohexol (mGFR), urine albumin creatinine ratio (ACR), epidermal growth factor to the urine creatinine concentration (EGF-Cr).

Fig. 3. Population pyramid showing the frequency of mGFR by sex.



Measured GFR (mGFR): GFR as single-sample plasma iohexol clearance(mL/min/1,73m2)

Table 1. Characteristics of the study population (n=1311).

Female sex, n (%)	679	(51.8)
Age, years	57.9	(3.8)
Waist-hip ratio women	0.86	(0.06)
Waist-hip ratio men	0.95	(0.06)
Body mass index, kg/m2	26.8	(3.8)
Cholesterol, mmol/L	5.6	(0.9)
HDL cholesterol, mmol/L	1.6	(0.4)
LDL cholesterol, mmol/L	3.7	(0.9)
Triglycerides, mmol/L	1.2	(0.6)
HbA1c, %	5.5	(0.4)
U-Na, mmol/L	54.0	(33-89)
U-K, mmol/L	45.0	(26-74)
U-Na/K-ratio	1.43	(0.76)
Serum creatinine (µmol/L)	66.5	(12.0)
Cystatin C, mg/L	0.73	(0.11)
Estimated GFR (mL/min/1,73m2)	103.7	(11.2)
GFR as single-sample plasma iohexol clearance(mL/min/1,73m2)	94.3	(14.2)
ACR (left censored at limit of detection), mg/mmol	0.75	(0.49-1.16)
EGF, µg/L	12.3	(6.2-23.3)
EGF-Cr, µg/mmol	1.91	(0.73)

Data are mean (SD), median (interquartile range) or number (%) as appropriate.
Estimated GFR: glomerular filtration rate, calculated using the creatinine-cystatin C based CKD-EPI equation. ACR: urinary albumin to creatinine ratio. EGF: Urinary excretion of epidermal growth factor. EGF-Cr: Urinary excretion of epidermal growth factor to creatinine ratio.

Table 2. Blood pressure characteristics of the study population (n=1311).

Office systolic blood pressure, mmHg	127.7	(17.5)
Office diastolic blood pressure, mmHg	82.3	(9.6)
Ambulatory systolic blood pressure, 24 h mean, mmHg	122.2	(12.3)
Ambulatory diastolic blood pressure, 24 h mean, mmHg	75.8	(7.9)
Ambulatory systolic blood pressure, daytime mean, mmHg	129.2	(13.2)
Ambulatory diastolic blood pressure, daytime mean, mmHg	81.4	(8.5)
Ambulatory systolic blood pressure, night-time mean, mmHg	110.0	(12.2)
Ambulatory diastolic blood pressure, night-time mean, mmHg	65.8	(8.2)
Night-time systolic blood pressure dip (%)	14.7	(6.2)
Night-time diastolic blood pressure dip (%)	19.1	(7.2)
Normotensive, n (%)	756	(57.7)
White coat hypertension, n (%)	103	(7.9)
Masked hypertension, n (%)	167	(12.7)
Sustained hypertension, n (%)	285	(21.7)

Data are mean (SD), or number (%) as appropriate.

Normotensive: Office BP <140/90 and 24 h mean ABP <130/80

White coat hypertension: Office BP≥140/90 and 24 h mean ABP <130/80

Masked hypertension: Office BP≤140/90 and 24 h mean ABP ≥130/80

Sustained hypertension: Office B≥140/90 and 24 h mean ABP ≥130/80

Table 3. Mediation analysis for the urinary Na/K-ratio for the associations with ABP (n=1311).

	Total effect	P value	Direct effect	P value	Relationship	Indirect effect	t-statistic	Conclusion
24 h mean ABP as dependent variable								
Systolic BP	0.93	0.035	0.94 (0.31-1.56)	0.033	M1	-0.00 (-0.04-0.23)	-0.08	No mediation
					M2	0.02 (-0.00-0.09)	0.83	No mediation
					M3	-0.02 (-0.11-0.06)	-0.57	No mediation
Diastolic BP	0.37	0.072	0.37 (-0.03-0.77)	0.070	M1	-0.00 (-0.02-0.01)	-0.19	No mediation
					M2	0.00 (-0.02-0.06)	0.15	No mediation
					M3	-0.01 (-0.06-0.05)	-0.20	No mediation
Mean daytime ABP as dependent variable								
Systolic BP	0.93	0.007	0.94 (0.27-1.62)	0.006	M1	-0.00 (-0.04-0.03)	-0.23	No mediation
					M2	0.02 (-0.00-0.09)	0.62	No mediation
					M3	-0.02 (-0.11-0.06)	-0.55	No mediation
Diastolic BP	0.41	0.063	0.41 (-0.03-0.85)	0.065	M1	0.00 (-0.02-0.02)	0.04	No mediation
					M2	0.00 (-0.02-0.07)	0.07	No mediation
					M3	-0.00 (-0.05-0.05)	0.06	No mediation
Mean night-time ABP as dependent variable								
Systolic BP	0.89	0.006	0.90 (0.26-1.54)	0.006	M1	-0.00 (-0.03-0.02)	-0.09	No mediation
					M2	0.03 (-0.00-0.09)	0.97	No mediation
					M3	-0.04 (-0.12-0.04)	-0.81	No mediation
Diastolic BP	0.29	0.18	0.30 (-0.13-0.73)	0.17	M1	-0.00 (-0.02-0.02)	-0.18	No mediation
					M2	0.01 (-0.00-0.06)	0.45	No mediation
					M3	-0.02 (-0.07-0.03)	0.67	No mediation

The total effect is the direct and indirect effect of urinary Na/K-ratio on ABP**. The direct effect** is the effect of urinary Na/K-ratio on ABP, and **the indirect effect** of Na/K-ratio is through the mediators mGFR, ACR and EGF-Cr on ABP. Direct and indirect effects are reported beta coefficients (95% confidence intervals based on 5,000 bootstrap samples) for the Na/K-ratio per SD.

Relationships: M1: Na/K-ratio (SD)-> mGFR-> ABP (mmHg). **M2**: Na/K-ratio (SD)->ACR-> ABP (mmHg).

M3: Na/K-ratio (SD)->EGF-Cr -> ABP (mmHg).

Covariates: age, sex (male or female), waist-hip-ratio, HbA1c, triglycerides and HDL- cholesterol.

Ambulatory blood pressure (ABP), glomerular filtration rate as a single-sample plasma clearance of iohexol (mGFR), Urine albumin creatinine ratio (ACR), epidermal growth factor to the urine creatinine concentration (EGF-Cr).

Table 4. Mediation analysis for the urinary Na/K-ratio for the associations with office BP and the white coat effect as dependent variables (n=1311).

	Total effect	P value	Direct effect	P value	Relation-ship	Indirect effect	t-statistic	Conclusion
Office BP								
Systolic BP	3.68	<0.001	3.68 (2.81-4.54)	<0.001	M1	0.01 (-0.03-0.06)	0.33	No mediation
					M2	0.01 (-0.01-0.09)	0.51	No mediation
					M3	-0.02 (-0.14-0.10)	-0.30	No mediation
Diastolic BP	1.39	<0.001	1.39 (0.91-1.86)	<0.001	M1	0.01 (-0.03-0.05)	0.42	No mediation
					M2	0.00 (-0.08-0.05)	0.16	No mediation
					M3	-0.01 (-0.06-0.05)	-0.22	No mediation
Ambulatory white coat effect								
Systolic BP	2.74	<0.001	2.72 (2.10-3.34)	<0.001	M1	0.01 (-0.03-0.06)	0.33	No mediation
					M2	0.01 (-0.01-0.09)	0.51	No mediation
					M3	-0.02 (-0.14-0.10)	-0.30	No mediation
Diastolic BP	0.98	<0.001	0.98 (0.63-1.33)	<0.001	M1	0.01 (-0.03-0.05)	0.42	No mediation
					M2	0.00 (-0.08-0.05)	0.16	No mediation
					M3	-0.01 (-0.06-0.05)	-0.22	No mediation

The total effect is the direct and indirect effect of urinary Na/K-ratio on office BP and the white coat effect**.** **The direct effect** is the effect of urinary Na/K-ratio on office BP and the white coat effect, and **the indirect effect**

of Na/K-ratio is through the mediators mGFR, ACR and EGF-Cr on office BP and the white coat effect.

(95% confidence intervals based on 5,000 bootstrap samples) for the Na/K-ratio per SD.

Relationships: M1: Na/K-ratio (SD)-> mGFR-> Office BP (mmHg) and white coat effect (mmHg). **M2**: Na/K-ratio (SD)->ACR-> Office BP (mmHg) and White coat effect (mmHg). **M3**: Na/K-ratio (SD)->EGF-Cr -> Office BP (mmHg) and white coat effect (mmHg).

Covariates: age, sex (male or female), waist-hip-ratio, HbA1c, triglycerides and HDL- cholesterol.

Ambulatory blood pressure (ABP), glomerular filtration rate as a single-sample plasma clearance of iohexol (mGFR),

Urine albumin creatinine ratio (ACR), epidermal growth factor to the urine creatinine concentration (EGF-Cr).

Table 5: Mediation analysis for the urinary Na/K-ratio for the associations with hypertension phenotypes (n=1311)

Direct effect	P value	Relationship	Indirect effect	Conclusion
Normotension vs white coat hypertension (n=824)				
1.31 (1.06-160)	0.011	M1	1.0 (0.99-1.01)	No mediation
		M2	1.0 (0.99-1.02)	No mediation
		M3	1.04 (1.01-1.10)	Partial mediation
Normotension vs masked hypertension (n=889)				
0.88 (0.71-1.07)	0.21	M1	1.0 (0.98-1.01)	No mediation
		M2	1.02 (0.99-1.09)	No mediation
		M3	1.01 (0.99-1.04)	No mediation
Normotension vs sustained hypertension (n=1041)				
1.27 (1.09-1.48)	0.012	M1	1.0 (0.99-1.01)	No mediation
		M2	1.0 (0.99-1.02)	No mediation
		M3	1.0 (0.99-1.02)	No mediation

The direct effect is the effect of urinary Na/K-ratio on hypertension phenotype, and the **indirect effect** of Na/K-ratio is through the mediators mGFR, ACR and EGF-Cr on hypertension phenotype.

Effects are expressed as odds ratios (95% confidence intervals based on 5,000 bootstrap samples) for the Na/K-ratio per SD.

Relationships: **M1**: Na/K-ratio (SD)-> mGFR-> hypertension phenotype.

M2: Na/K-ratio (SD)->ACR-> hypertension phenotype. **M3**: Na/K-ratio (SD)->EGF-Cr -> hypertension phenotype.

Covariates: age, sex (male or female), waist-hip-ratio, HbA1c, triglycerides and HDL- cholesterol. Ambulatory blood pressure (ABP), glomerular filtration rate as a single-sample plasma clearance of iohexol (mGFR), urine albumin creatinine ratio (ACR), epidermal growth factor to the urine creatinine concentration (EGF-Cr).

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Paper 2

NOVEL BIOMARKERS IN PATIENTS WITH UNCONTROLLED HYPERTENSION WITH AND WITHOUT KIDNEY DAMAGE

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ABSTRACT

Introduction

Estimated glomerular filtration rate (eGFR) and urine albumin/creatinine ratio (ACR) are insensitive biomarkers for early detection of hypertension-mediated organ damage (HMOD). In this nationwide cross-sectional study, we assessed potential biomarkers for early HMOD in healthy persons and patients with hypertension. We hypothesised that plasma levels of biomarkers: (1) are different between healthy controls and patients with hypertension, (2): can classify patients with hypertension according to the degree of hypertension severity.

Design and methods

Patients with hypertension prescribed ≥2 antihypertensive agents were selected from a multicenter study. Healthy controls were selected from an ongoing study of living kidney donor candidates. Uncontrolled hypertension was defined as systolic daytime ambulatory blood pressure ≥135 mmHg. Kidney HMOD was defined by ACR >3.0 mg/mmol or eGFR <60 mL/min/1.73m^2. Patients with hypertension were categorized into three groups: (1) controlled hypertension; (2) uncontrolled hypertension without kidney HMOD; (3) uncontrolled hypertension with kidney HMOD. Fifteen biomarkers were analysed using a Luminex bead-based immunoassay, and nine fell within the specified analytical range.

Results

Plasma levels of IL-1RA, NGAL and uromodulin were significantly different between healthy controls (n=39) and patients with hypertension (n=176). In regression models, with controlled hypertension (n=55) as the reference category, none of the biomarkers were associated with uncontrolled hypertension without (n=59) and with (n=62) kidney HMOD. In models adjusted for cardiovascular risk factors and eGFR, osteopontin (OPN) was associated with uncontrolled hypertension without kidney HMOD (OR 1.77 (1.05-2.98), P=0.03), and Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES) with uncontrolled hypertension with kidney HMOD (OR 0.57 (0.34-0.95), P=0.03)

Conclusion

None of the biomarkers could differentiate our hypertension groups when established risk factors were considered. Plasma OPN may identify patients with uncontrolled hypertension at risk for kidney HMOD.

Plain Language Summery

What is the context?

In order to tailor individualized hypertension treatment, a risk assessment for cardiovascular disease must be performed. This includes evaluation of established hypertension mediated organ damage (HMOD), such as the presence of kidney damage and associated risk factors. Today, kidney function is assessed by blood and urine samples. However, today's blood and urine samples are not sensitive enough to capture kidney damage due to hypertension at a stage when prevention may be most effective.

What is new?

In this study we evaluated plasma levels of biomarkers related to endothelial and kidney cell pathology, inflammation and fibrosis in healthy patients and patients with hypertension. We hypothesized that plasma levels of biomarkers could differentiate between different degrees of hypertension severity.

Healthy controls had lower Interleukin 1 receptor antagonist (IL-1RA) and Neutrophil Gelatinase-Associated Lipocalin (NGAL) levels, but higher uromodulin compared to patients with hypertension. Except for osteopontin (OPN), all biomarkers showed significant trends in median biomarker levels across study groups. However, as hypertension severity increased, the median plasma OPN levels also rose. None of the biomarker could consistently differentiate the hypertension severity groups after considering established risk factors. However, OPN may be an early biomarker for kidney damage in hypertension.

What is the impact?

Biomarkers for early detection of organ damage in hypertension may guide targeted treatment. Plasma OPN may have potential to identify those at risk for hypertensive kidney damage. However, the studied biomarkers lack consistent discrimination across hypertension severity levels.

INTRODUCTION

Arterial hypertension affects about 35% of the adult population and is an important modifiable risk factor for cardiovascular and progressive kidney disease [1-3]. Still, 30 % of treated patients have uncontrolled hypertension, remaining at risk for developing hypertension-mediated organ damage (HMOD) [3, 4]. In order to tailor individualized hypertension treatment, a risk assessment for cardiovascular disease must be performed. This includes evaluation of established HMOD, such as e.g the presence of kidney damage [1]. Low-grade inflammation and fibrosis may play a crucial role in the pathogenesis of hypertension and HMOD, and biomarkers may help to identify patients with the highest risk of complications [5].

Inflammation usually follows three stages: increased vascular permeability, leukocyte recruitment and activation of tissue-repair processes. Inflammation is necessary for an acute response to injury and further healing. However, it can become harmful when the acute response does not resolve and becomes chronic [6]. Hypertension is associated with chronic inflammation in key tissues and organs that regulate blood pressure, such as the kidneys and blood vessels [6, 7], which might induce further organ damage and increased blood pressure [8, 9]. Hypertension induces arterial changes including smooth muscle cell transition to myofibroblasts, augmented collagen and arterial wall thickening, and kidney changes including mesenchymal transition of tubular epithelial cells, tubulointerstitial injury and fibrosis [7, 10]. In kidney biopsy studies, tubulointerstitial injury and fibrosis are highly prognostic for subsequent kidney failure, but cannot be reliably detected by standard clinical measures [11]. Biomarkers that captures HMOD at an early stage might improve risk stratification and form the basis for targeted individual treatment. Several biomarkers of kidney and endothelial cell pathology, and markers of inflammation and fibrosis are of interest (Figure 1 and Table 1). In this cross-sectional nationwide multicenter study, we aimed to assess the plasma levels of biomarkers in patients with hypertension and in healthy controls. Further, we aimed to study the associations between biomarkers, severity of hypertension and kidney HMOD. We hypothesised that: (1) plasma levels of biomarkers are different between healthy controls and patients with hypertension, and (2): plasma levels of biomarkers can classify patients with hypertension according to the degree of hypertension severity.

DESIGN AND METHODS

Study population

We selected patients with hypertension from a large nationwide multicenter study that recruited participants between 2017 and 2022 (identifier: NCT03209154) [12]. They were ≥18 years old, being prescribed ≥2 antihypertensive agents (or ≥1 fixed-dose combination pill), on a stable treatment regimen for at least four weeks, with estimated glomerular filtration rate (eGFR) >30 mL/min/1.73m^2 and urine albumin/creatinine ratio (ACR) <300 mg/mmol (Supplementary Table 1) [12]. Healthy controls were selected from an ongoing study of living kidney donors (identifier: NCT03729557) [13]. They were ≥18 years old, either accepted as living kidney donors, or evaluated for donation, and not found eligible due to immunological incompatibility, donor withdrawal or other non-medical causes, or family members related to donors or recipients and blood donors evaluated and fulfilling the Norwegian transplantation protocol for living kidney donors. Individuals accepted as kidney donors were examined prior to donation. An established collaboration with all regional and university hospitals in Norway allowed all Norwegian living kidney donors to participate (Supplementary Table 1) [13].

The healthy controls and patients with hypertension were age- and sex matched to the greatest extent possible, and the preanalytical blood sampling conditions were the same. Patients with hypertension were selected into three groups based on the severity of hypertension and presence of kidney HMOD; 1) controlled hypertension, 2) uncontrolled hypertension without kidney HMOD or 3) uncontrolled hypertension with kidney HMOD. Uncontrolled hypertension was defined as systolic daytime ambulatory blood pressure ≥135 mmHg, and kidney HMOD as ACR >3.0 mg/mmol or eGFR <60 mL/min per 1.73 m^2. Patients using systemic *i*mmunosuppressive medications were excluded.

The study was approved by the Regional Ethical Committee and conducted in accordance with the Declaration of Helsinki and consistent with ICH/Good Clinical Practice. All participants provided a signed written informed consent. Registration of patient data followed national personal data laws and

was approved by local data safety officers. Review of the data supporting these findings is possible upon reasonable request to the corresponding author.

Demographic and clinical characteristics

All patients underwent a structured physician-patient interview collecting information about demographic and lifestyle data, socioeconomic factors, and medical and family history [12, 13]. We recorded the patients' weight, height and calculated body mass index (BMI). Diabetes was defined as self-reported diabetes, HbA1c ≥48 mmol/mol, or the use of antidiabetic agents. Cardiovascular disease was defined as prior myocardial infarction, angina, stroke or peripheral artery disease.

Office blood pressure was measured using a validated automated oscillometric device, following the 2018 European Society of Hypertension/European Society of Cardiology guidelines [1]. Ambulatory blood pressure monitoring (ABPM) was programmed to automatic readings every 20 minutes during daytime (6:00 to 22:00) and every 30 minutes at night (22:00 to 6:00). The device was removed after 25 hours of recording. Recordings with less than 70% of the expected blood pressure readings, or two or more consecutive hours without valid readings, were repeated. We adjusted the readings to the patient-reported day and night periods [12].

Blood sample collection and biomarker analyses

Blood and morning urine samples were collected. The following parameters were measured at the time of collection at each study center: creatinine, HbA1c, cholesterol (HDL, LDL, total) and triglycerides in the blood, and albumin and creatinine in urine. We calculated ACR. To calculate creatinine-based eGFR, we applied the 2009 Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [14-17].

For individuals with hypertension, we collected a 5 mL Vacutainer tube (BD, Franklin Lakes, NJ) without additives for analyses of antihypertensive agents. Adherence to antihypertensive treatment was confirmed by pharmacological evaluation based on serum drug concentrations measured by Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry [18]. We present the number

of antihypertensive medications based on self-reported data, and the number of non-adherent patients based on the pharmacological evaluation.

EDTA plasma biobank samples were taken. For potential kidney donors the EDTA plasma samples were obtained at the evaluation visit prior to potential kidney donation. Immediately after phlebotomy, all plasma tubes were placed in icewater and within 30 minutes centrifuged for 20 minutes at 2500G at 4 °C and transferred to Sarstedt tubes. All biobank samples were then frozen to -80 °C within 2 hours of sampling and transported to the core laboratory biobank at Oslo University Hospital Ullevål. All biomarker analyses were performed at the Department of Medical Biochemistry, Oslo University Hospital Ullevål, Oslo. Biomarker plasma concentrations were determined using immunoassay technology with the commercial instrument Luminex IS 200 (Bio-Plex xMap; Luminex Corp., Austin, TX, USA). Samples were thawed, vortexed and spun down at 16 000G for 5 min at 4^{o}C. In accordance to the manufacture recommendations, supernatants were diluted 1:1 and analysed with a custom-made eleven plex (www.biotechne.com/l/rl/c2TCU6j3) containing targets against monocyte chemoattractant protein-1 (MCP-1), interferon gamma (IFN-γ), interleukin 1 receptor antagonist (IL-1RA), interleukin-18 (IL-18), T cell immunoglobulin and mucin domain 1 (TIM-1), von Willebrand factor A2 (vWF-A2), granulocyte-macrophage colony stimulating factor (GM-CSF), IL-1β, IL-6, osteopontin (OPN) and tumour necrosis factor (TNF). Samples were further diluted 1:50 and analysed with a custom-made 4 plex (www.biotechne.com/l/rl/QQyEg7ca) containing targets against regulated upon activation normal T-cell expressed and secreted (RANTES), neutrophil gelatinase-associated lipocalin (NGAL), cystatin C and uromodulin (Tamm-Horsfall protein). Patient and healthy control samples were evenly distributed on each assay plate. Four in-house controls per plate were used to observe both intra and inter percent coefficients of variation. Cytokine concentrations outside the reference limits that were extrapolated by the analysis software were also included in the statistical analysis. The investigator was blinded to clinical information when performing the analysis. Cystatin C was not assayed with an internationally traceable standard reference method, and was not further examined [16]. For the specified biomarkers IFN-γ, TIM-1, GM-CSF, IL-1β, and IL-6, a substantial portion of the conducted analyses yielded results below the threshold for either detection or quantitation, for both the healthy controls and the hypertensive patients. Consequently, these markers

were not subjected to further investigation, as detailed in supplementary table 2 and 3. Subsequent to the exclusion of patients undergoing systemic immunosuppression treatments, 98.5% or more of the biomarker analyses fell within the specified analytical range (supplementary table 3).

Statistical analyses

Descriptive statistics were used to summarize participant characteristics using mean and standard deviation (SD) or median and interquartile range (IQR) for continuous variables and frequency distribution and percentage for categorical variables. Characteristics were compared using a t-test, Mann-Whitney test, chi-square test, Fisher´s exact test of independence and Kendall´s rank correlation (Kendall´s tau), as appropriate. Kendall´s rank correlation is a non-parametric test for a monotonic tendency between two variables measured on a continuous or ordinal scale. The biomarkers were non-normally distributed and were transformed on a natural logarithmic (LN) scale. All biomarkers had outliers and influential cases after LN transformation that changed the magnitude of regression coefficients. We performed a symmetric winsorization and replaced the smallest and the largest data values. The number of adjusted measurements in the upper distribution corresponds to the number of adjusted measurements in the lower distribution for each biomarker. Extreme measurements above three SD from the mean were adjusted to the nearest measured value below this threshold [19].

We used univariable and multivariable logistic and multinomial logistic regression analyses to assess the associations between the plasma biomarkers as independent variables and the hypertension groups as dependent variables. Controlled hypertension was the reference group in the multinomial logistic regression analyses, and uncontrolled hypertension without kidney HMOD was the reference group in logistic regression analyses. In multivariable models, we added cardiovascular risk factors (sex, age, BMI, diabetes and cardiovascular disease), and finally eGFR. Only one of the three hypertension groups included individuals with elevated ACR, and the regression models were not adjusted for this variable due to complete separation. The biomarkers were analyzed as continuous variables, with odds ratio for belonging to one of the uncontrolled hypertensions groups reported per 1 SD higher LN–transformed biomarker concentration.

Participants with missing data were excluded only from analyses for which the case had missing data.

Multiple significance tests were used for descriptive purposes, and multiplicity corrections were not performed [20]. A 2-sided P value <0.05 was considered statistically significant.

RESULTS

Demographic and clinical characteristics

Two hundred and twenty-two individuals, 183 with hypertension and 39 healthy controls were initially examined for a panel of 15 biomarkers. After excluding 7 patients (with hypertension) using systemic immunosuppressive therapy, and 5 biomarkers, we ended up with a total of 215 individuals, 176 patients with hypertension and 39 healthy controls, and 9 biomarkers (Table 2A, Supplementary Table 2 and 3).

As expected from selection, the office blood pressure was higher in patients with hypertension than in healthy controls; mean (SD) 152±20/87±13 mmHg with median (IQR) 3 (2-4) antihypertensive agents daily for the participants with hypertension, and 123±12/76±9 mmHg for the healthy controls (Table 2A). The participants with hypertension had lower eGFR and higher urine ACR. As expected, the patients with hypertension had more cardiovascular risk factors than the healthy controls. They were older, had higher BMI, more frequently used lipid-lowering therapy and 27.8% were diagnosed with diabetes (Table 2A).

Among patients with hypertension, 55 patients had controlled hypertension, 59 had uncontrolled hypertension without kidney HMOD, and 62 had uncontrolled hypertension with kidney HMOD (Table 2B).

Compared to the controlled hypertension group, the uncontrolled hypertension group without kidney HMOD had a higher BMI. The patients with uncontrolled hypertension with kidney HMOD were older, more frequently had diabetes and cardiovascular disease, and reported a higher number of daily antihypertensive agents (Table 2B). Serum concentration assessment for adherence did not differ

between the two groups with uncontrolled hypertension compared to the controlled hypertension group (Table 2B).

Compared to the uncontrolled hypertension group without kidney HMOD, the group with kidney HMOD, had lower daytime diastolic and higher nighttime systolic blood pressure (Table 2B). They also reported using more antihypertensive agents, and they had a higher rate of adherence, evaluated pharmacologically (Table 2B). Additionally, the group with kidney HMOD was older, had a higher BMI, included more participants with diabetes and were more frequently using lipid-lowering treatment (Table 2B).

Plasma biomarker levels between healthy controls and patients with hypertension

Plasma levels of IL-1RA, NGAL and uromodulin were significantly different between healthy controls and patients with hypertension. IL-1RA and NGAL were lower and uromodulin was higher in the healthy controls (Table 3). All biomarkers, with the exception of OPN, exhibited a monotonic tendency in the medians across all study groups (Table 4). Plasma OPN levels were highest in healthy controls and in patients with uncontrolled hypertension with kidney HMOD. All biomarkers, with the exception of RANTES, exhibited a monotonic tendency in the medians across the hypertension severity groups (Supplementary Table 4).

Associations between plasma biomarkers and hypertension groups

In multinomial logistic regression models including only patients with hypertension and using controlled hypertension as the reference category, none of the biomarkers were associated with both groups with uncontrolled hypertension (Figure 2). Uromodulin and OPN were associated with uncontrolled hypertension without kidney HMOD in multivariable models with cardiovascular risk factors (Figure 2A). Only OPN was significantly associated after adjustment for eGFR (Figure 2B). RANTES and NGAL were associated with uncontrolled hypertension with kidney HMOD in multivariable models (Figure 2C), but only RANTES was significantly associated with hypertension with kidney HMOD in models also adjusted for eGFR (Figure 2D).

In logistic regression models including only participants with uncontrolled hypertension (n=126), vWF-A2, NGAL and uromodulin were associated with kidney HMOD in multivariable models adjusted for cardiovascular risk factors (Figure 3A), but not in models with additional adjustment for eGFR (Figure 3B).

DISCUSSION:

According to our knowledge, this is the first study to assess possible associations between selected plasma biomarkers of inflammation, kidney and endothelial dysfunction, and controlled and uncontrolled hypertension without and with kidney HMOD. Subclinical kidney disease is linked to hypertension development in the general population, potentially creating a harmful cycle of elevated blood pressure and worsening kidney damage [21, 22]. Kidney HMOD acts as a crucial intermediate stage between cardiovascular risk factors and advanced cardiovascular disease and chronic kidney disease (CKD) [23]. A biomarker that captures kidney HMOD at an early stage might improve risk stratification and form the basis for targeted individual treatment. Our hypertension groups were well characterized, defined by ABPM, on stable medication regimes with adherence to the number of reported antihypertensive agents assessed by pharmacological evaluation of serum drug concentrations. ABPM has been shown to correlate with HMOD, and predict end-stage kidney disease and cardiovascular morbidity and mortality better than office blood pressure [24-29]. Furthermore, lowering blood pressure can exaggerate an early GFR decline [1]. The eGFR was not significantly different between those who had controlled hypertension and those who had uncontrolled hypertension without evidence of HMOD. The latter group also had significantly higher urine ACR, although within the normal range. As hypertension-related structural abnormalities in the kidney can never completely regress [30-33], these data clearly demonstrate that our hypertension groups represent different stages of disease severity.

In this study levels of IL-1RA and NGAL were significantly lower and uromodulin was significantly higher in the healthy controls compared to patients with hypertension. All biomarkers, except OPN, exhibited a significant monotonic tendency in the medians across all four study groups. However,

OPN exhibited a significant monotonic tendency in the median values across all three hypertension groups. None of the biomarkers examined in this study could consistently differentiate all three hypertension groups when established risk factors were considered.

Higher circulating OPN levels have previously been associated with hypertension, HMOD and CKD [34-38]. Our study indicates that OPN may be a timely biomarker of kidney HMOD in hypertension. Among patients with hypertension, OPN was the only biomarker significantly associated with uncontrolled hypertension without kidney HMOD, at a potentially pivotal juncture in the progression of hypertension. No differences in usage of blood pressure or lipid lowering medication were found between those with controlled and uncontrolled hypertension without kidney HMOD, except for calcium channel blockers (Table 2 and Supplementary Table 5). Calcium channel blockers have not been found to exert an influence on plasma OPN levels [36]. However, humans express multiple isoforms of OPN with different functional effects, and larger and longitudinal studies are required to determine if individual OPN isoforms or total OPN levels may be useful biomarkers in hypertension [39, 40].

In our study, healthy controls had the highest OPN levels, but as hypertension severity increased, the median plasma OPN levels also rose. A previous study comparing individuals with and without hypertension reported elevated plasma OPN levels among participants with hypertension [36]. In that study, a smaller proportion of the hypertension patients were treated with ACEi and ARBs and the use of lipid lowering therapy was not reported. In a post hoc study from a double-blinded, multicenter trial in patients with hypertension, therapy with angiotensin II receptor blocker (ARB) and co-therapy with statin reduced circulating OPN levels [37, 41]. In our study the majority of hypertension patients were prescribed ACEi or ARBs, and about half were receiving lipid-lowering therapy, in contrast to a mere 4% of the healthy controls.

Among patients with hypertension, higher plasma level of uromodulin was independently associated with uncontrolled hypertension without kidney HMOD. This group also had the highest level of plasma uromodulin (Table 4). Our data are consistent with previous studies indicating that high

plasma uromodulin may protect against developing hypertensive kidney damage [42-45]. In a study on high-risk patients with hypertension and CKD stage 3 and 4 at baseline, higher urine uromodulin was associated with a slower eGFR decline and lower cardiovascular disease risk, and the association with eGFR decline was weakened by intensive blood pressure control [44, 45]. In our study, the association with uncontrolled hypertension without kidney HMOD was no longer statistically significant when adjusting for eGFR.

Among patients with hypertension, lower circulating RANTES was the only biomarker associated with uncontrolled hypertension with kidney HMOD in fully adjusted models. Higher plasma RANTES levels have been associated with hypertension, and studies have found significant correlations between higher circulating RANTES and vascular function [46-48]. However, conflicting results exist regarding RANTES' role in generating renal fibrosis [49, 50]. We have not found other studies examining the association between RANTES, degree of blood pressure control and kidney HMOD, and the effects of treatment on RANTES levels are unclear.

Among patients with hypertension, higher plasma NGAL was associated with uncontrolled hypertension with kidney HMOD after adjusting for cardiovascular risk factors. The association was not significant after further adjustment for eGFR. These results are in line with previous studies suggesting that NGAL may be an augmenting factor for kidney interstitial fibrosis [51-53]. In a general population cohort with ten years of follow-up, plasma NGAL added to the Framingham risk score improved risk prediction for all-cause mortality and major adverse cardiovascular events, and correctly reclassified ≈15% into more appropriate cardiovascular risk groups [54]. In a cohort of adult patients with non-dialysis–dependent CKD stages 3–5, higher plasma NGAL was independently associated with a greater risk for end-stage kidney disease, but not cardiovascular events or death [53]. In our study, when considering established risk factors, NGAL did not differentiate the hypertension groups.

IL-1RA has previously been associated with hypertension, possibly through modulation of the renin-angiotensin system, and may modulate hypertensive kidney disease [55-59]. Treatment with IL-1RA

led to a decrease in systolic blood pressure among obese patients with features of the metabolic syndrome [60, 61]. However, in our study, when considering established risk factors, IL-1RA did not separate the different hypertension groups.

A biomarker is a measurable indicator to; (1) detect or confirm a medical condition; (2) identify subtypes of a condition; (3) assess the status of a condition; (4) assess the biological response after treatment; (5) identify individuals likely to have a favourable or unfavourable effect from treatment; and (6) identify the likelihood of a clinical event or progression in the condition of interest [62]. Our study touches on all these points. In these exploratory analyses, our limited sample size may explain the lack of discriminatory abilities of the biomarkers for the hypertension groups. The confidence intervals for the odds ratios in the multivariable models were wide, reflecting a high dispersion and low statistical power. Additionally, biomarker performance can be inflated when studying only extreme disease cases [63]. This "spectrum effect" arises when both healthy individuals and those in advanced disease stages are examined together, potentially also masking differences in test performance [63]. Age-based matching can also introduce the spectrum effect, as blood pressure tends to rise with age. By age-based matching we risked selecting the oldest and healthiest controls and the youngest and sickest hypertension patients. However, we only assessed the associations between biomarkers and hypertension severity within the various hypertension groups, excluding healthy controls from the analysis. Inclusion of an intermediate group, as done in this study, may mitigate some of the spectrum effect. Our biomarker analyses were performed on frozen-thawed samples. However, all the chosen biomarkers appear to be stable after prolonged frozen storage and after freeze–thaw analysis [64-73]. However, the cross-sectional design of our study precludes the ability to obtain information on the temporal associations, including duration of treatment and blood pressure control. Serum drug measurement only reflects adherence at the time of measurement. The state of inflammation and signs of organ damage may depend on a time factor we were not able to account for, and longitudinal studies are needed to determine whether OPN, uromodulin, RANTES and NGAL are adequate biomarkers for risk stratification in hypertension. Further limitations include residual confounding, an issue that never can be ruled out in cohort studies. Finally, patients with hypertension

were selected based on blood pressure and standard clinical kidney function measurements, and we did not stratify patients based on other HMODs.

CONCLUSION

Finding biomarkers related to hypertension and HMOD at an early stage may aid targeted treatment. Plasma OPN may be an early biomarker for identifying patients with hypertension at risk for kidney HMOD. However, none of the biomarkers could consistently differentiate our hypertension groups when established risk factors were included in the models. The selected biomarkers may have a place in the field of hypertension, but further longitudinal analyses are needed.

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DISCLOSURE OF INTREST

The authors report no conflict of interest.

Table 1. Panel of plasma biomarkers included in the present study		
Name	**Family**	**Biological processes**
Interleukin 1 receptor antagonist (IL-1RA)	Cytokine-Interleukin	Inhibits and modulates interleukin 1 related immune and inflammatory responses. IL-1RA concentration is considered to indicate immune activation, rather than a net anti-inflammatory state.
Interleukin-18 (IL-18)	Cytokine-Interleukin	A pro-inflammatory cytokine cleaved into its active form by NLR family pyrin domain containing 3 inflammasome (NLRP3) upon sensing damage or pathogenic signals.
Tumour necrosis factor (TNF)	Tumor necrosis factor superfamily	The TNF activated nuclear factor kappa B (NF-κB) pathway is a master regulator of the inflammatory response involving up-regulation of NLRP3 inflammasome, pro-IL-1β and pro-IL-18 gene expression.
Monocyte chemoattractant protein-1 (MCP-1)	Cytokine-Chemokine	The NLRP3 inflammasome with secretion of IL-1ß synergizes with angiotensin- II to promote NF-kB activation and a proinflammatory response characterized by increased MCP-1 production. MCP-1 is a chemotactic protein for monocytes and macrophages, implicated in tissue repair and fibrosis.
Osteopontin (OPN)	Cytokine-non-structural extracellular matrix protein	Pro-inflammatory stimuli and activation of the NLRP3 inflammasome leads to increased tissue levels of osteopontin. Osteopontin is expressed in activated macrophages, T cells, smooth muscle, endothelial and epithelial cells and regulates cell adhesion, migration, proliferation and promotes macrophage and T cell infiltration.
Regulated upon activation normal T-cell expressed and secreted (RANTES)	Cytokine-Chemokine	RANTES is produced by several cells implicated in the development of hypertension, including vascular endothelium, smooth muscle, perivascular adipocytes and renal epithelial cells. RANTES is a chemotactic molecule for monocytes and T cells.
von Willebrand factor A2 (vWF-A2)	Glycoprotein involved in both hemostasis and thrombosis	An endothelial ligand for platelet glycoproteins and a suggested marker for endothelial injury and activation. Plasma levels of vWF-A2 shows the amount of protein, but not its functional status.
Neutrophil gelatinase-associated lipocalin (NGAL)	Lipocalin family	NGAL is upregulated in injured kidney epithelial cells. NGAL is a marker of tubular damage. NGAL may be an augmenting factor for kidney interstitial fibrosis.
Uromodulin (Tamm-Horsfall protein)	Glycoprotein produced by tubular cells of the thick ascending limb and the early distal tubule.	Plasma uromodulin is a suggested marker of intact tubular cells and the number of remaining functional nephrons/renal tissue. Plasma uromodulin may also modulate the immune response.

Table 2A. Characteristic subgroup (n=215)	Healthy controls (n=39)		Patients with hypertension (n=176)	
Female sex, n (%)	20	(51)	79	(45)
Age, years	55.6	(9.4)	61.1[a]	(11.1)
Cardiovascular disease, n (%)	0	0	39[a]	(22)
Active smoker, n (%)	5	(13)	28	(16)
Office systolic blood pressure, mmHg	123	(12)	152[a]	(20)
Office diastolic blood pressure, mmHg	76	(9)	87[a]	(13)
Number of antihypertensive agents per day, n	0	NA	3[a]	(2-4)
Body mass index, kg/m^2	25.8	(2.9)	30.1[a]	(5.5)
Lipid lowering treatment, n (%)	4	(10)	89[a]	(51)
Diabetes, n (%)	0	NA	49[a]	(28)
HbA1c, mmol/mol	34	(33-36)	38[a]	(35-43)
Serum creatinine (µmol/L)	74.0	(11.7)	79.9[a]	(24.4)
eGFR (mL/min/1,73m^2)	89.5	(12.6)	82.4[a]	(18.5)
ACR, mg/mmol	0.2	(0.2-0.4)	1.3[a]	(0.6-5.7)

Data are mean (SD), median (interquartile range) or number (%) as appropriate. Uncontrolled hypertension was defined as systolic daytime ambulatory blood pressure ≥135 mmHg, and kidney HMOD as ACR >3.0 mg/mmol or eGFR <60 mL/min per 1.73 m2.

[a] Significant differences between healthy controls and hypertensive patients.

Adherence to all self-reported antihypertensive agents: A pharmacologist evaluated the serum concentration analyses and found the patient adherent to all self-reported antihypertensive agents. Cardiovascular disease: Prior myocardial infarction, angina, stroke or peripheral artery disease. Diabetes: Self-reported, or HbA1C ≥ 48 mmol/mol, or use of antidiabetic agents. eGFR: Glomerular filtration rate, calculated using the 2009 creatinine-based CKD-EPI equation. Hypertension-mediated organ damage (HMOD), urine albumin to creatinine ratio (ACR).

Table 2B. Characteristic subgroup (n=176)	Patients with hypertension (n=176)					
	Controlled hypertension (n=55)		Uncontrolled hypertension without Kidney HMOD (n=59)		Uncontrolled hypertension with kidney HMOD (n=62)	
Female sex, n (%)	27	(49)	28	(47)	24	(39)
Age, years	58.4	(10.4)	59.9	(10.8)	64.7[b,c]	(11.2)
Cardiovascular disease, n (%)	7	(13)	11	(19)	21[b]	(34)
Active smoker, n (%)	11	(20)	11	(19)	12	(19)
Office systolic blood pressure, mmHg	132	(11)	161[b]	(16)	161[b]	(18)
Office diastolic blood pressure, mmHg	81	(9)	92[b]	(13)	88[b]	(13)
Ambulatory systolic blood pressure, 24 h mean, mmHg	120	(9)	149[b]	(9)	148[b]	(10)
Ambulatory diastolic blood pressure, 24 h mean, mmHg	74	(6)	85[b]	(9)	81[b]	(9)
Ambulatory systolic blood pressure, daytime mean, mmHg	125	(9)	154[b]	(10)	152[b]	(10)
Ambulatory diastolic blood pressure, daytime mean, mmHg	78	(6)	89[b]	(9)	85[b,c]	(10)
Ambulatory systolic blood pressure, nighttime mean, mmHg	108	(12)	133[b]	(13)	139[b,c]	(14)
Ambulatory diastolic blood pressure, nighttime mean, mmHg	64	(7)	72[b]	(10)	74[b]	(10)
Number of antihypertensive agents per day, n	3	(2-3)	3	(2-3)	4[b,c]	(3-4)
Adherence to all self-reported antihypertensive agents, n	44	(80.0)	37	(62.7)	50[c]	(80.6)
Body mass index, kg/m^2	27.9	(4.1)	29.9[b]	(5.8)	32.1[b,c]	(5.5)
Cholesterol, mmol/L	5.0	(1.1)	4.9	(1.1)	4.9	(1.2)
HDL cholesterol, mmol/L	1.6	(0.5)	1.5	(0.5)	1.4[b]	(0.4)
LDL cholesterol, mmol/L	3.1	(1.0)	3.1	(1.0)	3.1	(1.1)
Triglycerides, mmol/L	1.5	(0.9)	1.6	(0.8)	2.0[b,c]	(1.0)
Lipid lowering treatment, n (%)	27	(49)	23	(39)	39[c]	(63)
Diabetes, n (%)	6	(11)	9	(15)	33[b,c]	(53)
HbA1c, mmol/mol	38	(35-39)	37	(34-41)	43[b,c]	(38-53)
Serum creatinine (µmol/L)	75.7	(15.0)	70.7	(13.8)	92.6[b,c]	(32.6)
eGFR (mL/min/1,73m^2)	69.6	(13.8)	70.8	(11.3)	53.5[b,c]	(18.7)
ACR, mg/mmol	0.5	(0.3-1.1)	0.9[b]	(0.5-1.4)	15.1[b,c]	(5.5-43.0)

Data are mean (SD), median (interquartile range) or number (%) as appropriate. Uncontrolled hypertension was defined as systolic daytime ambulatory blood pressure ≥135 mmHg, and kidney HMOD as ACR >3.0 mg/mmol or eGFR <60 mL/min per 1.73 m^2. [b] Significant differences between the group with controlled hypertension compared to uncontrolled hypertension without and with kidney HMOD. [c] Significant differences between the group with uncontrolled hypertension without kidney HMOD compared to uncontrolled hypertension with kidney HMOD. Adherence to all self-reported antihypertensive agents: A pharmacologist evaluated the serum concentration analyses and found the patient adherent to all self-reported antihypertensive agents. Cardiovascular disease: Prior myocardial infarction, angina, stroke or peripheral artery disease. Diabetes: Self-reported, or HbA1C ≥ 48 mmol/mol, or use of antidiabetic agents. eGFR: Glomerular filtration rate, calculated using the 2009 creatinine-based CKD-EPI equation. Hypertension-mediated organ damage (HMOD), urine albumin to creatinine ratio (ACR).

Table 3. Levels of biomarkers between healthy controls and hypertensive patients (n=215)					
Biomarker	**Healthy controls (n=39)**		**Hypertensive patients (n=176)**		**P value**
IL-1 RA (ng/ml)	0.32	(0.28-0.39)	0.42	(0.31-0.64)	0.002
IL-18 (ng/ml)	0.10	(0.07-0.16)	0.12	(0.09-0.17)	0.13
TNF (pg/ml)	4.17	(3.54-5.50)	4.6	(3.7-6.0)	0.20
MCP-1 (ng/ml)	0.11	(0.09-0.14)	0.12	(0.10-0.15)	0.18
OPN (ng/ml)	41.7	(28.5-50.5)	36.6	(27.1-49.9)	0.23
RANTES (ng/ml)	14.5	(4.8-32.2)	8.5	(3.9-19.0)	0.12
vWF-A2 (ng/ml)	0.81	(0.54-0.95)	0.83	(0.59-1.11)	0.60
NGAL (ng/ml)	104	(92-115)	113	(93-144)	0.020
Uromodulin (ng/ml)	475	(370-593)	369	(239-506)	<0.001

Median levels (interquartile range) of plasma biomarkers.
Independent-Samples Mann-Whitney U Test.
Interleukin 1 receptor antagonist (IL-1RA), interleukin-18 (IL-18), tumour necrosis factor (TNF), monocyte chemoattractant protein-1 (MCP-1), osteopontin (OPN), regulated upon activation normal T-cell expressed and secreted (RANTES), von Willebrand factor A2 (vWF-A2), neutrophil gelatinase-associated lipocalin (NGAL), uromodulin (Tamm-Horsfall protein).

Table 4. The trend in biomarker medians in relation to all study groups (n=215)						
Biomarkers	**Healthy controls (Median (IQR) (n=39)**	**Controlled hypertension (Median (IQR) (n=55)**	**Uncontrolled hypertension without kidney HMOD (Median (IQR) (n=59)**	**Uncontrolled hypertension with kidney HMOD (Median (IQR) (n=62)**	**T_b**	**P value**
IL-1 RA (ng/ml)	0.32 (0.28-0.39)	0.39 (0.29-0.56)	0.37 (0.26-0.57)	0.51 (0.36-0.74)	0.20	<0.001
IL-18 (ng/ml)	0.10 (0.07-0.16)	0.12 (0.09-0.15)	0.11 (0.08-0.14)	0.15 (0.11-0.19)	0.15	0.003
TNF (pg/ml)	4.17 (3.54-5.50)	4.18 (3.60-4.79)	4.30 (3.23-5.50)	5.86 (4.71-7.84)	0.25	<0.001
MCP-1 (ng/ml)	0.11 (0.09-0.14)	0.11 (0.09-0.13)	0.12 (0.10-0.16)	0.13 (0.11-0.17)	0.17	0.001
OPN (ng/ml)	41.7 (28.5-50.5)	33.6 (22.4-38.2)	38.0 (27.6-50.7)	41.5 (31.4-54.7)	0.09	0.09
RANTES (ng/ml)	14.5 (4.8-32.2)	9.31 (5.42-19.56)	8.80 (4.17-21.45)	7.08 (3.43-17.57)	-0.10	0.049
vWF-A2 (ng/ml)	0.81 (0.54-0.95)	0.78 (0.55-0.99)	0.68 (0.47-0.97)	0.95 (0.71-1.29)	0.12	0.025
NGAL (ng/ml)	104 (92-115)	101 (90-136)	106 (87-128)	138 (109-195)	0.25	<0.001
Uromodulin (ng/ml)	475 (370-593)	400 (272-538)	467 (342-529)	248 (178-396)	-0.27	<0.001

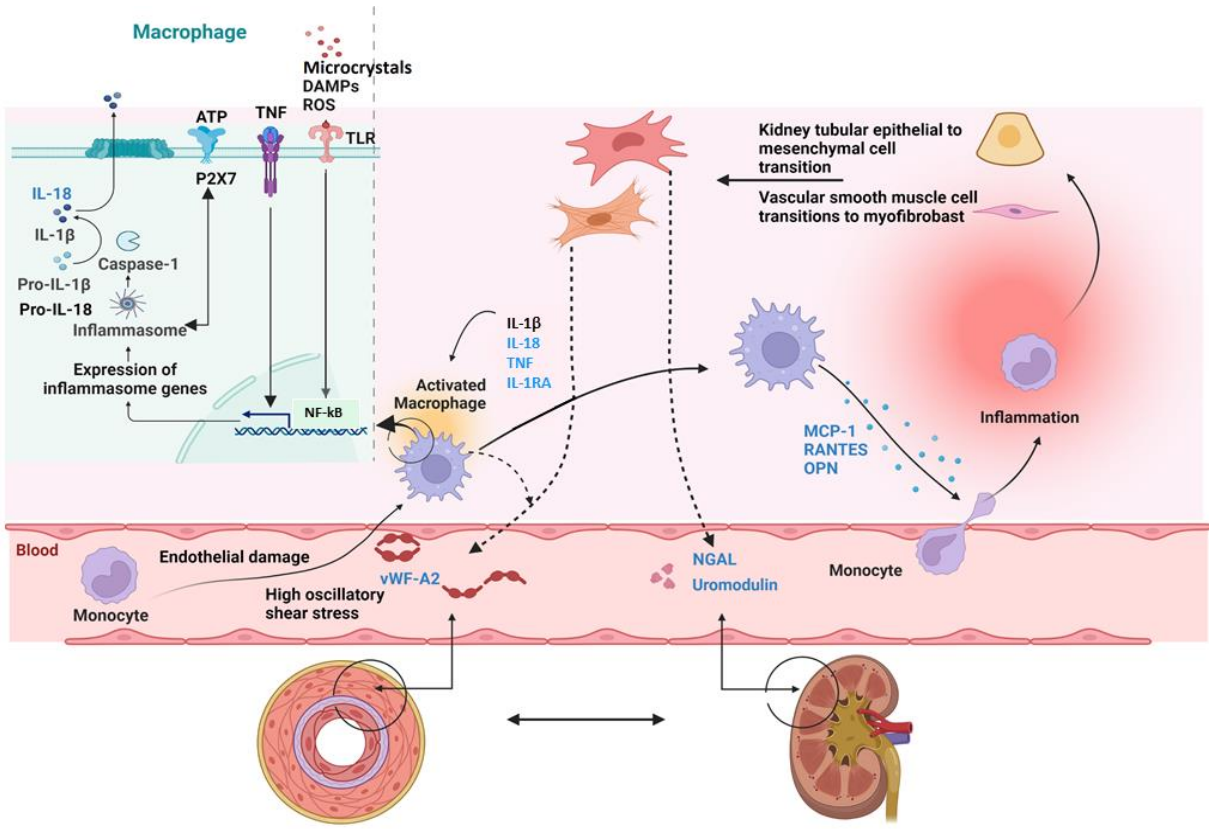
Kendall´s rank correlation (Kendall´s tau) for a monotonic tendency in biomarker medians across study groups.

Uncontrolled hypertension was defined as systolic daytime ambulatory blood pressure ≥135 mmHg, and kidney HMOD as ACR >3.0 mg/mmol or eGFR <60 mL/min per 1.73 m^2.

Nonparametric correlation coefficient (T_b),

Hypertension-mediated organ damage (HMOD), urinary albumin to creatinine ratio (ACR), glomerular filtration rate, calculated using the creatinine-based CKD-EPI equation (eGFR), interleukin 1 receptor antagonist (IL-1RA), interleukin-18 (IL-18), tumour necrosis factor (TNF), monocyte chemoattractant protein-1 (MCP-1), osteopontin (OPN), regulated upon activation normal T-cell expressed and secreted (RANTES) von Willebrand factor A2 (vWF-A2), neutrophil gelatinase-associated lipocalin (NGAL), uromodulin (Tamm-Horsfall protein).

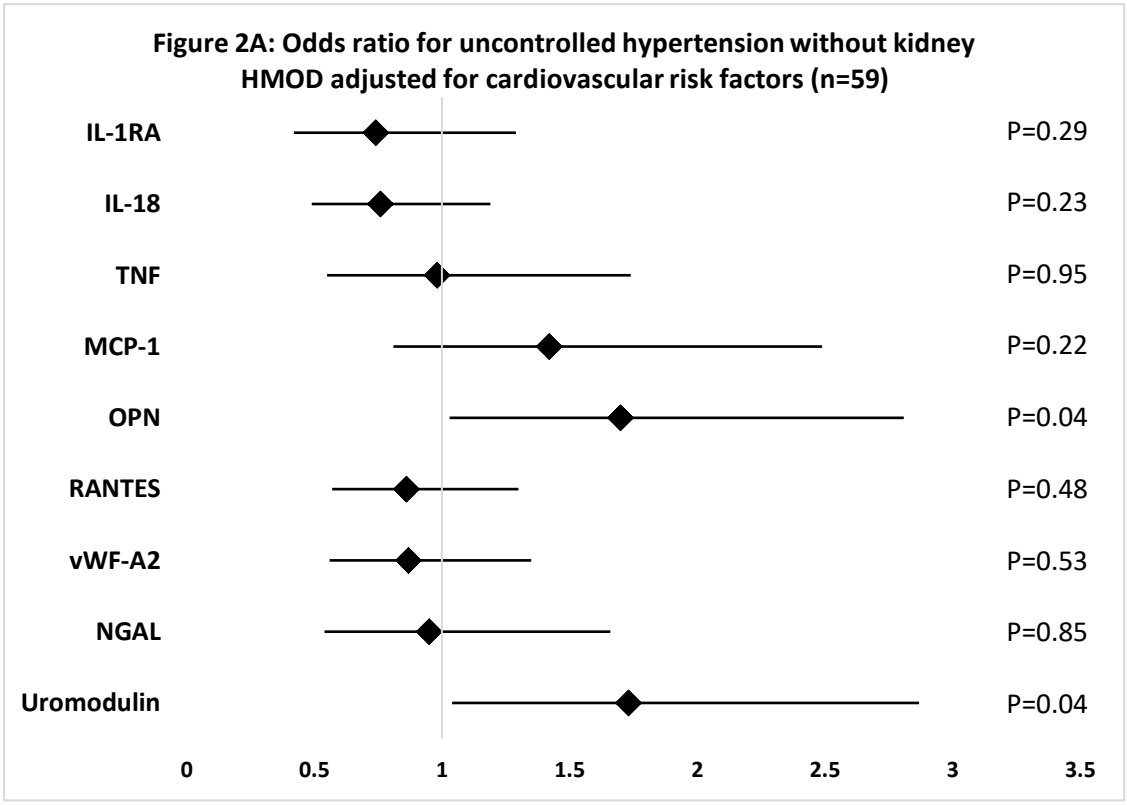
Figure 1: Biomarkers of kidney and endothelial cell pathology, and markers of inflammation and fibrosis



The NLRP3 inflammasome initiates inflammation through two signals: Signal I, triggered by DAMPS and PAMPS on TLR and cytokine receptors such as TNF-receptor, and Signal II, activated by DAMPs like microcrystals, ROS and ATP via P2X7 receptor. Once activated, the inflammasome activates caspase-1, which converts proinflammatory IL-1ß and IL-18 into their active forms. IL-1ß and IL-18 are primarily produced by monocytes and macrophages and bind to receptors on immune and vascular cells, causing inflammation. The anti-inflammatory cytokine IL-1RA antagonizes IL-1ß. Activation of TLR, NLRP3 inflammasome and the TLR MyD88 dependent and independent pathways leads to increased NF-kB activity and to the release of chemokines and extracellular matrix proteins, such as IL-1β, TNF, MCP-1, RANTES, and OPN. Chronic inflammations result in tissue damage over time. Endothelial injury, inflammation, and shear stress causes the release of vWF, while NGAL and uromodulin are markers of kidney injury. We investigated the biomarkers in blue font. IL-1β was investigated, but not detectable in the majority of examined individuals. Adenosine triphosphate (ATP), damage-associated molecular patterns (DAMPs), interleukin 1 receptor antagonist (IL-1RA), interleukin-1ß (IL-1ß), interleukin-18 (IL-18), monocyte chemoattractant protein-1 (MCP-1), neutrophil gelatinase-associated lipocalin (NGAL), nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing 3 (NLRP3) inflammasome, nuclear factor-κB (NF-kB), osteopontin (OPN), pathogen-associated molecular patterns (PAMPs), P2X purinoceptor 7 (P2X7), regulated upon activation normal T-cell expressed and secreted (RANTES), reactive oxygen species (ROS), toll-like receptors (TLR), tumour necrosis factor (TNF), von Willebrand factor (vWF).

(Figure created with BioRender.com).

Figure 2. Multinomial logistic regression among patients with hypertension, controlled hypertension as reference category (n=176)



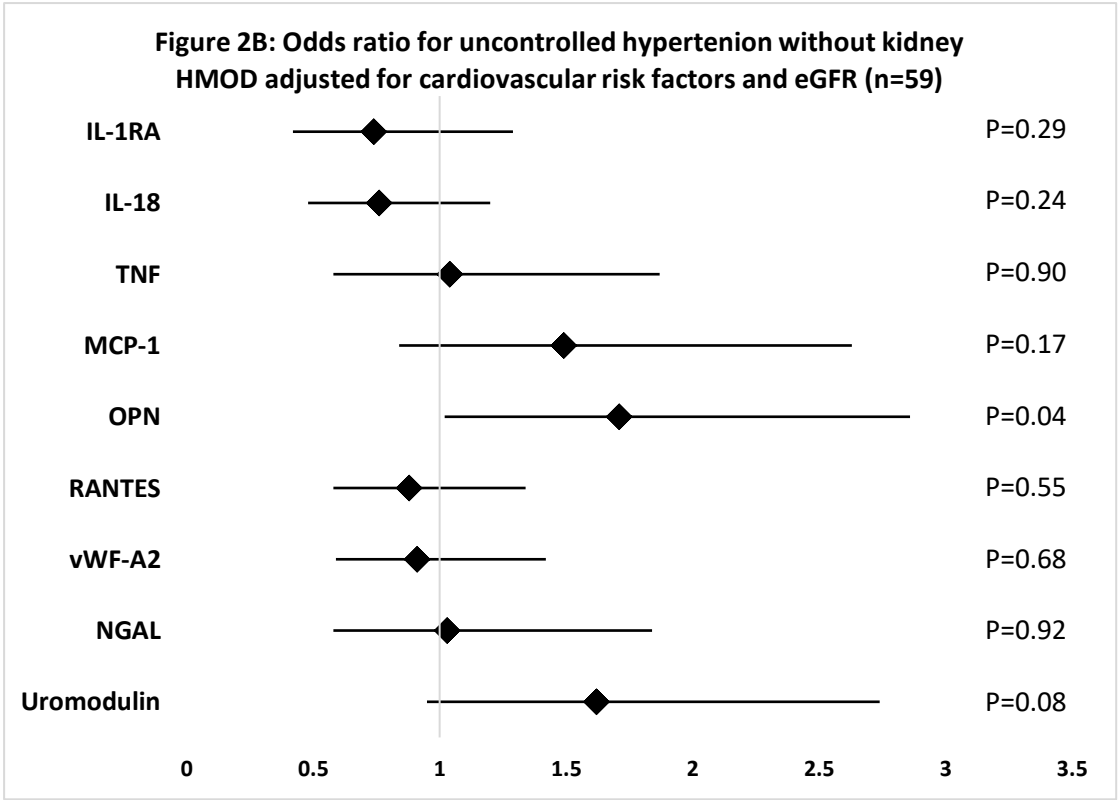


Figure 2C: Odds ratio for uncontrolled hypertension with kidney HMOD adjusted for cardiovascular risk factors (n=62)

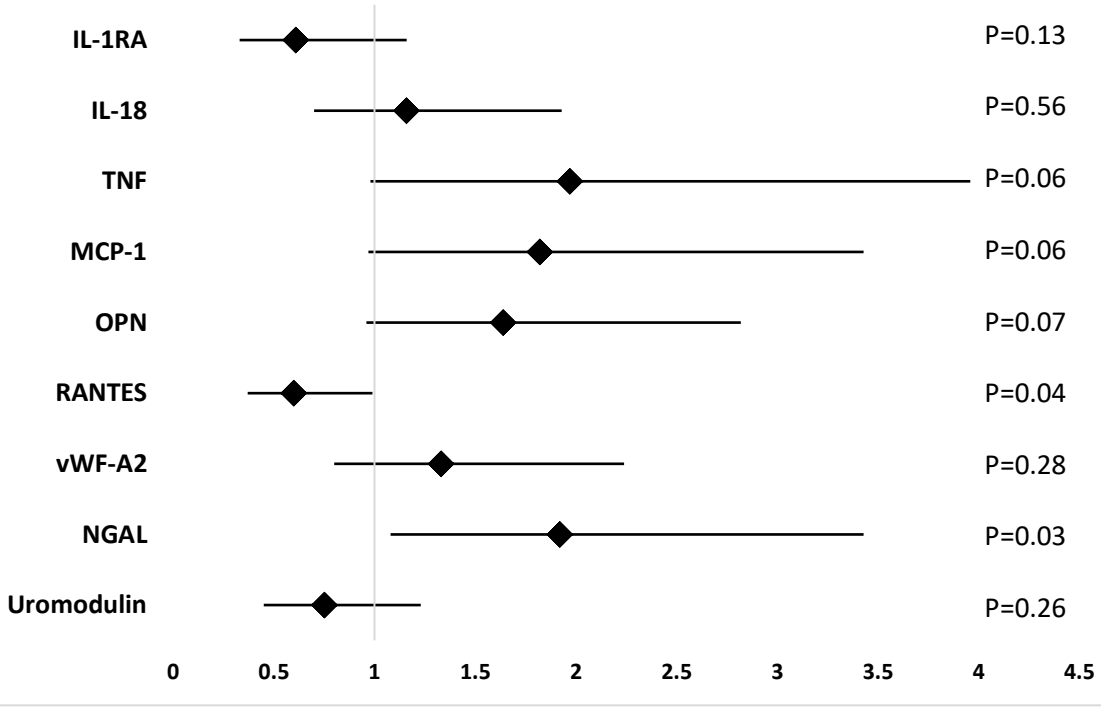
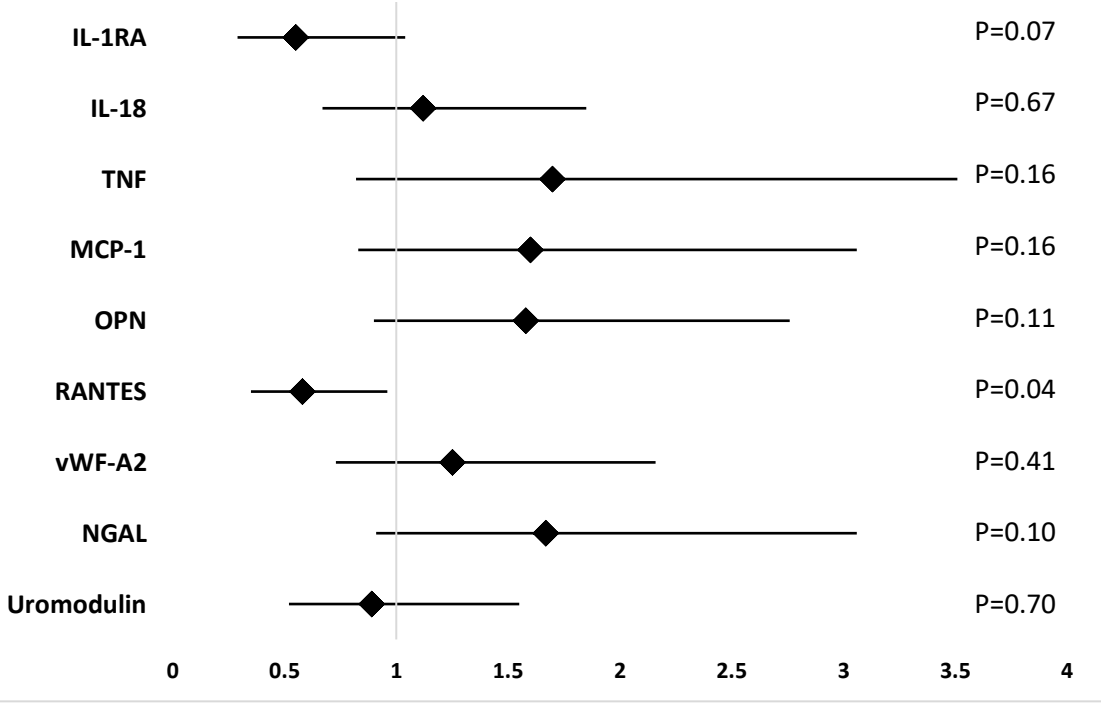
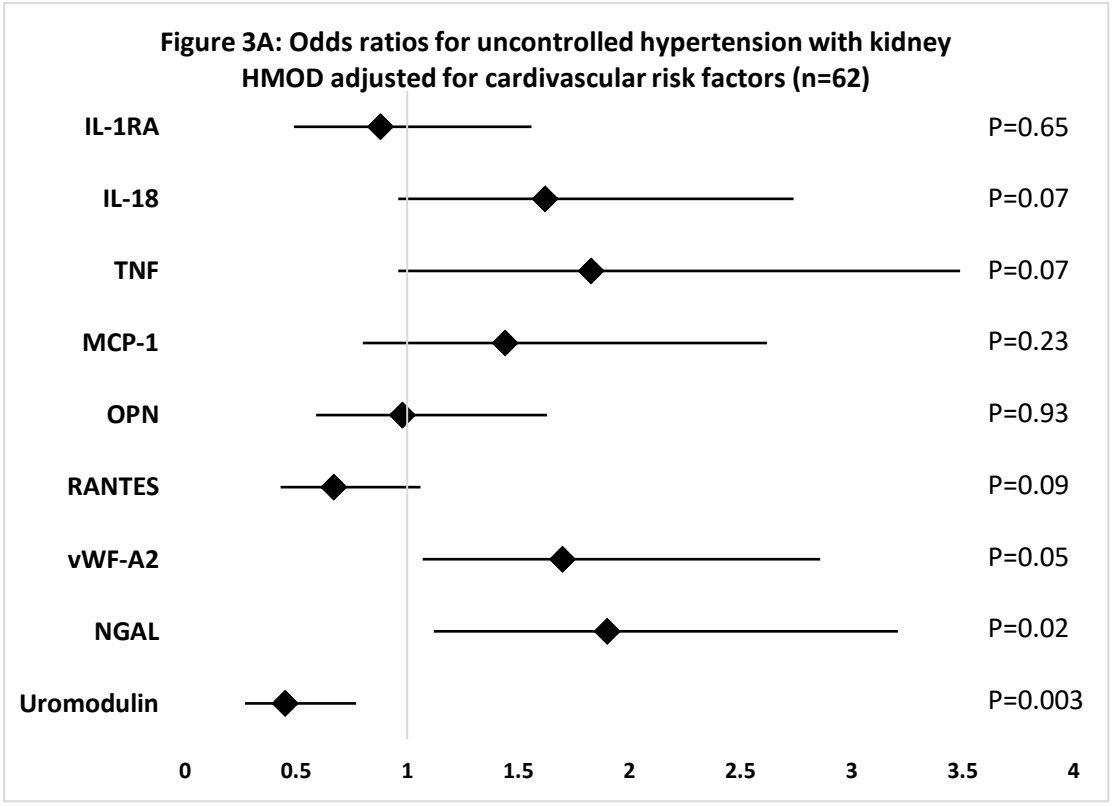


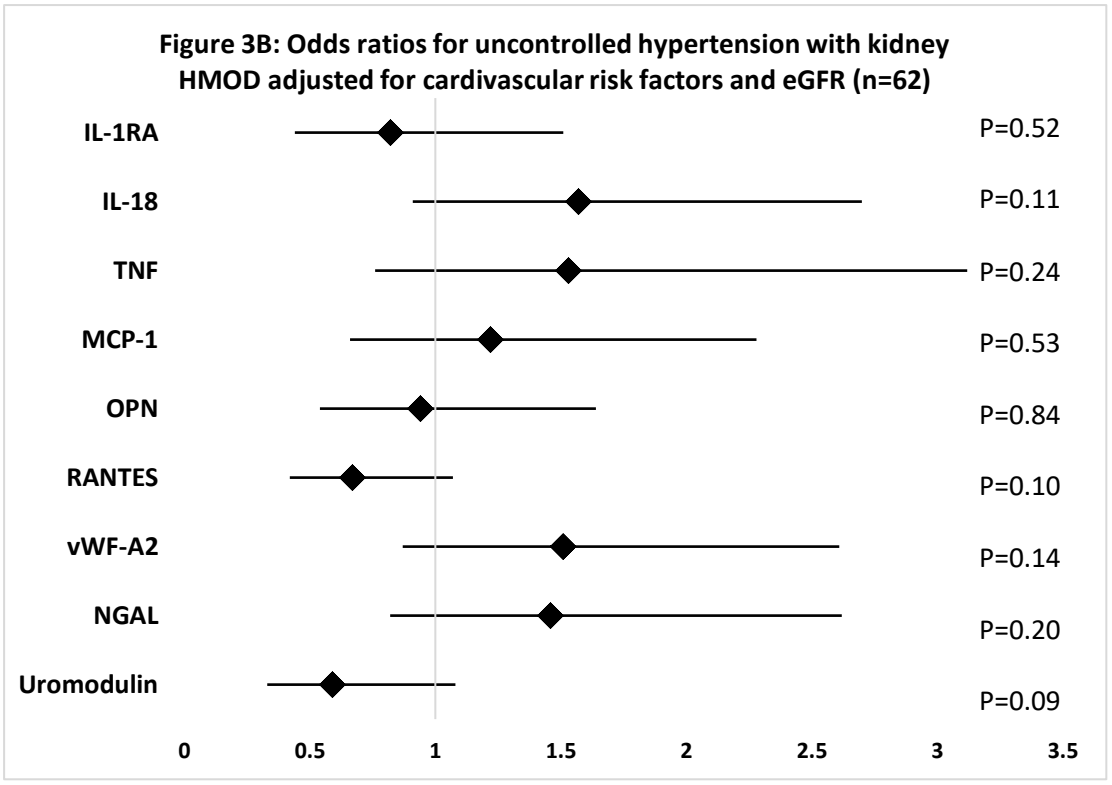
Figure 2D: Odds ratios for uncontrolled hypertension with kidney HMOD adjusted for cardivascular risk factors and eGFR (n=62)



Plasma biomarkers as natural logarithm transformation per SD unit: Odds ratio with 95% CI. Uncontrolled hypertension was defined as systolic daytime ambulatory blood pressure ≥135 mmHg, and kidney HMOD as ACR >3.0 mg/mmol or eGFR <60 mL/min per 1.73 m^2.
Cardiovascular risk factors: Sex, age, BMI, diabetes (self-reported, or HbA1C ≥48 mmol/mol, or us of antidiabetic agents), prior cardiovascular disease (prior myocardial infarction, angina, stroke or peripheral artery disease). eGFR: Glomerular filtration rate, calculated using the 2009 creatinine CKD-EPI equation. Hypertension-mediated organ damage (HMOD), urinary albumin to creatinine ratio (ACR), estimated glomerular filtration rate (eGFR), body mass index (BMI), interleukin 1 receptor antagonist (IL-1RA), interleukin-18 (IL-18), tumour necrosis factor (TNF), monocyte chemoattractant protein-1 (MCP-1), osteopontin (OPN), regulated upon activation normal T-cell expressed and secreted (RANTES), von Willebrand factor A2 (vWF-A2), neutrophil gelatinase-associated lipocalin (NGAL), uromodulin (Tamm-Horsfall protein).

Figure 3. Binary logistic regression among patients with uncontrolled hypertension, uncontrolled hypertension without kidney HMOD as reference category (n=121).





Plasma biomarkers as natural logarithm transformation per SD unit: Odds ratio with 95% CI. Uncontrolled hypertension was defined as systolic daytime ambulatory blood pressure ≥135 mmHg, and kidney HMOD as ACR >3.0 mg/mmol or eGFR <60 mL/min per 1.73 m^2.
Cardiovascular risk factors: Sex, age, BMI, diabetes (self-reported, or HbA1C ≥48 mmol/mol, or us of antidiabetic agents), prior cardiovascular disease (prior myocardial infarction, angina, stroke or peripheral artery disease). eGFR: Glomerular filtration rate, calculated using the 2009 creatinine CKD-EPI equation. Hypertension-mediated organ damage (HMOD), urinary albumin to creatinine ratio (ACR), estimated glomerular filtration rate (eGFR), body mass index (BMI), interleukin 1 receptor antagonist (IL-1RA), interleukin-18 (IL-18), tumour necrosis factor (TNF), monocyte chemoattractant protein-1 (MCP-1), osteopontin (OPN), regulated upon activation normal T-cell expressed and secreted (RANTES), von Willebrand factor A2 (vWF-A2), neutrophil gelatinase-associated lipocalin (NGAL), uromodulin (Tamm-Horsfall protein).

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Paper 3

BLOOD PRESSURE

Blood Pressure

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Associations of urinary orosomucoid, N-acetyl-β-D-glucosaminidase, and albumin with blood pressure and hypertension after 7 years. The Tromsø Study

Karl M. Brobak, Runa M. Andreassen, Toralf Melsom, Aud Høieggen, Jon V. Norvik & Marit D. Solbu

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ORIGINAL ARTICLE

Associations of urinary orosomucoid, N-acetyl-β-D-glucosaminidase, and albumin with blood pressure and hypertension after 7 years. The Tromsø Study

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ABSTRACT

Purpose: Subclinical chronic kidney disease is known to exacerbate hypertension and progression of kidney damage. In order to initiate timely interventions, early biomarkers for this vicious circle are needed. Our aim was to describe the cross-sectional associations of urinary orosomucoid and urinary N-acetyl-β-D-glucosaminidase (NAG) with blood pressure and the longitudinal associations of urinary orosomucoid and NAG to hypertension after 7 years, and to compare the strength of these associations to the urinary albumin excretion (UAE).

Material and methods: The Tromsø Study is a population-based, prospective study of inhabitants of the municipality of Tromsø, Northern Norway. Morning spot urine samples were collected on three consecutive days in the Tromsø 6 survey (2007–2008). We assessed the cross-sectional associations of urinary orosomucoid, NAG and UAE with blood pressure in Tromsø 6. In a cohort of participants attending Tromsø 6 and Tromsø 7 (2015–2016), we studied whether urinary biomarkers were longitudinally associated with hypertension.

Results: A total of 7197 participants with a mean age of 63.5 years (*SD* 9.2), and a mean blood pressure of 141/78 mmHg (*SD* 23.0/10.6), were included in the study. Orosomucoid and UAE, but not NAG, was significantly associated with systolic and diastolic blood pressure in all the crude and multivariable cross-sectional analyses. Orosomucoid had consistently, although marginally, stronger associations with blood pressure. Incident hypertension at follow-up (Tromsø 7) was consistently significantly associated with urinary orosomucoid, but not urinary NAG or UAE. However, the standardized regression coefficients for orosomucoid were only marginally stronger than the standardized regression coefficients for ACR.

Conclusion: In a cohort from the general population urine orosomucoid had a stronger cross-sectional association with blood pressure than UAE. After 7 years, urine orosomucoid showed the strongest association with incident hypertension. There were varying and weak associations between U-NAG, blood pressure and hypertension.

PLAIN LANGUAGE SUMMARY

- **What is the context?**

  There is a relationship between high blood pressure and cardiovascular and kidney disease. Hypertension is defined as the level of blood pressure at which the benefits of treatment outweigh the risks of treatment.

  Hypertension is a risk factor for developing kidney disease, and kidney disease is a risk factor for developing hypertension.

  Today, kidney function is assessed by blood and urine samples (estimated glomerular filtration rate and urinary albumin excretion). However, today's blood and urine samples are not sensitive enough to capture kidney damage due to hypertension at a stage when prevention may be most effective.

- **What is new?**

  In this study, we assessed if urine orosomucoid and N-acetyl-β-D-glucosaminidase (NAG) are more strongly associated with blood pressure and hypertension than urinary albumin excretion.

  In the population-based study of residents in Tromsø, Northern Norway, we assessed the relationship between the urine biomarkers and blood pressure, and the development of hypertension after 7 years.

  In the general population urine orosomucoid had a stronger relationship with blood pressure

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than urinary albumin excretion.
After 7 years, urine orosomucoid had the strongest relationship with the development of hypertension.
There were only varying and weak relationships between NAG, blood pressure and hypertension.

- **What is the impact?**
Orosomucoid showed a stronger relationship with blood pressure and the development of hypertension than urinary albumin excretion. Urine orosomucoid may aid targeted prevention and treatment in hypertension, but further prospective clinical studies are needed to assess if orosomucoid is a clinically useful biomarker in hypertension.

Introduction

According to the World Health Organization, hypertension is the most significant single contributor to overall morbidity and mortality [1]. An estimated 1.4 billion people worldwide have high blood pressure [2]. Subclinical kidney disease is associated with developing hypertension in the general population [3,4]. Therefore, the development of subclinical kidney damage can initiate a vicious circle with exacerbated hypertension and further progression of kidney damage. The underlying mechanism leading to kidney damage in hypertension is not settled, but endothelial dysfunction, afferent arteriolopathy, damage to the glomerular filtration barrier and tubular ischemia/dysfunction may be important and early steps [4].

Many observational studies have shown that a reduced GFR and albuminuria are independently and multiplicatively associated with an increased risk of cardiovascular events [5]. However, since a significant reduction in kidney function is needed to detect a decrease in estimated GFR (eGFR) [6], it is considered an insensitive marker of kidney damage. Futhermore, only a minority of patients with hypertension have elevated UAE to a level detected by most assays [7–10]. Overall, both eGFR and UAE are insensitive biomarkers of early renal damage in hypertension, at a stage when prevention may be most effective [4,11,12]. Further, lowering of UAE per se in hypertensive patients has not been shown to reduce cardiovascular disease (CVD) risk [13], making UAE a deficient surrogate therapeutic target in hypertension [14]. Finding a panel of biomarkers related to hypertension and hypertension-mediated organ damage at an early stage, that varies with modifiable risk factors, may aid targeted prevention and treatment [12,15].

Similar to UAE, urinary orosomucoid (α-1-acid glycoprotein) is considered a marker of general endothelial dysfunction and a damaged glomerular filtration barrier [16–18]. Orosomucoid is a constituent of the endothelial surface layer and maintains permselectivity [18,19]. Damage to this layer is considered a cause for atherosclerosis [20]. As tubular dysfunction and damage may increase UAE [21], urinary orosomucoid may be a more specific marker of endothelial dysfunction and glomerular damage in hypertension [18,19,22,23].

N-acetyl-β-D-glucosaminidase (NAG) is a lysosomal enzyme that, in the kidney, is found predominantly in lysosomes of proximal tubular cells [11]. The NAG present in the urine is secreted from proximal tubular cells by exocytosis [24]. NAG is thus exclusively a marker of tubular cell function [25]. Myocardial infarction, ischemic stroke, and mortality in the general population have been associated with NAG levels [26]. A small study of patients with newly diagnosed hypertension without microalbuminuria found a significantly higher NAG activity in hypertensive subjects compared to healthy controls [27]. Whereas UAE is dependent on alterations in the glomerular filtration barrier and impaired proximal tubular function [21], NAG may be an earlier and more specific marker of proximal tubular dysfunction in hypertension [27,28].

In this study, we aimed to assess if urinary orosomucoid and NAG have stronger associations cross-sectionally to blood pressure and longitudinally to hypertension than UAE.

Study population

The Tromsø Study is a population-based, prospective study of residents of the municipality of Tromsø, Northern Norway. Since 1974, seven surveys (Tromsø 1–7) have been conducted. The participants in Tromsø 6 (2007–2008) were recruited from four invited groups: Those who took part in the special study in the Tromsø 4, a random 10% sample of residents aged 30–39, everyone aged 40–42 or 60–87 and a random 40% sample of residents aged 43–59 years. A total of 12,984 attended (65.7% of the invited population) [29]. Common CVD risk factors were mapped in the first visit.

Further, 7955 of the participants were invited to undergo an extensive examination. The population eligible for the second visit included first visit participants aged 50–62 and 75–84 years, a 20% random sample aged 63–74, and finally all subjects who had also attended the second visit of Tromsø 4 (1994–1995). The attendance rate for this second visit was 91.8% ($n = 7306$). Morning spot urine samples from 7197 participants on three consecutive days were collected. UAE was available in 7195 participants (3090 men and 4105 women), urinary orosomucoid was available in 7181 participants (3086 men and 4095 women), and urinary NAG measurements were available in 7170 participants (3082 men and 4088 women).

The invited population in Tromsø 7 (2015–2016) consisted of all individuals aged 40 and older. A total of 21,083 attended (64.7% of the invited population) [30].

A total of 5114 attended both the second visit of the Tromsø 6 and the Tromsø 7 surveys (Figure 1). UAE was available in 5046 participants, urinary orosomucoid was available in 5033 participants and urinary NAG measurements were available in 5025 participants of the Tromsø 7.

The UiT, The Arctic University of Norway conducted the survey in cooperation with The National Health Screening Service. The Regional Committee for Medical and Health Research Ethics approved the study, and all participants gave their written consent [29,30].

Methods

In both Tromsø 6 and 7, the participants returned a self-administered questionnaire, including information about current medication, diabetes, CVD, and smoking habits. Tobacco use was dichotomized into current smokers or not (all others). Blood samples and time since the last meal were obtained. We defined fasting as time since the previous meal of at least eight hours. We defined the presence of diabetes as self-reported diabetes, self-reported use of glucose-lowering drugs, HbA1c ≥6.5%, non-fasting glucose ≥11 mmol/L, or a fasting blood glucose >7.0 mmol/L. We calculated the body mass index (BMI; kg/m^2) from height and weight measured by study personnel.

Blood pressure was recorded in triplet by trained personnel, using an automatic device (the Dinamap Pro care 300 Monitor (GE Healthcare). The cuff was chosen after the circumference of the upper arm was measured. After a 2-min rest, three attended readings on the upper right arm were taken in a sitting position, separated by a 1-min interval. We used the mean of the second and third readings in the analyses [31]. Several antihypertensive agents may affect the levels of urinary biomarkers independently from their blood pressure lowering effect [32], and in the cross-sectional design the cohort was dichotomized into those with and without treated hypertension. We defined treated hypertension as using one or more blood pressure-lowering drugs. In the longitudinal analyses, we defined incident hypertension as being normotensive in Tromsø 6 and having started with one or more blood pressure-lowering drugs or an increase in blood pressure ≥140/90 mmHg at follow-up (Tromsø 7). In the longitudinal analysis, we further defined controlled hypertension at baseline (Tromsø 6) as blood pressure <140/90 mmHg while using one or more blood pressure-lowering drugs. Among those with controlled hypertension at baseline, we defined progressive hypertension as an increase in the number of blood

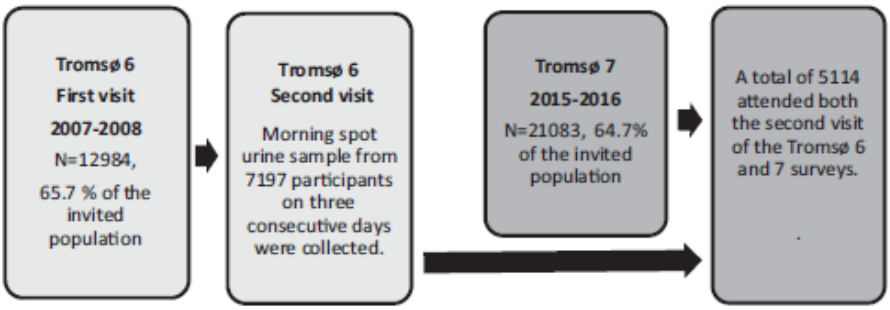

Figure 1. Selection of study participants from the Tromsø Study, 6th and 7th wave. Figure 1 depicts a range of change in predicted probabilities. A standardized coefficient of 0.01 represents 1% of the maximum change possible in predicted probability of incident hypertension. NAG: (Log urinary N-acetyl-β-D-glucosaminidase to creatinine ratio per *SD* unit): standardized beta coefficients at different probability reference value; ACR: (Log urinary albumin to creatinine ratio per SD unit): standardized beta coefficients at different probability reference value; OCR: (Log urinary orosomucoid to creatinine ratio per SD unit): standardized beta coefficients at different probability reference value. Fully Adjusted Model: Adjusted for CVD risk factors and NAG and ACR and OCR. CVD risk factors: Sex, Age, BMI, Current smoker, Diabetes, Prior cardiovascular event (stroke or heart attack).

pressure-lowering drugs or blood pressure ≥140/90 mmHg while on the same blood pressure-lowering drugs at follow-up (Tromsø 7).

We used morning urine samples collected on three consecutive days at baseline (Tromsø 6). We measured creatinine, albumin, and NAG in fresh urine samples at the time of collection. Urine creatinine was measured using colorimetric methods (Jaffes reaction) and an autoanalyser (ABX PENTRA, Horiba ABX, Montpellier, France). We measured the urine albumin concentration by an immunoturbidimetric method with the ABX Pentra Micro-albumin CP (Horiba ABX, Montpelier, France). A colorimetric method (with 3-cresolsulfonphthaleinyl-N-acetyl-β-d-glucosaminide; Boehringer Mannheim, Germany) was applied for the NAG measurements. Urine samples were frozen at −20 °C, shipped to the Department of Quality and Research, Regional Hospital of Randers, University of Aarhus, Denmark and analyzed for orosomucoid by a highly sensitive EU-labelled fluorometric immunoassay (DELFIA) [33]. We added Tween 20, stored the urine samples at −20 °C and analyzed thawed samples at 37 °C based on a self-developed application in the AutoDELFIA system [33,34]. For each urine sample, we calculated the ratios of albumin, NAG, and orosomucoid concentrations with the urine creatinine concentration (NAG-creatinine ratio [NAG-Cr], albumin-creatinine ratio [ACR], orosomucoid-creatinine ratio [OCR]), respectively). The median values of these biomarkers assessed on days 1,2 and 3 were used in the analyses.

Serum creatinine was measured with an enzymatic method (Modular P; Roche Diagnostics). Cystatin C was measured with a particle-enhanced turbidimetric immunoassay using reagents from Gentian on a Modular P800 analyzer (Roche Diagnostics). We calculated creatinine-cystatin C based eGFR by applying the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [35,36]. Serum glucose, triglycerides, and cholesterol were measured on a Modular P800 (Roche Diagnostics). HbA1c was measured with a liquid chromatographic method (Variant II in-strument, Bio-Rad Laboratories, Hercules, CA).

Statistical analyses

Baseline characteristics were summarized for the entire cohort and subgroups with or without treated hypertension and men and women with a mean (*SD*) for normally distributed variables, median for skewed variables, and *n* (percentage) for categorical variables. Student's *t*-test, Mann-Whitney tests, Wilcoxon-signed-rank test and chi-square test were used for comparison, as appropriate.

In the cohort of participants with morning spot urine samples from Tromsø 6, second visit, we used univariable and multivariable linear regression analyses to assess the associations between the urinary biomarkers and the dependent variables systolic and diastolic blood pressure in the whole cohort and subgroups with and without treated hypertension. The creatinine adjusted urinary biomarkers were non-normally distributed and were log-transformed. As standardized regression coefficients are context dependent, we did the crude analysis to address which biomarker explains the most variance, ignoring the effect of the other independent variables. We did multivariable and fully adjusted models to address which biomarker had the most unique variance (i.e. the largest beta weight) when the other independent variables were included in the regression models. In crude linear models, we added one urinary biomarker ([NAG-Cr] or [ACR] or [OCR]). In multivariable models, we added CVD risk factors (sex, age, BMI, current smoker, diabetes and prior cardiovascular event (stroke or heart attack)) and one urinary biomarker ([NAG-Cr] or [ACR] or [OCR]). In fully adjusted models, we added CVD risk factors and all urinary biomarkers ([NAG-Cr] and [ACR] and [OCR]). We included eGFR in all multivariable models but excluded this variable from the models if it had no impact on the standardized regression coefficients. In the same crude and multivariable linear models, we also used the log-transformed median concentration of the urinary biomarkers without the urine creatinine concentration ratio. The strengths of the associations were compared and presented as standardized b-coefficients (std beta) in the whole cohort and in those with and without treated hypertension.

In a cohort of participants who delivered urine samples in Tromsø 6 and later participated in Tromsø 7, we assessed the association between the biomarkers and the dependent variable incident hypertension in the normotension cohort in Tromsø 6. We further evaluated the association between the biomarkers and the dependet variable progressive hypertension in the cohort with controlled hypertension in Tromsø 6. We used univariable and multivariable logistic regression analyses and the same regression models described in the cross-sectional analyses. The logistic regression analysis reports unstandardized regression coefficients, *p* values, the odds ratio with corresponding 95% confidence intervals and the semi-standardized regression

coefficients using the Kaufman formula to compare the strength of the associations [37,38]. The semi-standardized coefficient given by the Kaufman formula measures the change in predicted probability associated with a one standard deviation change in the predictor and is restricted to the interval −1 to 1 [38]. The semi-standardized regression coefficients were calculated at the mean predicted probability for the outcome in the population for the two longitudinal analyses (mean predicted probability 0.32 for incident hypertension and mean predicted probability 0.55 for progressive hypertension). The range of estimated semi-standardized regression coefficients for different probability reference values was calculated for the urine biomarkers and displayed graphically for the fully adjusted models.

Participants with missing data were excluded only for analyses for which the case had missing data.

The urine albumin assay's limit of detection in Tromsø 6 is given at 4 mg/L, but the PENTRA instrument reports albumin concentrations as low as 1 mg/L. These results were published in previous publications from the Tromsø Study [39–41] and in the main analyses of the present study. The limit of detection for the albumin concentration almost corresponds to the median albumin concentration in Tromsø 6. Given a large number of observations below the limit, we further treated all observations of albumin concentration lower than 4 mg/L as left-censored and repeated the linear and logistic regressions for the association with blood pressure and incident hypertension using the deletion method [7,42].

We explored interaction effects between the urinary biomarkers, age, and sex. We present the most pronounced interaction effects in a supplementary table and display the mean associations with standardized beta coefficients in the main tables. Significant interactions with sex are presented as standardized beta coefficients for men and women. Significant interactions with age are presented as centered scores expressed as *SD* (i.e. standardized regression coefficients) corresponding to the mean age ± 1 *SD*.

Statistical significance was defined as two-sided $p < 0.05$ in all analyses. We used SPSS Statistics for Windows, Version 27.0 (IBM SPSS Statistics for Windows, Version 27 .0. Armonk, NY: IBM Corp).

Results

Baseline characteristics

A total of 7197 participants with a mean age of 63.5 years (*SD* 9.2), a mean blood pressure of 141/78 mmHg (*SD* 23.0/10.6), and a mean BMI of 27.3 kg/m^2 (*SD* 4.2) were included in the cross-sectional study (Figure 1).

Of the 7197 participants, 2326 (32%) used one or more blood pressure-lowering drugs. Subjects using blood pressure-lowering drugs were older and had higher blood pressure, BMI, and more frequently diabetes and CVD compared to participants without antihypertensive treatment. On the other hand, current smoking was observed more frequently among those not using blood pressure-lowering drugs (Table 1). The participants using blood pressure-lowering drugs had lower eGFR and higher OCR, NAG-Cr, and ACR (Table 2). The average number of blood pressure-lowering drugs in the treated cohort was 2.

Women had lower eGFR and OCR but higher NAG-Cr. Women not using blood pressure-lowering drugs had higher ACR compared to men (Supplementary Table S1).

The correlation coefficient between the log-transformed median ACR and OCR was 0.55. The correlation coefficient between the log-transformed median ACR and NAG-Cr was 0.13. The corresponding correlations between the urinary biomarker concentrations (not adjusted for the urine creatinine concentration) were essentially the same ($p < 0.001$ for all correlations).

The cohort of participants who delivered urine samples in Tromsø 6 and later participated in Tromsø 7 consisted of 5114 participants (Figure 1). During this time period mean serum creatinine increased from 69.2 µmol/L (*SD* 15.1) to 76.2 µmol/L (*SD* 24.0) and accordingly eGFR decreased from 93.8 mL/min/1.73 m^2 (*SD* 14.6) to 80.1 mL/min/1.73 m^2 (*SD* 18.9) (Supplementary Table S2).

Cross-sectional associations

OCR and ACR, but not NAG-Cr, was significantly associated with systolic and diastolic blood pressure in all the crude and multivariable cross-sectional analyses. OCR had a consistently, although marginally, stronger association with blood pressure than ACR in crude and multivariable models. In fully adjusted models with cardiovascular risk factors and all urinary biomarkers included, OCR was the only urine biomarker significantly associated with blood pressure in all groups (i.e. the groups with and without treated hypertension and the entire cohort) (Table 3). Adjustment for eGFR did not affect the standardized regression coefficients in the multivariable models and was therefore excluded from the analyses. OCR exhibited the most pronounced significant

Table 1. Characteristics. Individuals with and without treated HT in the Tromsø 6–2 study.

	Without treated HT		With treated HT		
	($n = 4871$)		($n = 2326$)		*p* Value
Female sex, *n* (%)	2810	(57.6)	1297	(55.9)	0.12
Age, years	61.7	(8.8)	67.5	(8.7)	<0.001
Systolic blood pressure, mmHg	138	(22.3)	147	(22.9)	<0.001
Diastolic blood pressure, mmHg	78	(10.6)	79	(10.6)	<0.001
Body mass index, kg/m^2	26.4	(3.9)	28.5	(4.5)	<0.001
No. blood pressure lowering drugs, *n*	0		2	(1.0–2.0)	
Cholesterol, mmol/L	5.9	(1.1)	5.4	(1.1)	<0.001
HDL cholesterol, mmol/L	1.6	(0.5)	1.5	(0.4)	<0.001
LDL cholesterol, mmol/L	3.8	(0.9)	3.4	(1.0)	<0.001
Triglycerides, mmol/L	1.2	(0.9–1.7)	1.4	(1.1–2.0)	<0.001
HbA1c, %	5.6	(5.4–5.8)	5.8	(5.5–6.1)	<0.001
Current smoking, *n* (%)	976	(20.4)	340	(14.9)	<0.001
Diabetes, *n* (%)	251	(5.4)	397	(18.5)	<0.001
Physically active, *n* (%)	1475	(31.4)	479	(22.0)	<0.001
Self-reported previous cardiovascular event, *n* (%)	129	(2.7)	493	(22.4)	<0.001

($n = 7197$).

Data are mean (*SD*), median (interquartile range) or number (%) as appropriate. Treated HT was defined as use of one or more blood pressure lowering drug. Blood pressure lowering drugs: Drugs containing beta blocking agents, calcium channel blockers, ACE inhibitors, angiotensin II antagonists or diuretics, self-reported. Smoking habits were defined as current smoking or no smoking; the nonsmoking group consisted of never smokers and previous smokers. Diabetes: self-reported diabetes, self-reported use of glucose lowering drugs, HbA1c ≥6.5%, non-fasting glucose ≥11 mmol/L, fasting blood glucose >7.0. Physically active: at least 2 times per week with exercise duration >30 min with sweating and/or breathlessness, self-reported. Cardiovascular event: prior stroke or heart attack.

interactions with sex and age. The age interaction was most prominent for OCR in men, and OCR showed a stronger association with blood pressure in the youngest category (men ≤60 years) (Supplementary Table S3).

The associations of urinary albumin and orosomucoid concentrations with blood pressure did not differ substantially from the corresponding associations with ACR and OCR, respectively (Supplementary Table S4). Orosomucoid had the highest unique variance with blood pressure in the cohort with treated hypertension. Although NAG concentration showed a stronger association with blood pressure than NAG-Cr, urinary NAG concentration was not significantly associated with blood pressure in fully adjusted models and explained the least variance in crude and multivariable models. (Supplementary Table S4).

Incident hypertension in longitudinal analysis

The number of participants with normotension in Tromsø 6 was 2744, among these, 681 participants were classified with incident hypertension in Tromsø 7. OCR and ACR, but not NAG-Cr, were significantly associated with incident hypertension after seven years in crude and multivariable models. The semi-standardized regression coefficients showed that OCR had the marginally strongest association with incident hypertension. In fully adjusted models, OCR was the only urine biomarker with a significant association with incident hypertension. In these models, the difference between the semi-standardized regression coefficients for ACR and OCR increased due to the correlation between these biomarkers and displaying a higher unique variance between OCR and incident hypertension (Table 4 and Figure 2). Adjustment for baseline eGFR did not affect the semi-standardized regression coefficients, and there were no interactions between the biomarker-creatinine ratios and age or sex.

The associations with incident hypertension did not change for albumin or orosomucoid in analysis without adjustment for the urine creatinine concentration, but NAG showed a stronger association with incident hypertension than NAG-Cr. Orosomucoid was the only significant urine biomarker in the fully adjusted model. The difference between the semi-standardized regression coefficients in the fully adjusted model increased, displaying a higher unique variance between orosomucoid and incident hypertension (Supplementary Table S5). Inclusion of baseline eGFR in these models did not alter the standardized regression coefficients.

Progressive hypertension in longitudinal analysis

The number of participants with controlled hypertension in Tromsø 6 was 353, and 193 were classified as having progressive hypertension in Tromsø 7. In the longitudinal analyses of progressive hypertension, only OCR and ACR, but not the NAG-Cr, yielded significant or borderline significant results in crude and multivariable models. OCR and ACR showed equally strong associations with progressive

Table 2. Markers of kidney damage.

	Without treated HT ($n = 4871$)		With treated HT ($n = 2326$)		p Value
Serum creatinine (µmol/L)	68.1	(13.3)	74.5	(22.2)	<0.001
Cystatin C, mg/L	0.82	(0.15)	0.94	(0.25)	<0.001
Estimated GFR (mL/min/1.73 m^2)	94.7	(13.9)	83.7	(18.9)	<0.001
ACR, mg/mmol	0.32	(0.18–0.65)	0.47	(0.23–1.10)	<0.001
OCR, g/g	0.38	(0.21–0.80)	0.62	(0.30–1.7)	<0.001
NAG/Cr, U/g	1.76	(1.05–2.78)	2.02	(1.20–3.12)	<0.001

Individuals with and without treated HT in the Tromsø 6–2 study ($n = 7197$).
Data are mean (*SD*) or median (interquartile range) as appropriate. Treated HT was defined as use of one or more blood pressure lowering drug. Estimated GFR: glomerular filtration rate, calculated using the creatinine-cystatin C based CKD-EPI equation. ACR: urinary albumin to creatinine ratio; OCR: urinary orosomucoid to creatinine ratio; NAG-Cr: urinary N-acetyl-β-D-glucosaminidase to creatinine ratio.

Table 3. Urine biomarkers as ratios with urine creatinine concentration cross sectional analysis of the Tromsø 6–2 Study.

		Crude			Multivariable model			Fully adjusted model		
Groups		Std beta	p	95% CI	Std beta	p	95% CI	Std beta	p	CI
Systolic BP as dependent variable										
All	NAG-Cr	0.01	0.60	(−0.02–0.03)	−0.01	0.23	(−0.04–0.01)	−0.03	0.006	(−0.06 to −0.01)
	ACR	0.18	<0.001	(0.16–0.20)	0.10	<0.001	(0.07–0.12)	0.06	<0.001	(0.04–0.09)
	OCR	0.21	<0.001	(0.18–0.23)	0.11	<0.001	(0.08–0.13)	0.08	<0.001	(0.05–0.11)
Without treated HT	NAG-Cr	−0.01	0.60	(−0.04–0.02)	−0.01	0.47	(−0.04–0.02)	−0.03	0.04	(−0.06 to −0.00)
	ACR	0.18	<0.001	(0.15–0.21)	0.11	<0.001	(0.08–0.14)	0.08	<0.001	(0.05–0.11)
	OCR	0.21	<0.001	(0.18–0.23)	0.11	<0.001	(0.08–0.14)	0.08	<0.001	(0.05–0.11)
With treated HT	NAG-Cr	0.003	0.87	(−0.03–0.04)	−0.02	0.42	(−0.06–0.02)	−0.03	0.12	(−0.07–0.01)
	ACR	0.10	<0.001	(0.06–0.14)	0.08	<0.001	(0.04–0.12)	0.05	0.09	(−0.01–0.10)
	OCR	0.11	<0.001	(0.07–0.15)	0.08	<0.001	(0.04–0.13)	0.06	0.03	(0.01–0.12)
Diastolic BP as dependent variable										
All	NAG-Cr	−0.05	<0.001	(−0.08 to −0.03)	−0.01	0.38	(−0.03–0.01)	− 0.03	0.02	(−0.05 to −0.01)
	ACR	0.07	<0.001	(0.05–0.10)	0.10	<0.001	(0.07–0.12)	0.06	<0.001	(0.03–0.09)
	OCR	0.11	<0.001	(0.08–0.13)	0.11	<0.001	(0.08–0.13)	0.08	<0.001	(0.05–0.11)
Without treated HT	NAG-Cr	−0.06	<0.001	(−0.09 to −0.03)	− 0.01	0.51	(−0.04–0.02)	−0.03	0.04	(−0.06–0.00)
	ACR	0.08	<0.001	(0.05–0.11)	0.11	<0.001	(0.09–0.14)	0.08	<0.001	(0.05–0.11)
	OCR	0.13	<0.001	(0.10–0.16)	0.11	<0.001	(0.08–0.14)	0.08	<0.001	(0.05–0.11)
With treated HT	NAG-Cr	−0.04	0.04	(−0.08 to −0.001)	−0.01	0.69	(−0.05–0.04)	−0.02	0.27	(−0.07–0.02)
	ACR	0.04	0.07	(−0.01 to −0.08)	0.07	0.002	(0.03–0.11)	0.03	0.22	(−0.02–0.09)
	OCR	0.05	0.03	(0.01–0.09)	0.08	<0.001	(0.04–0.13)	0.07	0.01	(0.02–0.13)

Linear regression with standardized beta coefficients for urine biomarkers ($n = 7197$).
Systolic and diastolic blood pressure (BP) as dependent variable (mmHg).
Treated HT: defined as use of one or more blood pressure lowering drug.
CVD risk factors: Sex. Age. BMI. Current smoker. Diabetes. Prior cardiovascular event (stroke or heart attack).
Crude Model: NAG or ACR or OCR.
Multivariable Model: Adjusted for CVD risk factors and NAG, or ACR, or OCR.
Fully Adjusted Model: Adjusted for CVD risk factors and NAG and ACR and OCR.
NAG-Cr: (Log urinary N-acetyl-β-D-glucosaminidase to creatinine ratio per *SD* unit): standardized beta coefficients. *p*-Value and 95% CI.
ACR: (Log urinary albumin to creatinine ratio per *SD* unit): standardized beta coefficients. *p*-Value and 95% CI.
OCR: (Log urinary orosomucoid to creatinine ratio per *SD* unit): standardized beta coefficients. *p*-Value and 95% CI.

hypertension in the multivariable models. However, none of the biomarkers were significantly associated with progressive hypertension in the fully adjusted model (Table 5, Figure 3). Adjustment for baseline eGFR affected the standardized regression coefficients, and eGFR was included in these multivariable models (Supplementary Table S6). There were no interactions between the biomarkers-creatinine ratios and age or sex.

The analyses with progressive hypertension and biomarkers without the urine creatinine ratios yielded no significant associations.

Left censored analysis for ACR

The analyses with ACR left-censored, including participants with urine albumin concentration ≥4 mg/L only, comprised 2553 participants in the cross-sectional analysis with blood pressure, and 752 participants in the longitudinal analysis with incident hypertension, which affected the regression coefficients for ACR in the univariable and multivariable models. The analysis reinforced the previously described pattern with the strongest associations between OCR, blood pressure and incident hypertension (Supplementary Table S7, S8).

Table 4. Urine biomarkers as ratios with urine creatinine concentration.

Longitudinal analysis: Incident hypertension ($n = 2744$).

	Unstandardized beta coefficient (*p*-value)	95% CI for odds ratio			Standardized regression coefficient at mean predicted probability 0.32
		Lower	Odds ratio	Upper	
Crude					
NAG-Cr	0.07 (p 0.48)	0.89	1.07	1.29	0.007
ACR	0.53 (p < 0.001)	1.32	1.69	2.16	0.044
OCR	0.52 (p < 0.001)	1.35	1.68	2.08	0.050
Multivariable Model					
NAG-Cr	0.08 (p 0.45)	0.88	1.08	1.33	0.009
ACR	0.39 (p 0.005)	1.13	1.48	1.95	0.033
OCR	0.36 (p 0.003)	1.13	1.43	1.81	0.034
Fully Adjusted Model					
NAG-Cr	0.03 (p 0.80)	0.84	1.03	1.26	0.003
ACR	0.26 (p 0.08)	0.97	1.29	1.73	0.022
OCR	0.28 (p 0.03)	1.02	1.32	1.7	0.027

Baseline population Tromsø Study 6_2 without blood pressure lowering drug and blood pressure <140/90 (mmHg).
Dependent variable: Incident Hypertension defined as use of one or more blood pressure lowering drug or blood pressure ≥140/90 (mmHg) at T7.
CVD risk factors: Sex, Age, BMI, Current smoker, Diabetes, Prior cardiovascular event (stroke or heart attack).
Crude model: NAG or ACR or OCR.
Multivariable model: Adjusted for CVD risk factors and NAG, or ACR, or OCR.
Fully adjusted model: Adjusted for CVD risk factors and NAG and ACR and OCR.
NAG-Cr: (Log urinary N-acetyl-β-D-glucosaminidase to creatinine ratio per *SD* unit): unstandardized beta coefficients (*p*-value), odds ratio with 95% CI and standardized regression coefficient.
ACR: (Log urinary albumin to creatinine ratio per *SD* unit): unstandardized beta coefficients (*p*-value), odds ratio with 95% CI and standardized regression coefficient.
OCR (Log urinary orosomucoid to creatinine ratio per *SD* unit): unstandardized beta coefficients (*p*-value), odds ratio with 95% CI and standardized regression coefficient.

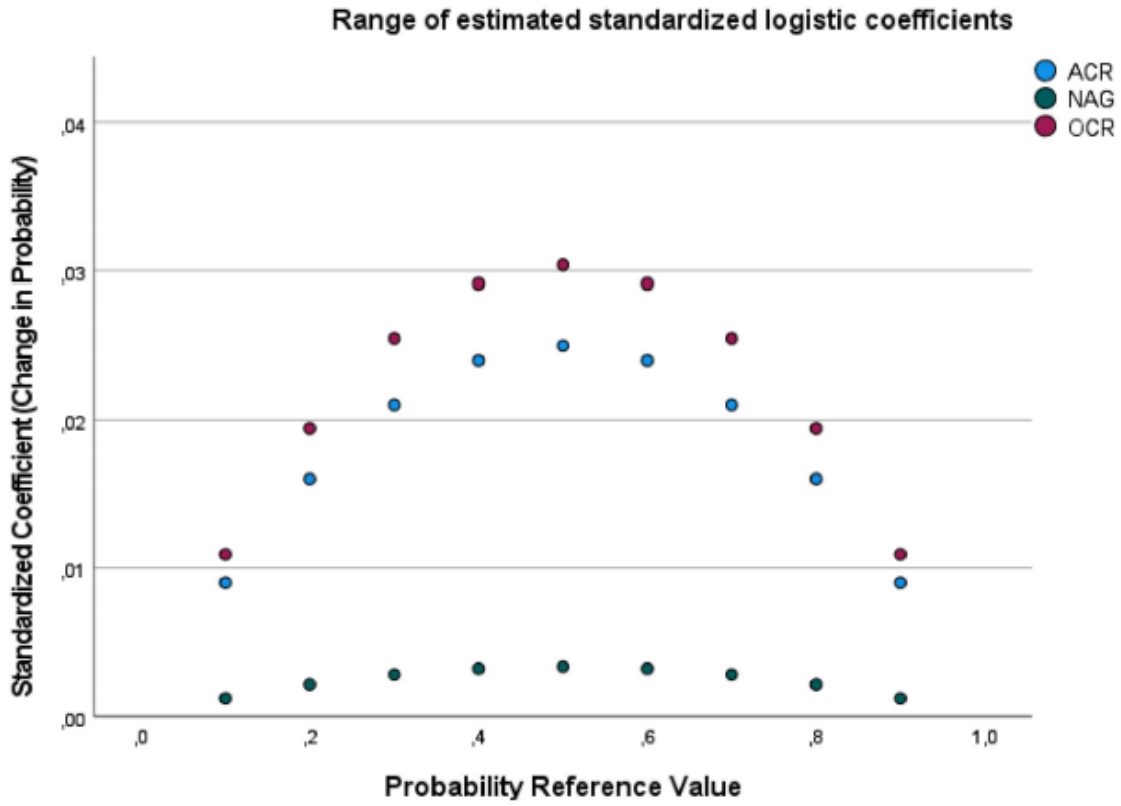

Figure 2. Fully adjusted model without eGFR. Range of change in predicted probabilities in incident hypertension. Figure 2 depicts a range of change in predicted probabilities. A standardized coefficient of 0.01 represents 1% of the maximum change possible in predicted probability of incident hypertension. NAG: (Log urinary N-acetyl-β-D-glucosaminidase to creatinine ratio per SD unit): standardized beta coefficients at different probability reference value; ACR: (Log urinary albumin to creatinine ratio per SD unit): standardized beta coefficients at different probability reference value; OCR: (Log urinary orosomucoid to creatinine ratio per SD unit): standardized beta coefficients at different probability reference value. Fully Adjusted Model: Adjusted for CVD risk factors and NAG and ACR and OCR. CVD risk factors: Sex, Age, BMI, Current smoker, Diabetes, Prior cardiovascular event (stroke or heart attack).

Table 5. Urine biomarkers as ratios with urine creatinine concentration.

Longitudinal analysis: progressive hypertension ($n = 353$).

	Unstandardized beta coefficient (p-value)	95% CI for odds ratio			Standardized regression coefficient. at mean Predicted Probability 0.55
		Lower	Odds ratio	Upper	
Crude					
NAG-Cr	−0.02 (p 0.94)	0.62	0.98	1.56	−0.002
ACR	0.45 (p 0.024)	1.06	1.57	2.32	0.06
OCR	0.38 (p 0.058)	0.99	1.47	2.18	0.05
Multivariable model with eGFR					
NAG-Cr	−0.13 (p 0.06)	0.53	0.88	1.46	−0.014
ACR	0.49 (p 0.048)	1.00	1.63	2.65	0.069
OCR	0.51 (p 0.044)	1.01	1.67	2.74	0.070
Fully adjusted model with eGFR					
NAG-Cr	−0.29 (p 0.28)	0.44	0.75	1.27	−0.033
ACR	0.36 (p 0.22)	0.81	1.43	2.53	0.051
OCR	0.39 (p 0.20)	0.82	1.47	2.64	0.053

Baseline population Tromsø Study 6–2 with ≥ 1 blood pressure lowering drugs and blood pressure <140/90 (mmHg).
Dependent variable: Progressive Hypertension defined as an increase in one or more blood pressure lowering drug and/ or blood pressure ≥140/90 (mmHg) at T7.
CVD risk factors: Sex, Age, BMI, Current smoker, Diabetes, Prior cardiovascular event (stroke or heart attack), eGFR calculated using the creatinine-Cystatin C based CKD-EPI equation.
Crude Model: NAG or ACR or OCR.
Multivariable Model: Adjusted for CVD risk factors and NAG, or ACR, or OCR.
Fully Adjusted Model: Adjusted for CVD risk factors and NAG and ACR and OCR.
NAG-Cr: (Log urinary N-acetyl-β-D-glucosaminidase to creatinine ratio per *SD* unit): unstandardized beta coefficients (p-value), odds ratio with 95% CI and standardized regression coefficient.
ACR: (Log urinary albumin to creatinine ratio per *SD* unit): unstandardized beta coefficients (p-value), odds ratio with 95% CI and standardized regression coefficient.
OCR: (Log urinary orosomucoid to creatinine ratio per *SD* unit): unstandardized beta coefficients (p-value), odds ratio with 95% CI and standardized regression coefficient.

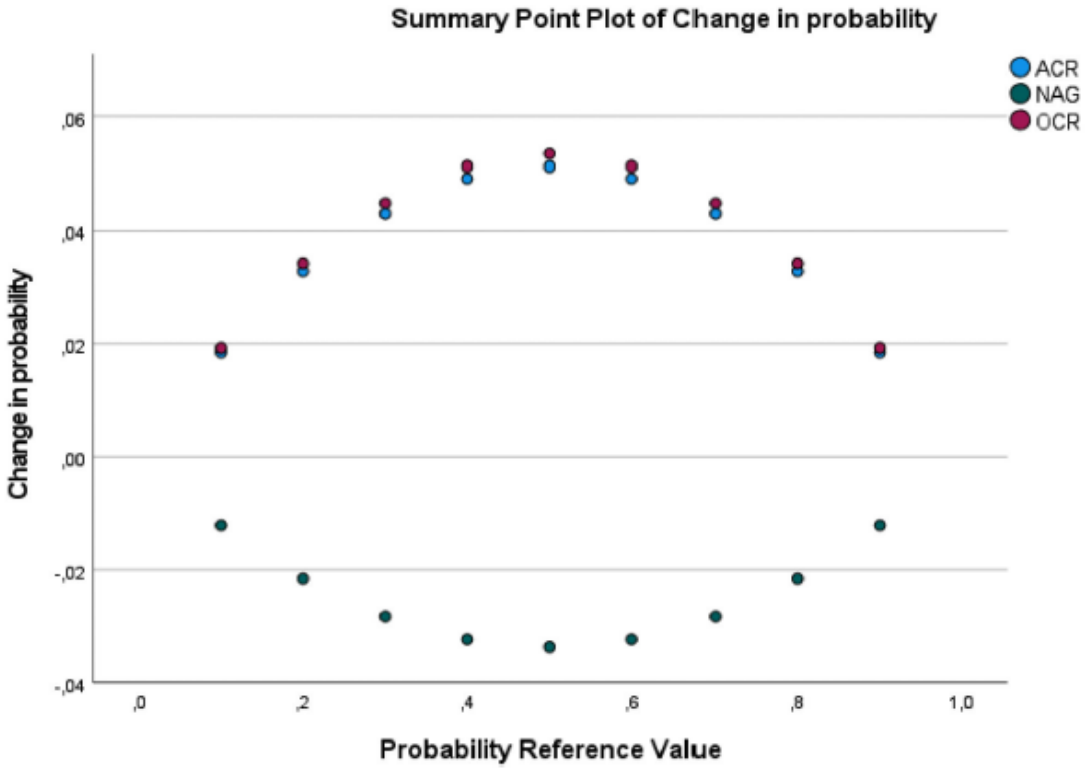

Figure 3. Fully adjusted modell with eGFR. Range of change in predicted probabilities in progressive hypertension. Figure 3 depicts a range of change in predicted probabilities. A standardized coefficient of 0.01 represents 1% of the maximum change possible in predicted probability of progressive hypertension. NAG: (Log urinary N-acetyl-β-D-glucosaminidase to creatinine ratio per SD unit): standardized beta coefficients at different probability reference value; ACR: (Log urinary albumin to creatinine ratio per SD unit): standardized beta coefficients at different probability reference value; OCR: (Log urinary orosomucoid to creatinine ratio per SD unit): standardized beta coefficients at different probability reference value. Fully Adjusted Model: Adjusted for CVD risk factors and NAG and ACR and OCR. CVD risk factors: Sex, Age, BMI, Current smoker, Diabetes, Prior cardiovascular event (stroke or heart attack), eGFR calculated using the creatinine-Cystain C based CKD-EPI equation.

Discussion

The present population-based, cross-sectional and prospective observational study is, according to our knowledge, the first study that aims to compare UAE, urinary orosomucoid and NAG in relation to blood pressure and the development and progression of hypertension. Associations between urinary orosomucoid and urinary NAG with kidney function decline, CKD, and CVD have been described [26,43–48]. Mechanisms for these associations are unclear, and correlation coefficients between UAE and these biomarkers are small to moderate, between 0.1 and 0.5 [26,49,50], and traditional cardiovascular risk factors such as hypertension may modify the associations. In our study, urine orosomucoid excretion had a marginally stronger association than UAE with blood pressure in the cross-sectional analysis and with incident hypertension in the longitudinal analysis. These findings may be of clinical implication because both eGFR and ACR are insensitive biomarkers of early kidney and endothelial damage in hypertension at a stage when prevention may be most effective [4,11,12].

Orosomucoid is a 41–43 kDa acute phase glycoprotein, synthesized mainly by hepatocytes [51], but also produced in endothelial cells [16]. Similar to albumin, it is negatively charged due to the presence of sialic acids [51]. In addition to functioning as a serum transport protein [52,53], it modulates immune and inflammatory responses [54,55], and it is a constituent of the endothelial surface layer and maintains permselectivity [18,19]. A study of plasma orosomucoid, and other inflammation-sensitive plasma proteins, showed a cross-sectional association with blood pressure and a longitudinal association with increased stroke risk [45]. In patients with type 2 diabetes and normal UAE, increased urinary orosomucoid independently predicted cardiovascular mortality [50,56,57]. This relationship is proposed to be caused by inflammation and early endothelial dysfunction [58]. Sun et al found an association between established markers of kidney dysfunction, orosomucoid and hypertension in a prospective study on people with prediabetes [48]. Urine, but not serum orosomucoid, may also be an early and predictive marker for lupus nephritis [22,59], indicating that urine orosomucoid may reflect a pathophysiological process in the kidneys, and not just systemic inflammation. Levels of urinary orosomucoid was significantly higher with active compared to inactive lupus nephritis, both for absolute levels and levels standardized by urine creatinine. Urine orosomucid was however not significantly different between lupus nephritis groups when corrected for nonselective proteinuria [22]. Due to its smaller molecular weight and possibly less efficient tubular reabsorption [60,61], urinary orosomucoid excretion may be a more specific marker of endothelial dysfunction and glomerular damage than UAE [18,19,22]. The pathophysiological mechanisms of orosomucoid in hypertension has not been clarified and we cannot make inferences about causality from observational studies. A cross sectional study of participants in Tromsø 6 second visit showed an association between urinary orosomucoid and diastolic dysfunction and carotid arteriopathy [62]. UAE was not significantly associated with diastolic dysfunction, but the associations with carotid arteriopathy were comparable to UOCR. In our study, urine orosomucoid showed a stronger relationship with blood pressure and the development of hypertension than UAE, but further prospective clinical studies are neede to assess if orosomucoid is a clinically useful biomarker in hypertension.

The proximal tubules are responsible for reabsorbing approximately 65% of filtered load and most low molecular weight proteins [63]. It is hypothesized that subclinical kidney injury, with the development of afferent arteriolopathy and tubulointerstitial disease, especially in the outer medulla, leads to kidney ischemia, inflammation and oxidative stress, which is associated with a decrease in sodium filtration and an increase in blood pressure [4]. Urinary NAG is a marker of proximal tubular cell function and is considered a sensitive marker related to inflammation and oxidative stress [25]. Earlier studies of urinary NAG in blood pressure and hypertension are limited in sample size or in the urine collection method [12,64–67]. A small study of patients with newly diagnosed hypertension without microalbuminuria found a significantly higher NAG activity in hypertensives compared to healthy controls [28]. Further, NAG, but not ACR, was significantly associated with incident hypertension among the HIV-uninfected women in the Women's Interagency HIV Study [64]. In this general population cohort, consisting of participants with overall normal kidney function, urinary NAG yielded only weak or non-significant associations with blood pressure and hypertension in our multivariable models with CVD risk factors.

Further, the limit of detection for the albumin concentration assay corresponded almost to the population's median albumin concentration, similarly to other studies [8–11]. Orosomucoid was analyzed by a highly sensitive immunoassay with a limit of detection

below the lowest measured value in our population [35]. Additional recommended analyses with ACR left-censored [8,35] reinforced the described pattern of associations between biomarkers, blood pressure and incident hypertension. In the left-censored analyses, OCR had the strongest association with blood pressure in treated and untreated subjects. Further, only OCR had significant associations with incident hypertension in the additional analysis.

The best measure of biomarker excretions in urine is likely to vary for glomerular and tubular biomarkers [26,68]. We used morning urine samples collected on three consecutive days. We reduced the impact of a day-to-day variation using the median values of the three specimens. There is a high correlation between 24-h urine albumin excretion and ACR [69,70]. Whether this correlation also applies to OCR is unknown. Timed urine collections may be problematic due to imprecision in timing and volume of the collections, especially in outpatients settings. But tubular biomarker excretion in urine may be more accurately estimated with timed collections than in spot urine samples. The excretion of a tubular biomarker may not correlate with the amount of filtered creatinine. The timed collections may further be expressed with ratios of urine creatinine to adjust for varying urine concentration and dilution [71]. To study the effect of the urine creatinine concentration, we analyzed the relationship between the biomarkers, blood pressure and hypertension both with and without adjustment for urine creatinine. The associations of urinary albumin and orosomucoid concentrations with blood pressure and incident hypertension did not differ substantially from the corresponding associations with ACR and OCR. NAG showed a stronger association with blood pressure and incident hypertension than NAG-Cr. The analysis without adjustment for urine creatinine did not affect the assessment of the strength of these associations compared to urinary albumin excretion (UAE). The functioning nephron mass may be another factor altering the urine biomarker excretion [72]. However, in our study, adjusting for eGFR did not change associations with blood pressure and hypertension for most analyses. The relatively healthy study population with a narrow distribution of eGFR may explain this.

Participants with the highest blood pressure at baseline, or the largest increase in blood pressure during follow-up, would be most likely to increase their antihypertensive treatment. To take this into account, we dichotomized blood pressure into incident hypertension and progressive hypertension in the longitudinal analysis, which led to a loss of statistical power.

The strengths of the present study includes the combination of a cross-sectional and prospective design, the inclusion of a large, well described cohort with a high attendance rate, and an extended follow-up. Moreover, using three urinary samples per participants made the estimation of the biomarkers more robust. However, there are several limitations. The population included in the Tromsø Study consists almost exclusively of North-European middle-aged and elderly persons, limiting broad generalizations. Information about drug dosing or adherence to prescribed hypertension treatment was lacking, which may bias the results, particularly in the analysis assessing progressive hypertension. We were unable to obtain information regarding detection limits for the NAG measurements. Finally, this study did not address the associations between the urine biomarkers and clinically relevant endpoints in hypertension.

Conclusions

In a cohort from the general population, urine orosomucoid excretion showed a stronger cross-sectional association with blood pressure in treated and untreated individuals and a stronger association with incident hypertension after 7 years than UAE. Urine orosomucoid excretion may be an earlier biomarker than UAE for kidney and endothelial damage in hypertension. However, further studies are needed to determine the pathophysiological mechanisms and the clinical value of orosomucoid as a biomarker in hypertension. The tubulointerstitial function is essential in blood pressure regulation, and tubulointerstitial damage appears early in CKD. However, we found only varying and weak associations between U-NAG, blood pressure and hypertension. The observational design limits suggestions about cause and effect and further prospective studies should be undertaken.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Appendix

Appendix

Table of contents

Abbreviations

ADAMTS-13	A disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13
CVD	Cardiovascular disease
CKD	Chronic kidney disease
sTNF	Circulating TNF
EGF	Epidermal growth factor
ENaC	Epithelial sodium channels
eGFR	Estimated glomerular filtration rate
GFR	Glomerular filtration rate
HMOD	Hypertension mediated organ damage
IL-1ß	Interleukin 1-beta
IL-1RA	Interleukin 1 receptor antagonist
IL-18	Interleukin-18
mTNF	Membrane bound tumor necrosis factor
MCP-1	Monocyte Chemoattractant Protein-1
NAG	N-acetyl-β-D-glucosaminidase
NGAL	Neutrophil Gelatinase-Associated Lipocalin
NLRP3	NOD-like receptors protein 3 inflammasome
NF-κB	Nuclear factor-kappa B
OPN	Osteopontin
RANTES	Regulated upon Activation Normal T-cell Expressed and Secreted
RNA	Ribonucleic acid
TNF	Tumour Necrosis Factor
TNFR1, CD120a, p55	TNF receptor 1
TNFR2, CD120b, p75	TNF receptor 2
UAE	Urinary albumin excretion
ACR	Urine albumin-to-creatinine ratio
Na/K-ratio	Urine sodium-potassium ratio
vWF-A2	Von Willebrand Factor A2

S1 Biomarkers included in the thesis

S1.1 Novel biomarkers of kidney damage in hypertension

S1.1.1 Urinary N-acetyl-β-D-glucosaminidase (NAG)

NAG is a lysosomal enzyme that, in the kidney, is found predominantly in lysosomes of proximal tubular cells. The NAG present in the urine is secreted from proximal tubular cells by exocytosis, and NAG is thus exclusively a marker of tubular cell function [1]. Increased urinary NAG is considered a sensitive tubular injury marker related to inflammation and oxidative stress [1]. Myocardial infarction, ischemic stroke, and mortality in the general population are associated with NAG levels [2]. A small prospective study of residents of a farming community in Japan showed an association between elevated serum NAG activity and the development of hypertension [3]. Among patients with CKD, risk prediction after adjusting for both ACR and eGFR did not improve with the addition of urinary NAG [4]. However, there are no major studies on the use of urinary NAG as a biomarker in hypertension, and NAG may be an early marker of proximal tubular dysfunction in hypertension [5-7].

S1.1.2 Uromodulin (Tamm-Horsfall protein)

Uromodulin is a glycoprotein produced in the tubular cells of the thick ascending limb and the early distal tubule and released into the blood as monomers and urine where it polymerizes [8, 9]. Reduced serum concentrations of uromodulin are found in persons with interstitial fibrosis or tubular atrophy [10, 11]. Plasma uromodulin is a suggested marker of intact tubular cells and may be used as a marker for the number of remaining functional nephrons/mass of functional kidney tissue [12-15]. Low serum uromodulin concentrations can signify early kidney injury, even when serum creatinine levels are within the normal range [12]. Low serum uromodulin has been estimated to outperform that from urine, and may be useful in stratifying patients at risk for CKD [9, 11, 12, 14, 16]. In high-risk patients with hypertension

and CKD, higher urine uromodulin was associated with slower eGFR decline and lower CVD risk, and the association with eGFR decline was weakened by intensive blood pressure control [17, 18]. Storage conditions, centrifugation and vortexing may adversely affect the stability of uromodulin in urine samples. In contrast, monomeric serum uromodulin appears to be stable over weeks, even at increased temperatures [19, 20].

S1.1.3 Neutrophil Gelatinase-Associated Lipocalin (NGAL)

NGAL is upregulated in injured kidney epithelial cells and a is marker of tubular damage [21]. NGAL may be an augmenting factor for kidney interstitial fibrosis [22-24]. In a general population cohort with ten years of follow-up, plasma NGAL added to the Framingham risk score, improved c-statistics for all-cause mortality and major adverse cardiovascular events, and correctly reclassified ≈15% into more appropriate risk groups [25]. In a cohort of adult patients with CKD stages 3–4, higher plasma NGAL was independently associated with a greater risk for end-stage kidney disease but not cardiovascular events or death [26]. Plasma NGAL has been shown to be stable after frozen storage and after up to three freeze-thaw cycles [27]

S1.1.4 Epidermal growth factor (EGF)

EGF is considered a marker of functional tubular mass and regeneration potential, and lower urinary EGF levels are associated with an increased risk of rapid GFR loss and incident CKD in populations without pre-existing CKD or diabetes [28-30]. Messenger RNA coding for EGF is expressed along the distal nephron [30]. A diet high in sodium and low in potassium can stimulate salt reabsorption along the distal nephron [31]. In animal studies, EGF has been shown to regulate epithelial sodium channels- (ENaC-) mediated sodium transport in the distal part of the kidney tubule [32]. An increased renal cortical expression of the EGF-receptor has been found in salt-sensitive prehypertensive and hypertensive rats [33]. However, it is unknown whether this expression is a cause or a consequence of salt-sensitive

hypertension [34]. Although increased tubular sodium reabsorption is generally accepted as one of the crucial steps in the genesis of essential hypertension, the renal abnormalities leading to this increased reabsorption have not yet been fully elucidated [35]. Moreover, salt restriction slows the deterioration of kidney function in patients with CKD, and low dietary consumption may also attenuate the age-related rise in blood pressure [36-38]. This suggests that dietary salt may play a significant role in the age-dependent decline in kidney function and age-dependent increase in blood pressure in healthy individuals [28]. In a prospective cohort study of young adults (mean age, 45 years) without hypertension, cardiovascular disease, or kidney disease at baseline, higher urine EGF was associated with lower risk of incident hypertension and lower 10-year blood pressure elevations [39]. Intrarenal EGF may be a potential molecular mechanism that may underlie kidney dysfunction and salt sensitivity [28, 39]. Urinary EGF appears to be stable after prolonged frozen storage and after freeze–thaw cycles [40, 41]

S1.2 Novel biomarkers of vascular and endothelial damage in hypertension.

S1.2.1 Urinary orosomucoid

Like urinary albumin excretion (UAE), urinary orosomucoid (α-1-Acid glycoprotein) is considered a marker of general endothelial dysfunction and a damaged glomerular filtration barrier [42, 43]. Orosomucoid is a 41-43-kDa acute phase glycoprotein synthesised mainly by hepatocytes [44], but it can also be produced in endothelial cells [45]. It is a serum transport protein [46, 47], it modulates immune and inflammatory responses [48-50] and is a constituent of the endothelial surface layer and maintains permeability selectivity [42, 51]. Damage to this layer is considered a direct cause of UAE and atherosclerosis. Urinary orosomucoid excretion has been associated with generalized endothelial dysfunction and

atherosclerosis among patients with diabetes [43, 52]. Cross-sectional association between orosomucoid and kidney function has previously been reported among patients with type-2 diabetes and sick-cell disease [53, 54]. In patients with type 2 diabetes and normal UAE, increased urinary orosomucoid independently predicted cardiovascular mortality [52, 55, 56]. An association between established markers of kidney dysfunction, orosomucoid and hypertension was found in a prospective study on people with prediabetes [57]. In a longitudinal study elevated levels of orosomucoid were associated with increased occurrence of carotid plaque and increased incidence of ischemic stroke [58]. Urinary orosomucoid appears to be stable after frozen storage and after freeze-thaw cycles [59, 60].

S1.2.2 von Willebrand Factor A2 (vWF-A2)

The glycoprotein vWF-A2 is an endothelial ligand for platelet glycoproteins and a suggested marker for endothelial injury and activation [61-64]. Inflammatory cytokines from macrophages activate endothelial cells to release vWF, leading to increased circulating levels [64]. The accumulation of platelets and vWF within capillaries, arterioles, and postcapillary venules represents a pivotal process that plays a crucial role in endothelial cell activation, leukocyte recruitment, trans-endothelial migration, tissue inflammation and subsequent injury to target organs. This phenomenon might serve as a unifying step, connecting various pathological events and contributing to the progression of diverse vascular disorders [65]. The released ultra-large, highly reactive vWF multimers can be reduced in size and reactivity via proteolytic cleavage at the A2 domain by a disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13 (ADAMTS-13) [66]. Plasma vWF-A2 reflects the amount of circulating protein but does not represent its functional status [67]. Higher plasma levels of vWF have been associated with hypertension [65, 68, 69]. Studies have been conflicting on the association between vWF levels and the risk of CVD [67, 70]. In cross-sectional studies, higher plasma levels of vWF have also been associated with CKD, and

longitudinally with increased mortality in CKD [71-76]. Plasma vWF appears to be stable after frozen storage and after freeze–thaw analysis [77, 78].

S1.3 Novel biomarkers of inflammation in hypertension

S1.3.1 Interleukin 1 receptor antagonist (IL-1RA)

The cytokine IL-RA inhibits and modulates various interleukin 1-related immune and inflammatory responses [79-82]. IL-1RA concentration indicates immune activation rather than a net anti-inflammatory state [83]. IL-1RA has been associated with hypertension, CVD and CKD [84-87], possibly through modulation of the renin-angiotensin system [81]. IL-1 antagonism with human recombinant IL-1RA (anakinra) led to a decrease in systolic blood pressure in two interventional trials in obese patients with features of the metabolic syndrome [80, 88]. IL-1RA may also modulate hypertensive kidney and vascular disease [82, 89, 90]. Plasma IL-1RA appears to be stable after prolonged frozen storage and after freeze-thaw analysis [41, 91]

S1.3.2 Interleukin-18 (IL-18)

The cytokine IL-18 is a pro-inflammatory cytokine cleaved into its active form by the NOD-like receptors protein 3 inflammasome (NLRP3) upon sensing damage or pathogenic signals [92]. IL-18 has been associated with hypertension and may be important for promoting renal and vascular inflammation and remodeling [93, 94]. An association between higher levels of circulating IL-18 and CVD have been found in longitudinal studies on general populations [95, 96]. In high-risk patients with hypertension and eGFR<60 ml/min/1.73m^2 at baseline, IL-18 was associated with increased risk for CKD progression [97]. Plasma IL-18 appears to be stable after prolonged frozen storage and after freeze-thaw analysis [41, 91]

S1.3.3 Tumour Necrosis Factor (TNF)

TNF is an important activator of the NLRP3 inflammasome involving nuclear factor-kappa B (NF-κB)-mediated up-regulation of NLRP3, pro-IL-1β and pro-IL-18 gene expression [92]. The TNF-activated NF-κB pathway is a master regulator of the inflammatory response [82]. TNF has key roles in proinflammatory cytokine regulation, expression of inflammation genes, oxidative stress, and antiapoptotic signaling pathways in virtually all types of cells [98, 99]. Serum TNF has been associated with hypertension and hypertension mediated organ damage (HMOD) [100-105], and circulating levels are affected by renin-angiotensin-aldosterone system blockade [106]. However, results have been conflicting [84, 102, 105, 107]. TNF can be bound to the cell membrane (mTNF) or found as a circulating ligand (sTNF) for two receptors, namely TNF receptor 1 (TNFR1, CD120a, p55) and TNF receptor 2 (TNFR2, CD120b, p75). The receptors are located on the cell membrane, but can also be shed and released in soluble forms with the ability to bind and neutralize the activity of sTNF [105]. The two receptors can activate different cell responses. TNFR1 can initiate caspase-mediated apoptosis and necroptosis. TNFR2 activation typically promotes cell proliferation and survival. However, the integrated activity of the TNFR1-TNFR2 signaling also depends on the combined effect with each other, and with other complex signaling systems [105, 108]. Plasma TNF appears to be stable after prolonged frozen storage and after freeze–thaw analysis [41, 91]

S1.3.4 Monocyte Chemoattractant Protein-1 (MCP-1)

Activation of the NLRP3 inflammasome with the secretion of IL-1ß synergizes with angiotensin-II to promote NF-kB activation and a proinflammatory response characterized by increased MCP-1 production [109, 110]. MCP-1 is a chemotactic protein for monocytes and macrophages, implicated in tissue repair and fibrosis [111]. Serum and plasma MCP-1 levels have been associated with hypertension, reduced GFR and albuminuria, and are suggested

markers of tubulointerstitial inflammation and fibrotic activity [13, 111, 112]. However, results have been conflicting [113, 114]. In high-risk patients with hypertension and eGFR<60 ml/min/1.73m^2 at baseline higher urine MCP-1 was associated with increased risk for CKD progression [97]. In a meta-analysis of population-based prospective cohort studies including over 17000 individuals with a mean follow-up time of 16 years, baseline MCP-1 was associated with increased risk of ischemic stroke after adjustment for cardiovascular risk factors [115]. Plasma MCP-1 appears to be stable after prolonged frozen storage and after freeze-thaw analysis [41, 91]

S1.3.5 Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES)

RANTES is a chemotactic molecule for monocytes and T cells [116]. RANTES is produced by several cells implicated in the development of hypertension, including vascular endothelium, smooth muscle cells, perivascular adipocytes and renal epithelial cells [117, 118]. RANTES may regulate migration and infiltration of monocytes/macrophages and T cells in hypertension [116]. Higher plasma RANTES levels have been associated with hypertension, and studies have found significant correlations between circulating RANTES and vascular function [119-121]. However, conflicting results exist regarding RANTES' role in CKD development and CVD [117, 122-124]. Plasma RANTES appears to be stable after prolonged frozen storage and after freeze-thaw analysis [41, 125]

S1.3.6 Osteopontin (OPN)

OPN is a non-structural extracellular matrix protein capable of interacting with cell surface receptors, growth factors, cytokines as well as structural matrix proteins [126, 127]. Pro-inflammatory stimuli and activation of the NLRP3 inflammasome leads to increased tissue levels of OPN. OPN is expressed in activated macrophages, T cells, smooth muscle, endothelial and epithelial cells and regulates cell adhesion, migration, and proliferation and

promotes macrophage and T cell infiltration [126, 128, 129]. OPN has been associated with hypertension, HMOD and CVD [130-138]. Humans express multiple isoforms of OPN with different functional effects, and it is unresolved whether individual OPN isoforms or total OPN levels are useful biomarkers in hypertension [126, 128]. Plasma OPN appears to be stable after prolonged frozen storage and after freeze-thaw analysis [139, 140]

S1.4 Urinary sodium-potassium ratio (Na/K-ratio)

A high sodium intake in the general population is associated with hypertension, CKD and CVD [141, 142]. Sodium restriction has a blood pressure lowering effect [143]. High potassium intake is associated with lower blood pressure and reduced risk of CVD [144, 145]. Replacing salt with a potassium chloride salt substitute has reduced the risk of stroke and cardiovascular disease mortality [146, 147]. The Na/K-ratio is a proxy for sodium and potassium intake [148, 149]. Despite the central role of the kidney in regulating sodium excretion to match sodium intake, recent research indicates that potassium can counteract the adverse vascular effects of excessive sodium intake by influencing the kidney's sodium handling [31, 150]. High sodium and low potassium consumption may also disrupt the endothelial glycocalyx barrier, induce endothelial stiffening, and trigger endothelial dysfunction leading to increasing blood pressure [151-153]. Further, a diet high in sodium and low in potassium may cause kidney glomerular and tubulointerstitial injury, deviant salt handling and salt-sensitive hypertension [154, 155].

S2 Standardized coefficients in logistic regression

In multiple regression each of the unstandardized coefficients is multiplied by the standard deviation of each of the respective independent variables, and divided by the standard deviation of the dependent variable. In logistic regression, the calculation of standardized coefficients is complicated by the fact the dependent variable is the probability, expressed as

the natural logarithm of the odds, of being in one as opposed to another category, the Logit(Y). The variance in the dependent variable will vary with different values of the independent variables, and the values for Logit(0) and Logit(1) go towards infinity and does not permit the calculations of means and standard deviations for the Logit(Y) [156]. In paper 1 we reported the semi-standardized regression coefficients from the Kaufman formula for the logistic regression analysis [157, 158]. The semi-standardized coefficient given by the Kaufman formula measures the change in predicted probability, that the dependent variable has one or the other of its possible values, associated with a one standard deviation change in the predictor and is restricted to the interval -1 to 1, in the context of a selected reference predicted probability analysis [157, 158]. The semi-standardized regression coefficients were calculated at the mean predicted probability for the outcome in the population for the two longitudinal analyses. A range of estimated semi-standardized regression coefficients for different probability reference values was calculated and displayed graphically for the fully adjusted models [157, 158].

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