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Prevalence, diagnosis, and pathophysiology

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Norwegian University of Science and Technology
Thesis for the Degree of
Philosophiae Doctor
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1 NORSK SAMMENDRAG

Translasjonsmedisinske studier av hypertensiv nefropati: Prevalens, diagnose og patofysiologi

Kronisk nyresjukdom defineres som nedsatt nyrefunksjon (estimert glomerulær filtrasjonsrate (eGFR) <60 mL/min/1,73m^2), og/eller avvik i urinfunn (proteinuri, hematuri) eller bildediagnostikk av nyrene, som varer i mer enn 3 måneder. Kronisk nyresjukdom finnes hos rundt én av ti voksne i industrialiserte land. Tilstanden utgjør en byrde på samfunn og helsevesen, spesielt på grunn av den økte risikoen for hjerte-/karsjukdom og slag som følger med kronisk nyresjukdom. Tidlig diagnose og behandling, med vekt på blant annet blodtrykk, proteinlekkasje i urin og kolesterol, bremser forverringa av nyrefunksjonen. Dagens diagnoseverktøy med blod- og urinprøver gir utslag først når nyrefunksjonen er moderat redusert, og nyresjukdommen er etablert. Vi trenger bedre diagnoseredskaper for å finne de som har tidlig nyresjukdom, og skille ut de som gradvis forverres til endestadium nyresvikt. Vi trenger også mer kunnskap om hvilke sjukdomsmekanismer som er viktigst i utviklinga av nyresvikt, for å kunne finne nye behandlinger.

Den vanligste årsaken til endestadium nyresvikt i Norge er nyresjukdom på grunn av høgt blodtrykk, såkalt hypertensiv nefropati. Etter sukkersyke er det den vanligste årsaken til endestadium nyresvikt i industrialiserte land. Sjøl om tilstanden er godt kjent, er det paradoksalt nok debatt rundt både definisjonen, utbredelsen, årsakene og de grunnleggende sjukdomsmekanismene bak sjukdommen.

Hypertensiv nefropati har ofte vært antatt årsak til kronisk nyresjukdom hos individer med *langvarig høgt blodtrykk*, kun *sparsomme urinfunn* (lite blod eller protein i urinen), og som ikke har tegn til andre sjukdommer som kan gi nyreskade (for eksempel sukkersyke, cystenyrer eller kronisk nyrebetennelse/glomerulonefritt). Hos disse har man tidligere ofte antatt diagnosen hypertensiv nefropati uten å ta vevsprøve fra nyrene (nyrebiopsi) for bekreftelse. Det har vært omdiskutert hvor presise disse kliniske kriteriene er. Dette har vært undersøkt hos afrikansk-amerikanere og i enkelte andre etniske grupper, men ikke så godt hos hvite europeere.

I artikkel 2 gjennomgikk vi 4920 nyrebiopserte pasienter fra Norsk nyreregister, hvorav 918 hadde biopsi-bekreftet hypertensiv nefropati. Blant de 918 hadde mange urinfunn, hvorav 34 % hadde blod i urinen og 57 % hadde protein i urinen. Vi fant at de tradisjonelle kliniske diagnosekriteriene var relativt upresise (sensitivitet 0,12, spesifisitet 0,96), og at mens fravær av disse kriteriene utelukket diagnosen relativt presist (negativ prediktiv verdi 0,83), klarte ikke disse kriteriene, de gangene de var tilstede, å forutsi diagnosen presist (positiv prediktiv verdi 0,41). Kriteriene som var sterkest assosiert med biopsi-bekreftet hypertensive nefropati var høg

alder, høgt diastolisk blodtrykk, lav proteinuri, fravær av blod i urin, hankjønn og fravær av sukkersyke. Vi fant at de kliniske kriteriene var mest presise hvis man brukte alder >50 år, proteinuri <1 gram/døgn, diastolisk blodtrykk >90 mmHg, og ingen blod i urinen.

I artikkel 1 beskreiv vi forekomsten av kronisk nyresjukdom i Norge på to tidspunkter med ti års mellomrom ved hjelp av tall fra tverrsnittsundersøkelsene HUNT2 i 1995-97 og HUNT3 i 2006-08. Hovedfunnet var at forekomsten av kronisk nyresjukdom holdt seg stabil på rundt 11 %. I denne perioden fant det sted en klar reduksjon av høgt blodtrykk, noe reduksjon av kolesterol og økt fysisk aktivitet, samt moderate økninger i både sukkersyke og overvekt. Vi tror at blodtrykksreduksjonen har bidratt til at nyresjukdom-tallene har holdt seg stabile, på tross av økt sukkersyke og overvekt.

I artikkel 3 undersøkte vi proteiner og peptider i urinen hos pasienter med langtkommen nyresjukdom av forskjellige typer, og fant at et sett av 273 forskjellige urin-proteiner og peptider skiller mellom nyresjuke og –friske. Urin-proteinene som var mest forskjellige, peker i retning mot forstyrrelser i bindevev- og arrvevsproduksjonen hos de nyresjuke.

I artikkel 4 viste kombinerte gen- og urin-analyser at individer med hypertensiv nefropati har endringer innen flere områder av metabolismen eller stoffomsetninga, med spesielt redusert utskillelse i urin av flere typer aminosyrer sammenliknet med friske. Disse forskjellene peker mot forstyrrelser av blodtrykksregulering, åreforkalkning, arrdannelse/fibrose og oksidativt stress, som er kjente mekanismer i dannelsen av hypertensiv nefropati og nyresjukdom generelt.

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Biveileder: Professor, ph.d. Per Bruheim, NTNU

2 ENGLISH SUMMARY

A translational medicine approach to hypertensive nephropathy: prevalence, diagnosis, and pathophysiology

Chronic kidney disease is defined as reduced kidney function (estimated glomerular filtration rate (eGFR) < 60 mL/min/1,73m^2), and/or pathological findings in urine (proteinuria, hematuria) or diagnostic imaging of the kidneys, lasting more than three months. Chronic kidney disease is found in one out of ten adults in industrialized countries, and represents a burden on society and health care systems, especially because of the increased risk of cardiovascular disease and stroke that comes with chronic kidney disease. Early identification and treatment, with focus on blood pressure, proteinuria and cholesterol, reduce the progression rate of chronic kidney disease. Today's diagnostic tools with blood and urine tests indicate disease only when the kidney function is already moderately reduced, and the kidney disease is established. We need better diagnostic tools to find early stages of chronic kidney disease, and single out those who will progress to end stage renal disease. We also need more knowledge on which disease mechanisms are at play in the development of renal failure, in order to find new treatments.

Chronic kidney disease due to hypertension, or hypertensive nephropathy, is the most common cause of end stage renal disease in Norway, and is second only to diabetes mellitus as cause of end stage renal disease in industrialized countries. Although it is a well known entity, there has been debate over its definition, prevalence, causes, and which are the basic pathophysiological mechanisms behind the disease.

Hypertensive nephropathy has often been the assumed cause of chronic kidney disease in individuals with long-standing hypertension, and only *sparse urinary findings* (little hematuria or proteinuria), and with no signs of other diseases that may cause renal damage (e.g. diabetes, polycystic kidney disease, or glomerulonephritis). The precision of these clinical criteria in identifying hypertensive nephropathy has been debated, and examined in African Americans and in certain other ethnic groups, but not extensively in Caucasian Europeans. In paper 2 we examined 4920 kidney-biopsied patients from the Norwegian Renal Registry, of which 918 had biopsy-proven hypertensive nephropathy. Among those 918 many had urinary findings: 34% had hematuria and 57% had proteinuria. We found that traditional clinical diagnostic criteria were relatively imprecise (sensitivity 0.12, specificity 0.96), and that while absence of these criteria excluded the diagnosis with relative precision (negative predictive value 0.83), the criteria, when present, were not able to accurately predict the diagnosis (positive predictive value 0.41). The criteria most strongly associated with biopsy-proven hypertensive nephropathy

were high age, high diastolic blood pressure, low proteinuria, absence of hematuria, male sex and no diabetes. We found that the clinical criteria were most accurate if the following cut-offs were used: age > 50 years, proteinuria < 1 gram/day, diastolic blood pressure > 90 mmHg, and no hematuria.

In paper 1 we described the prevalence of chronic kidney disease in Norway at two time points ten years apart by means of the cross-sectional studies HUNT2 in 1995-97 and HUNT3 in 2006-08. The main finding was that the prevalence of chronic kidney disease was stable at around 11%. During these years, a marked reduction in blood pressure took place, with moderate reductions of cholesterol and increased physical activity, as well as moderate increases in the prevalence of both diabetes and obesity.

In paper 3 we examined urinary proteins and peptides in patients with advanced kidney disease of many different etiologies, and found that a set of 273 different urinary proteins and peptides distinguish between healthy controls and chronic kidney disease. The urinary proteins with the greatest difference between the two groups point towards perturbations of fibrosis in those with kidney disease.

In paper 4 combined genetic and urine metabolomics analyses showed that individuals with hypertensive nephropathy exhibit perturbations of several facets of metabolism, with particularly reduced urinary excretion of several amino acids compared to healthy controls. These differences point towards perturbations in blood pressure regulation, atherosclerosis, fibrosis, and oxidative stress, which are familiar mechanisms in the pathophysiology of hypertensive nephropathy and chronic kidney disease in general.

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4 CONTENTS

5 ABBREVIATIONS

ACR	albumin:creatinine ratio
CKD	chronic kidney disease
CKD-EPI	Chronic Kidney Disease – Epidemiology Collaboration
DN	diabetic nephropathy
ESRD	end-stage renal disease
EI	electron ionization
GC-MS	gas chromatography - mass spectrometry
GFR	glomerular filtration rate
HD	hemodialysis
HN	hypertensive nephropathy
LC-MS	liquid chromatography - mass spectrometry
MDRD	Modification of Diet in Renal Disease
MS	mass spectrometry
m/z	mass to charge ratio
NMR	nuclear magnetic resonance
PCA	principal component analysis
PLS-DA	partial least squares – discriminant analysis
QC	quality control
Q-TOF	quadrupole time-of-flight
RAAS	renin-angiotensin-aldosterone system
RF	random forest
ROS	reactive oxygen species
SVM	support vector machine

6 LIST OF PAPERS

Paper 1

Hallan SI, Øvrehus MA, Romundstad S, Rifkin D, Langhammer A, Stevens PE, Ix JH. Long-term trends in the prevalence of chronic kidney disease and the influence of cardiovascular risk factors in Norway. *Kidney International*. 2016 Sep;90(3):665-73.

Paper 2

Øvrehus MA, Oldereid TS, Dadfar A, Bjørneklett R, Aasarød K, Hallan SI. Hypertensive nephrosclerosis in whites: clinical phenotypes, long-term prognosis, and diagnosis in the general population and the nephrology clinic (manuscript)

Paper 3

Øvrehus MA, Zürbig P, Vikse BE, Hallan SI. Urinary proteomics in chronic kidney disease: diagnosis and risk progression beyond albuminuria. *Clin Proteomics*. 2015 Aug 7;12(1):21.

Paper 4

Øvrehus MA, Bruheim P, Ju W, Zelnic L, Darshi M, Rise Langlo KA, Sharma K, de Boer I, Hallan SI. Gene expression studies and targeted metabolomics reveal disturbed serine, methionine, and tyrosine metabolism in early hypertensive nephrosclerosis (in press, *Kidney International Reports*, 2018)

7 INTRODUCTION

Chronic kidney disease affects an estimated 10 to 12% of the adult population in industrialized countries, and carries with it a large increase in cardiovascular morbidity and mortality, even in early stages (3, 4). This makes chronic kidney disease a heavy burden on public health (5, 6). Patients that progress to end-stage renal disease and require transplantation and particularly dialysis face costly treatments, reduced quality of life, and increased cardiovascular mortality (7, 8). Early recognition and nephrologist care has been shown to reduce loss of kidney function and increase life expectancy in patients with chronic kidney disease (9, 10). Today, kidney function is most often evaluated using serum creatinine-based estimations of glomerular filtration rate (eGFR) in combination with urine analysis, especially proteinuria. A central problem is that the most commonly used diagnostic test for chronic kidney disease, serum creatinine, only shows pathological values after a substantial part of the functional kidney tissue has already been damaged (11). Also, serum creatinine is imprecise in early stage CKD, in the elderly and in persons with very high body mass index (12, 13). It may also be affected by several non-renal factors, such as diet, drugs, muscle mass and laboratory measurement methods (14, 15). A combination of urinary albumin-to-creatinine ratio and serum creatinine-based eGFR has been the recommended biomarker for diagnosis, staging and prognosis by the Kidney Disease Improving Global Outcomes (KDIGO) group (1), and is widely regarded as the most precise diagnostic and prognostic marker in chronic kidney disease. However, there is significant within-subject variation of 20-50% in albumin excretion from day to day due to factors such as inflammation, exercise, upright posture, fever etc (16, 17), so repeated samples are recommended (1). Although an early marker with a graded response with increasing disease severity, albuminuria may be found only after a certain kidney damage has been established. Albuminuria is an excellent biomarker in for example diabetic nephropathy, but is not always present in all types of kidney disease, such as hypertensive nephropathy or tubulointerstitial diseases. Given the inherent weaknesses of creatinine-based GFR estimations and albuminuria, there is a need for supplemental diagnostic tests in detecting early chronic kidney disease, and for better assessing which patients are at risk of progression to advanced kidney disease.

Hypertensive nephrosclerosis is the most frequent cause of end-stage renal disease in Norway, and the second most frequent cause in the Western world (18, 19). Hypertensive nephropathy is clinically assumed in longstanding hypertension with gradual loss of kidney function and low/moderate albuminuria, and in the absence of hematuria and other known causes of kidney disease, such as diabetes mellitus, glomerulonephritis, interstitial nephritis etc. The diagnosis

is, however, often assumed solely on clinical criteria, and not always biopsy-verified. This makes it prone to misdiagnosis. There is a need for a more detailed phenotypical description of biopsy-verified hypertensive nephropathy, and evaluation of the precision of the clinical criteria commonly used to establish the diagnosis.

New analytical techniques have surfaced over the last decades, with potential to improve early diagnosis and risk stratification in chronic kidney disease. The omics, with studies of genes (genomics), gene products (transcriptomics), proteins (proteomics), and intermediary metabolite molecules (metabolomics), is a relatively new family of high-throughput analytical platforms in the biosciences. Coupled with modern biobanks, medical registries and evolving information technology, the omics have become important biomarker research tools. There is currently little data on hypertensive nephrosclerosis from the omics research platforms.

7.1 Chronic kidney disease

In 2013 the Kidney Disease Improving Global Outcomes (KDIGO) CKD Work Group defined chronic kidney disease (CKD) as the presence for more than three months of either

- a decreased glomerular filtration rate (GFR) of less than 60 mL/min/1.73m2, or
- in the case of GFR above 60 mL/min/1.73m2, kidney damage as defined by albuminuria (albumin:creatinine ratio >3mg/mmol), urine sediment abnormality, electrolyte disturbances due to tubular disorders, abnormalities detected by histology or imaging, or a history of kidney transplantation (1).

KDIGO offers a risk stratification into CKD stages according to a combination of GFR and albuminuria (Figure 1): Stages G1, G2, G3, G4 and G5 represent GFRs of >90, 89-60, 59-30, 29-15, and <15 mL/min/1.73m2, respectively, and stage G3 is further subdivided into G3a and G3b (45-59 and 30-44 mL/min/1.73m2, respectively). Albuminuria is staged as A1 (normal to mildly increased, <3 mg/mmol), or A2 (moderately increased, 3 to 30 mg/mmol) or A3 (severely increased, >30mg/mmol).

Prognosis of CKD by GFR and Albuminuria Categories: KDIGO 2012

GFR categories (ml/min/ 1.73 m²) 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			

Green: low risk (if no other markers of kidney disease, no CKD); Yellow: moderately increased risk; Orange: high risk; Red, very high risk.

Figure 1. Prognosis of CKD by GFR and albuminuria categories (KDIGO CKD Work Group, *Kidney Int Suppl* 2013 (1)). Reproduced with permission from the Kidney Disease Improving Global Outcomes CKD Work Group.

CKD is common, with a prevalence in the adult population for all stages CKD 1 to 5 of 10.2% in Norway, 11.7% in the USA (20), and ranging from 8 to 16% in data from 52 countries across all five continents (19).

The main causes of end-stage renal disease in Norway are hypertensive/vascular nephropathy, followed by diabetic nephropathy, glomerulonephritis, pyelonephritis/interstitial nephritis, and polycystic kidney disease (18). Globally, diabetes mellitus and hypertension are the most common causes of chronic kidney disease in all developed countries, and in many developing countries (19, 21). Also, glomerulonephritis, interstitial nephritis, polycystic kidney disease and HIV- and hepatitis-associated nephropathy are frequent causes of CKD globally.

Glomerular filtration rate is currently most widely estimated by means of serum creatinine (22). Equations that combine serum creatinine with demographic variables (sex, age, race) are used

to calculate the estimated glomerular filtration rate, eGFR, and the most used are the Modification of Diet in Renal Disease (MDRD) and Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equations (23, 24). New equations have been developed using other filtration markers, such as cystatin C, or a combination of cystatin C and creatinine, for improved precision of GFR estimation (25-27). Other measures of renal function are based on blood urea nitrogen (BUN), creatinine and urea clearance (require blood and 24-hour urine collection), and B2-microglobulin. The gold standard is measured GFR (mGFR) based on measured clearance of iothalamate or other exogenous filtration markers such as iohexol, Cr-EDTA or inulin.

It has been shown that CKD is an independent risk factor for cardiovascular disease (3, 28-30), peripheral arterial disease (31), and all-cause mortality (32, 33). It is likely that the increased cardiovascular risk stems from many combined processes, such as hypertension, chronic inflammation, endothelial dysfunction, oxidative stress and coagulation, among others.

Prognosis in established chronic kidney disease varies with the cause of CKD, kidney function, albuminuria, and concurrent comorbidity (1). Our most validated prognostic marker in chronic kidney disease is currently the CKD stage based on combined glomerular filtration and albuminuria level, published by KDIGO in 2012 (1). With higher CKD stage there is a graded increase in future risk of all-cause and cardiovascular mortality, end-stage renal disease, acute kidney injury and progressive kidney disease (Figure 2):

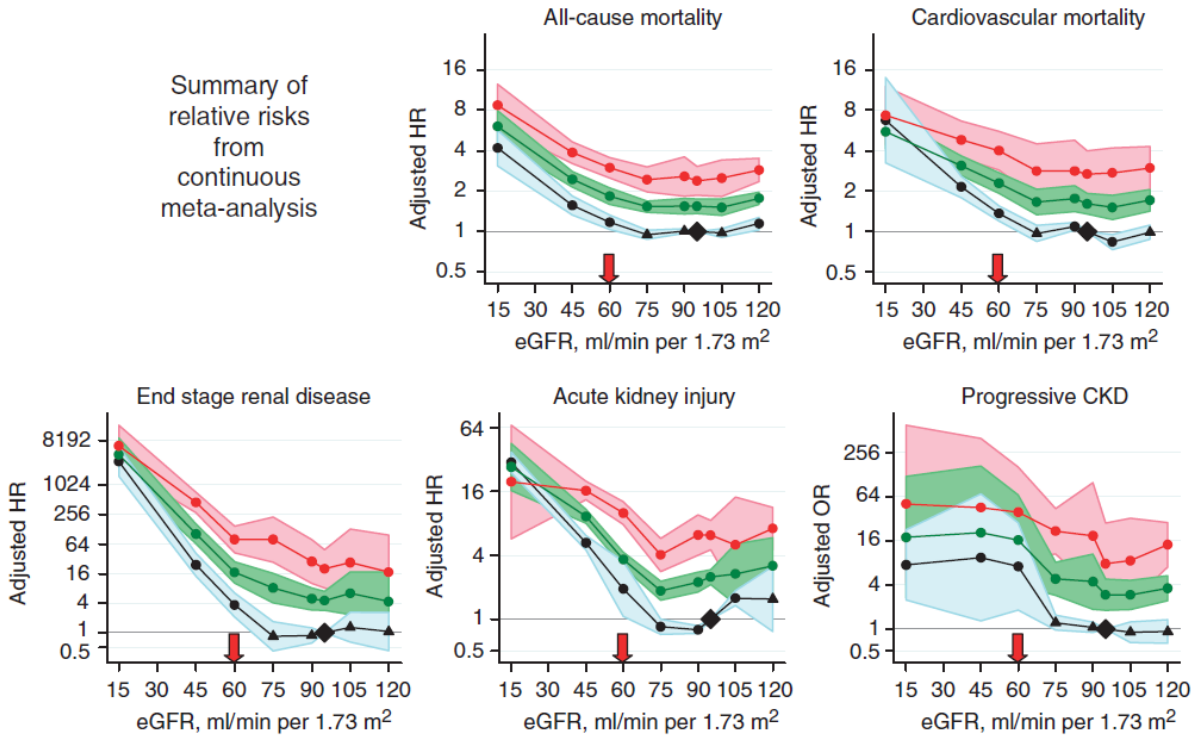

Figure 2. Risk of end-points according to eGFR and albuminuria (Levey, *Kidney International* 2011 (2)). Blue line: urine ACR<30mg/g or dipstick negative/trace. Green line: urine ACR 30-299mg/g or dipstick 1+. Red line: urine ACR ≥300mg/g or dipstick ≥2+. Reproduced with permission from Elsevier.

Newer prognostic markers have been investigated in later years, both in serum and urine. Among others are the *APOL1*, *CUBN*, *ELMO1*, and *PTPN6/PHB2* gene loci in genomics; u-/s-Neutrophil gelatinase associated lipocalin (NGAL), u-Kidney injury molecule-1 (KIM-1), Fibroblast growth factor-23 (FGF-23), and the CKD273 panel in proteomics; and tryptophane, urea cycle, nitric oxide synthesis, and oxidative metabolism intermediates in metabolomics (34-38).

From kidney biopsy studies we have learned that signs of tubulointerstitial disease, such as interstitial fibrosis and tubular atrophy, correlate better with disease prognosis than glomerular changes, even in glomerular diseases (39, 40). As a routine examination, however, it is not feasible or medically justifiable to obtain kidney biopsies in order to predict an individual's renal prognosis.

7.1.1 Hypertensive nephropathy

Hypertensive nephropathy, or arterionephrosclerosis, is assumed to be a common cause of chronic kidney disease and end-stage renal disease. The term nephrosclerosis is derived from Greek (Νεφρός, "nephros", kidney, and , σκλήρωσις "sklerosis", hardening/hardness), and was first coined by the German pathologist Theodor Fahr and the nephrologist Franz Volhard in the 1914 publication "Die Brightsche Nierenkrankenheit", and later in Fahr's publication "Uber Nephrosklerose" in 1919 (41, 42). Hypertensive nephropathy/arterionephrosclerosis is assigned as the cause in around 30% of end-stage renal disease in Norway, making it the most frequent cause of ESRD in Norway (18), the second most frequent in the US (19), and among the most frequent globally (19). Clinically, hypertensive nephropathy is suspected in CKD patients with longstanding hypertension and signs of blood pressure-related organ affection such as hypertensive left ventricular hypertrophy or hypertensive retinopathy, low proteinuria, and no signs of other kidney diseases like hematuria, diabetes, glomerulonephritis etc. (43, 44). The accuracy of traditional clinical criteria to predict biopsy-verified hypertensive nephrosclerosis was shown in several studies to be variable, with positive predictive values ranging from 50% to 85%, across several countries and ethnic backgrounds (45-48). Definitive diagnosis is made by kidney biopsy. Typical histopathological findings are arterial medial thickening and hyaline arteriolosclerosis in afferent arterioles, leading to narrowing of the lumen. Furthermore, arterial medial hypertrophy, intimal sclerosis and duplication of elastic laminae may be seen. Varying degrees of focal glomerular ischemic changes with thickening and wrinkling of basement membrane, mesangial matrix increase, capillary collapse and glomerulosclerosis along with tubular atrophy and interstitial fibrosis are also seen (49-51).

The treatment of hypertensive nephropathy consists of blood pressure control, adequate antiproteinuric treatment with ACE inhibitors or ATII blockers, and aggressive treatment of traditional cardiovascular risk factors, such as smoking, hypercholesterolemia, obesity, and diabetes mellitus (52).

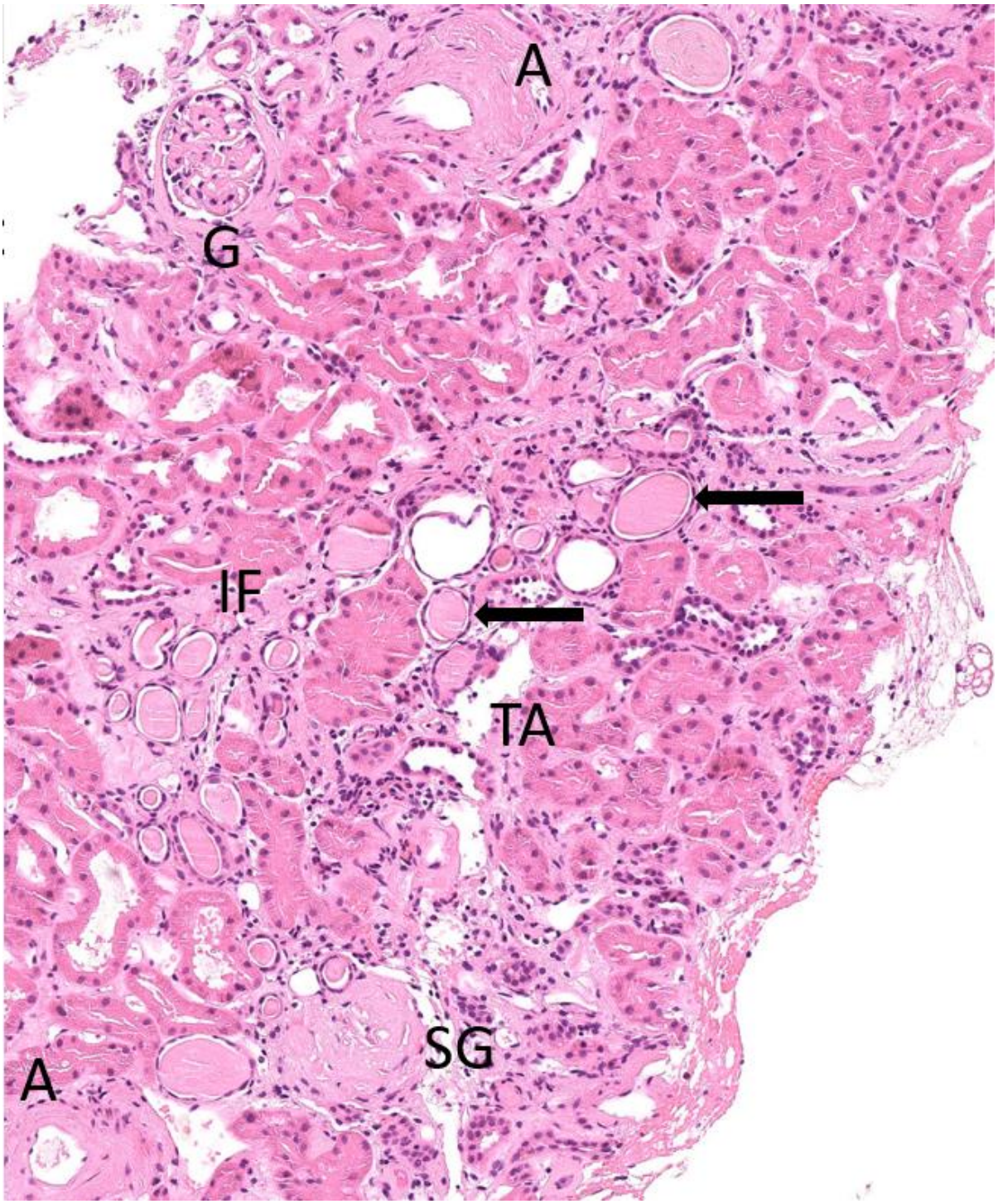

Figure 3. Light microscopy in nephrosclerosis. A: Artery with hyalinosis. G: Glomerulus with early signs of pericapsular fibrosis. SG: Sclerotic glomerulus. IF: Interstitial fibrosis. TA: Tubular atrophy with peritubular fibrosis. Arrows: Tubuli with proteinaceous content. Reproduced with kind permission from pathologist Tina Syvertsen Overrein, MD, St Olavs University Hospital, Trondheim.

Regarding prognosis, the incidence of ESRD is low in non-malignant hypertension without proteinuria. In the Multiple Risk Factor Intervention Trial the reported 7-year incidence of creatinine doubling to >2.0 mg/dL (equivalent to 176 µmol/L) was 0.2% (53). In the Hypertension Detection and Follow-up Program the reported 5-year incidence of creatinine doubling to >2.0 mg/dL (equivalent to 176 µmol/L) and at least 1.25 times the baseline value was around 2.2% (54). In the AASK trial with assumed hypertensive renal disease, the mean GFR slope from baseline through four years was around -2.0 mL/min/1.73m2/year, and no additional benefit of slowing progression was seen with intensive vs ordinary blood pressure goals (55). It is important to point out, however, that patient series with prognostic measures in biopsy-verified hypertensive nephropathy are scarce.

Several pathophysiological mechanisms contribute to hypertensive nephrosclerosis. Meyrier points to a loss of autoregulation in preglomerular arteries (56). Autoregulation is the ability of the afferent arteriole to vasoconstrict in a setting of high preglomerular blood pressure, to protect the glomerular capillaries from the high hydrostatic pressure of the systemic circulation. Loss of autoregulation has been shown in rat models with long-standing hypertension (57), and in humans in ageing and hypertension (58, 59). Animal models with genetic hypertension (Dahl salt-sensitive rats, Brown-Norway rats and fawn-hooded rats) suggest that the loss of autoregulation produces an intrarenal hypertension, paving way for glomerular lesions induced by hyperfiltration and shear-stress on podocytes (60-63). Subsequently, arteriolohyalinosis of the afferent glomerular arteriole, with resultant pressure-induced dilatation, leads to glomerular hypertrophy and focal segmental glomerulosclerosis (59). Second, the micromilieu of the kidneys is characterized physiologically by low oxygen tensions (64), and ischaemia/hypoxia are central in most renal diseases. It has been shown that chronic ischaemia/hypoxia induces fibrosis through many locally working factors, and has been proposed as common pathway for renal fibrosis and GFR loss (56, 65).

Assuming that end-stage renal disease stems from longstanding hypertension has been supported by registries of end-stage renal disease which utilize clinicians' diagnoses, often in the absence of kidney biopsy (47). Recent genomic studies have shown that African Americans with two risk alleles of the *APOL1* gene and a hypertensive nephropathy phenotype have a very high risk of kidney failure (66). It has been postulated that individuals of African descent with hypertension and CKD probably often have a separate disease belonging to the spectrum of focal segmental glomerulosclerosis (FSGS) which is not necessarily initiated by hypertension (67). In whites, on the other hand, clinical nephrosclerosis is associated with histological

arteriolar nephrosclerosis with resultant glomerular ischemia, a process more likely initiated by hypertension and cardiovascular risk factors (68).

7.2 The kidneys and the metabolism

7.2.1 Urine production

Urine is produced as the sum of three processes: glomerular filtration, tubular reabsorption, and tubular secretion. First, filtration from the glomerular capillaries into Bowman's capsule produces large quantities of fluid that is almost completely free of proteins, the so-called primary urine. Most substances, with the notable exception of proteins, are freely filtered from the glomeruli, so their concentrations in the primary urine are almost the same as in the blood. On average, an adult produces around 150 to 170 L of primary urine every day (69). The primary urine passes through the tubules where water and certain substances are reabsorbed, and re-enter the bloodstream via the peritubular capillaries. Third, some substances are secreted actively from the peritubular capillaries into the tubules and excreted in the final urine. Some waste products, such as creatinine, are freely filtrated and completely excreted without neither reabsorption nor secretion. Other substances, like salts, are freely filtered and partially reabsorbed back into the bloodstream, to varying degrees. Some substances are freely filtered and almost completely reabsorbed from the tubules, such as amino acids and glucose. Other substances are freely filtered and in addition actively secreted into the tubules for excretion (70).

The kidneys regulate water excretion according to hydration state, salt and water intake, and serum sodium level, to mention the most important variables. Accordingly, urinary volume and concentrations vary greatly, over many orders of magnitude, and so do the concentrations of all urinary biomarkers. It is important to adjust for these effects in urinary biomarker analyses.

7.2.2 The kidneys and amino acids

Amino acids have several important functions in the metabolism. They serve as building blocks for proteins, substrates for energy production, and as neurotransmitters. A 70 kg man holds around 12 kg of protein, and around 250 grams of free amino acids, of which only approximately 5 g are found in the circulation (71). With normal kidney function, around 50 to 70 grams of amino acids are freely filtered every day, of which almost everything (98%) is

reabsorbed (72, 73). Amino acids are reabsorbed from the tubules by several different transmembrane transporter proteins expressed on the brush border membrane of the proximal kidney tubule cells (74). The most important of these is the luminal B^0AT1 transporter, which has broad selectivity and reabsorbs more than 80% of all amino acids, by co-transport with Na^+. One amino acid and one Na^+ ion are bound simultaneously and imported into the cell, powered by the chemical gradient of Na^+, which is constantly exported out of the proximal tubular cells by the sodium-potassium ATPase. Once inside the cell, amino acids and glucose exit over the basolateral cell membranes into the interstitium along their chemical gradients, and are absorbed into the peritubular capillaries. The capacity of the amino acid-binding carrier proteins is so large that essentially all the amino acids are reabsorbed from the tubules (70).

The kidneys play a role in the normal amino acid metabolism in humans. In the physiological state, the kidneys not only filter and reabsorb, but also synthesize, degrade and excrete amino acids. In the postabsorptive state there is net uptake or release of certain amino acids by the kidneys. Renal synthesis is the major source of certain amino acids, such as serine, cysteine, arginine and tyrosine, to which the kidneys contribute 50% to 100% of the total body pool (71). The kidneys are also the main site of excretion of certain amino acids, such as proline and glutamine, the latter being used as substrate for ammonia production, which is central in the acid-base balance (75). The kidneys are central for the removal of the amino acids glutamine and proline (72), for removal of citrulline and the peptide Cysteine-Glycine derived from glutahionine metabolism (76), and for S-adenosylhomocysteine, a metabolite of homocysteine (77).

It has been shown that CKD leads to changes in plasma and urinary levels of amino acids, across a spectrum from early-stage CKD to end stage renal disease (ESRD). Whereas the urinary levels of some amino acids increase in CKD (such as homocysteine, glutamate, guanidoacetate), others decrease (such as leucine, serine, taurine, threonine and glutamate) (78, 79).

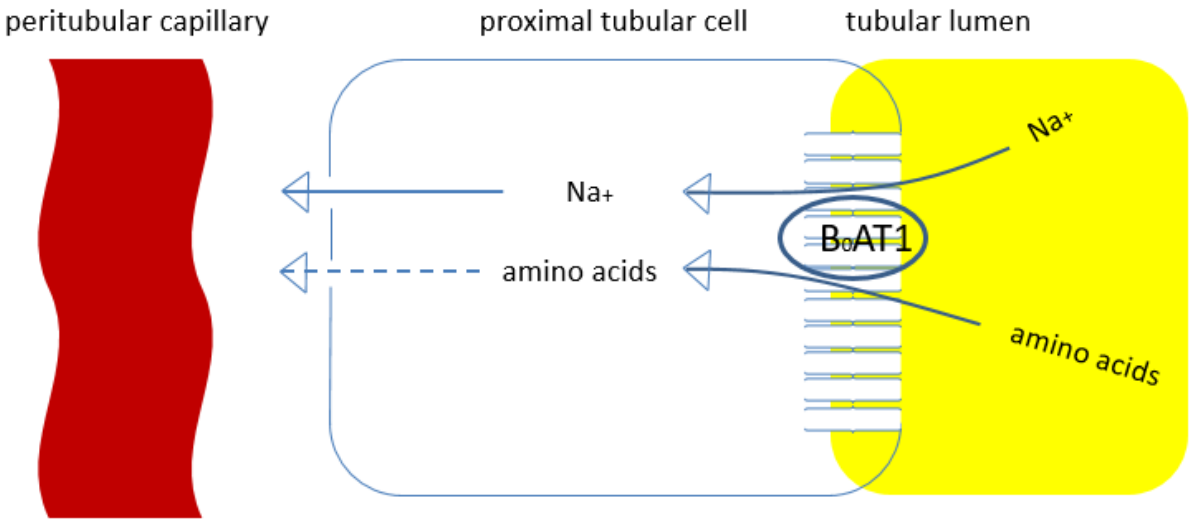

Figure 4. Tubular reabsorption of amino acids from the primary urine happens in the proximal tubule. An active Na+ pump on the basal side of the cell pumps out Na+ and sets up a gradient that drives passive import of Na+ with co-transport of amino acids on the apical surface. (Copyright PK Øvrehus)

7.2.3 The kidneys and urinary protein

Normally, only small amounts of protein are filtered across the glomeruli. Most of these proteins are reabsorbed in the proximal tubule after binding to two specific tubular receptors, megalin and cubulin. These transmembrane receptors recover a wide range of ligands from the tubules: vitamin binding proteins such as intrinsic factor vitamin B12, miscellaneous carrier proteins such as albumin, lipoproteins such as apolipoprotein E, hormones such as insulin, enzymes such as α-Amylase, and immune-related proteins such as IgG light chains.(80) These ligands bind to megalin and cubulin or both, and are internalized by way of invagination or endocytosis, producing endocytic vesicles that fuse with for example lysosomes, where the ligands are further processed.

In cases of disruption of the glomerular filtration barrier function with leakage of proteins into the glomerular ultrafiltrate (glomerular proteinuria), or an overproduction of certain filterable immune proteins (overflow proteinuria), the protein reabsorbing capacity in the proximal tubule may be exceeded, leading to proteinuria. In other cases there may be a tubular dysfunction because of kidney disease, inhibiting the proximal tubular reabsorption of proteins (tubular proteinuria).

Some urinary protein excretion is normal. The average daily urinary protein excretion in adults is 80 mg/day, with normal excretion considered to be <150 mg/day. The large part of this is uromodulin, formerly known as Tamm-Horsfall protein, constituting around 85% of daily urinary protein excretion. It is secreted from distal tubular cells, and is believed to have a role in calcium-binding to avoid urinary stones (81). Urinary albumin excretion is approximately 5-10 mg daily in young healthy adults (82).

Microalbuminuria is an early marker of kidney dysfunction and is a predictor of kidney and cardiovascular outcomes in CKD (2).

7.2.4 Kidney disease and global metabolism

That the systemic metabolism affects kidney function and kidney disease is well known from diabetes. Vice versa, chronic kidney disease also influences the extrarenal metabolism. CKD causes protein energy wasting and is implicated in reduced insulin sensitivity (83, 84). Also the levels of some circulating metabolites, such as amino acids, are significantly regulated by the kidneys (75, 85). Finally, the kidneys have regulatory roles in many types of circulating metabolites, some of which have surprisingly shown hormone-like effects, such as glutamate (86, 87) and citric cycle metabolites (88).

It is fair to say that our knowledge both on all facets of the human metabolism, and the way the kidneys interact with metabolism, is not complete.

7.3 Overview of translational medicine in kidney research

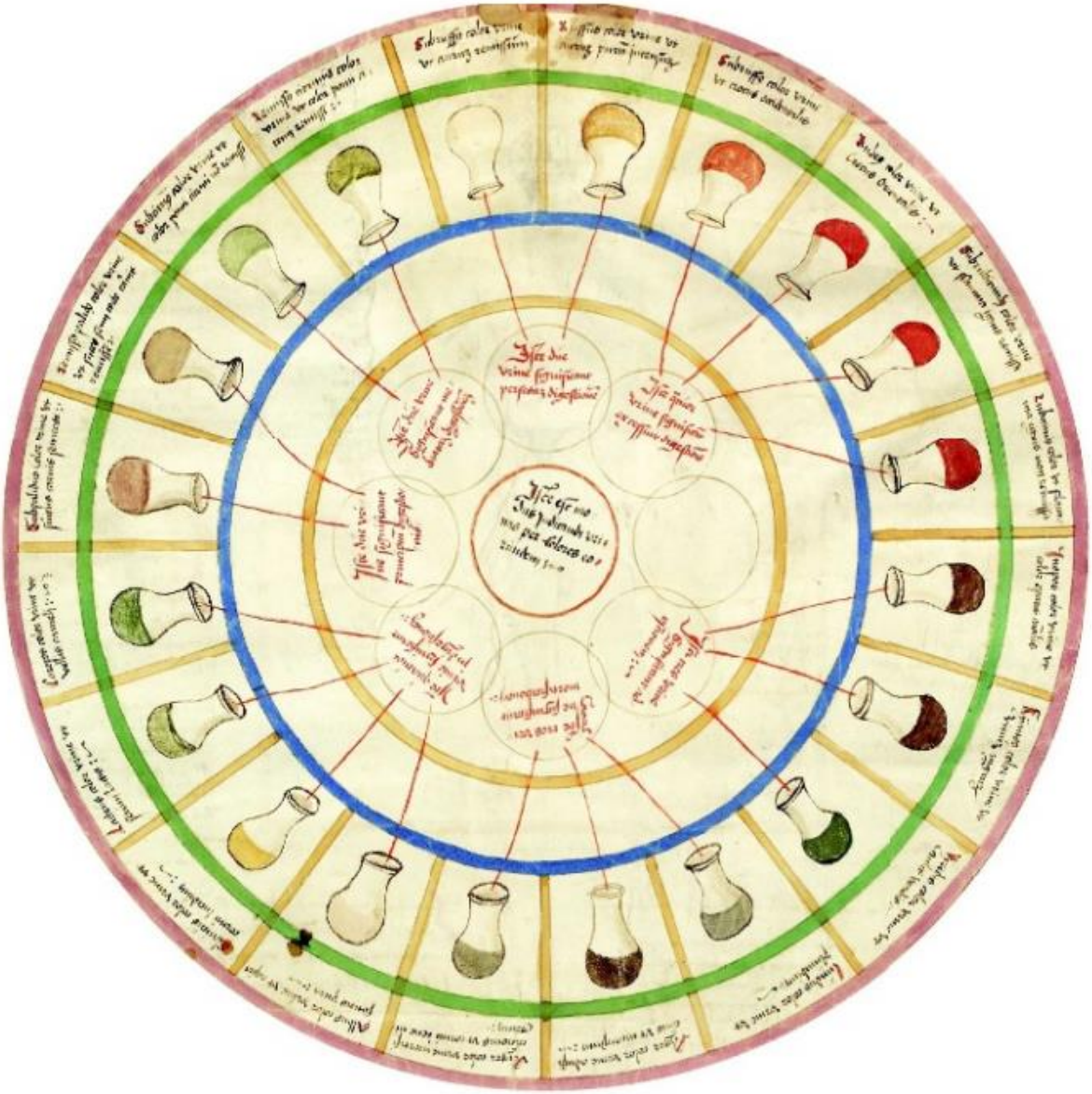

Figure 5. A typical urine wheel from the Middle Ages. Published in *Epiphanie Medicorum* by Ulrich Pinder, Nuremberg, in 1506.

Translational medicine is a term that encompasses many of the new analytical platforms known as the "omics". Genomics, transcriptomics, proteomics, and metabolomics are the principal ones.

The study of metabolites in kidney diseases has a long history, in one sense starting already with urine tests in the Middle Ages (89), where the colour of the urine was subject to systematic studies (Figure 5). Urine, which is easily obtained non-invasively and has intimate relation to

the kidneys, lends itself to metabolomics studies, and has already made possible large cohort and biobank studies in nephrology. Today, with the advent of high-throughput analytical platforms, coupled with modern biobanks, medical registries and ever evolving information technology features, it is an up-and-coming method for studying a broad range of biological questions in kidney disease.

Metabolomics, which is the study of low-molecular weight compounds in tissues or fluids, makes part of the "omics" family of systems biology research branches (90). Metabolomics aim to describe the presence of many metabolites at the same time, thus providing a kind of snap-shot of the end product of the ongoing metabolism. Whereas genomic techniques describe the expression of genetic material in disease, and transcriptomics and proteomics describe the RNAs and proteins that these genes code for, metabolite studies describe the total end product, or down-stream "net effect", of all these up-stream perturbations.

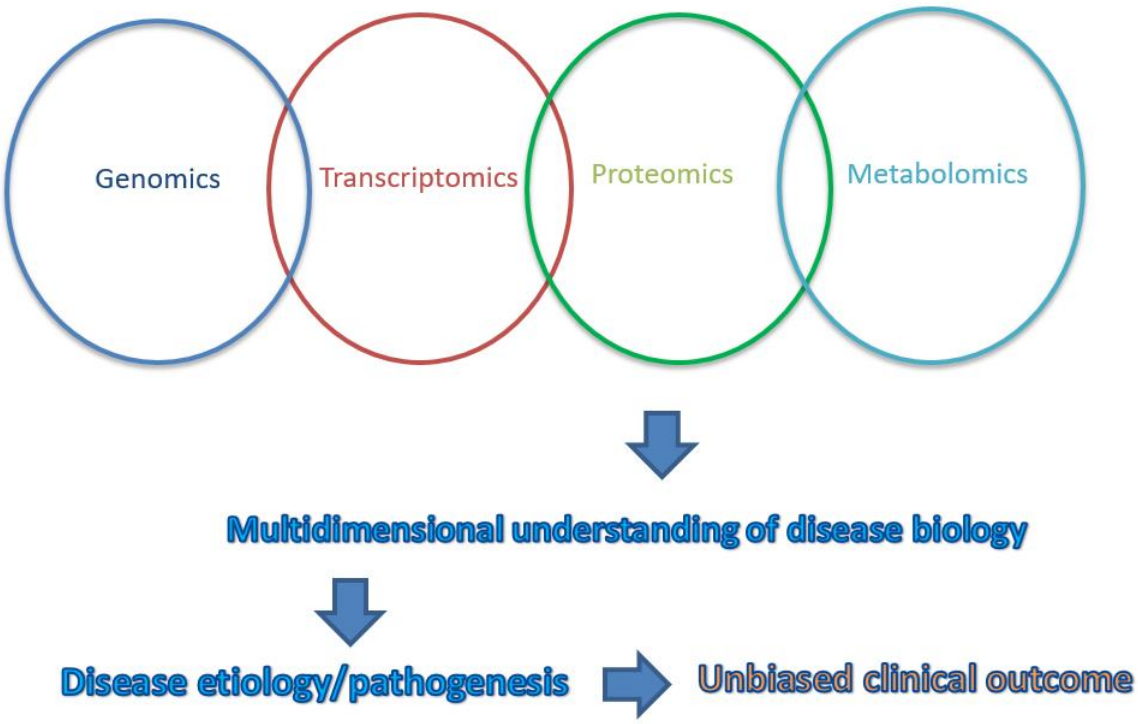

Figure 6. The family of omics. (Copyright PK Øvrehus)

Metabolomic analysis is a relatively recent development in renal research. Metabolomic studies have been undertaken in nephrology using blood and urine, and have been done in fields such as chronic kidney disease, diabetic nephropathy, acute kidney injury, transplantation nephrology, glomerulonephritis and renal cancer, amongst others. The two major metabolomics methodologies are nuclear magnetic resonance (NMR) spectrometry and mass spectrometry (MS), the latter in conjunction with a separation step, typically gas or liquid chromatography (GC-MS and LC-MS, respectively). Each of these techniques has strengths and weaknesses. NMR, while robust, reliable and with many years in use already, is hampered with low sensitivity. The MS techniques have high sensitivity and resolution, but have issues concerning sample pretreatment, and for LC-MS in particular, reproducibility and systems stability over time. There is no single and completely exhaustive technique for full metabolome coverage, but rather they should be used in complementary ways. The vast data produced in metabolomics poses challenges in data processing and biostatistics. This provided, metabolomic studies in kidney research have the potential to elucidate important pathophysiological pathways and contribute to diagnostic, prognostic and therapeutic steps forward.

Proteomics is the large-scale study of proteins and peptides in a biological system (91), and makes part of the omics family. Like metabolomics, proteomics analyses are relatively fast and high-throughput, and handle blood and urine samples by the use of LC-MS, CE-MS and NMR platforms. CE-MS has been the dominant platform for proteomics analysis in nephrology. Proteomics platforms have been utilized for diagnosis of CKD across a range of etiologies, such as ANCA associated vasculitis (92), kidney transplant rejection (93), IgA nephropathy (94), diabetic nephropathy (95, 96) and general CKD (95). Proteomics-based biomarkers have also been used for prognostication in CKD, both in diabetic nephropathy (97, 98) and general CKD (99-101).

Genomics is the large-scale study of genes and gene expression patterns in health and disease. In nephrology, one direction in genomics analyses has been the genomic characterization of kidney tissue from specific biopsy-proven renal diagnoses from biopsy registries, such as the European Renal cDNA Bank (102). Another direction has been the plasma-based genome-wide association studies (GWAS), where countless genetic loci or single nucleotide polymorphisms (SNPs) have been analyzed and associated to clinical traits, such as eGFR, hypertension, or CKD (103). Yet another genomics platform are the genome-wide association studies of metabolite concentrations (mGWAS), which bring together genomic and metabolomic data to provide genome-wide association analyses of metabolic compounds (104).

	Source	Method	Pathway	Reference
CKD 1-2				
Glutamate↑	S, U	CE-TOF-MS	Amino acids	134
Aspartate↑	S, U	CE-TOF-MS	Amino acids	134
Ornithine↑	S	CE-TOF-MS	Amino acids	134
Histidine↓	U	CE-TOF-MS	Amino acids	134
Carnosine↓	U	CE-TOF-MS		134
Hypotaurine↓	U	CE-TOF-MS		134
Hypotaurine↑	S	CE-TOF-MS		134
Hypoxanthine↑	S	CE-TOF-MS	Nucleotides	134
Adenosin↓	S	CE-TOF-MS	Nucleotides	134
Adenosin↓	U	CE-TOF-MS	Nucleotides	134
Lactate↑	S	CE-TOF-MS	Anaerobic glycolysis	134
Citrate↓	U	CE-TOF-MS	Krebs cycle	134
U-3-phosphoglycerate↓	U	CE-TOF-MS	Carbohydrate	134
CKD 2-4				
Citrulline↑	S	GC-MS/LC-MS	Arginine-NO	125
Ornithine↑	S	GC-MS/LC-MS	Arginine-NO	125
Arginine↑	S	GC-MS/LC-MS	Arginine-NO	125
Proline-hydroxyproline↑	S	GC-MS/LC-MS		125
Fibrinopeptide A↑	S	GC-MS/LC-MS	Coagulation, inflammation	125
CKD 1-5				
ADMA, SDMA↑	P	CE-MS/LC-MS	Nitric oxide	108
Indoxyl sulphate↑	P	CE-MS	Bacterial	108
Kynurenine, kynurenic acid↑	S	LC-MS	Tryptophan	108
Tryptophan↓	S	LC-MS	Tryptophan	108
Uric acid	S	LC-MS	Nucleotides	
ESRD				
Adipate↑	P	LC-MS	β-oxidation	108
ADMA, SDMA↑	P	CE-MS/LC-MS	Nitric oxide	108
Hippurate↑	P	LC-MS	Bacterial	108
Homovanillate↑	P	LC-MS	Serotonin	108
5-hydroxyindolacetic acid	P	LC-MS	Dopamine	108
Indoxyl sulphate↑	P	CE-MS	Bacterial	108
Kynurenine, kynurenic acid↑	S	LC-MS	Tryptophan	108
MOPEG↑	P	LC-MS	Norepinephrine	108
TMAO↑	P	LC-MS	Bacterial	108
Homocysteine↑	P	LC-MS	Serine-homocysteine	108

Table 1. Overview of metabolomics findings in CKD and ESRD. S: serum. P: plasma. U: urine. Adapted from (35).

7.3.1 Metabolomics in hypertensive nephropathy

The pathophysiology of hypertensive nephropathy is complex, involving glomerular, tubular, and interstitial changes due to endothelial dysfunction, activation of the renin-angiotensin-aldosterone system (RAAS), and oxidative stress, as well as genetic, metabolic, and inflammatory perturbations (50, 105). However, there is paucity of metabolomics data in the field of hypertensive nephropathy. Here is a short resume of where metabolomics stand in hypertensive nephropathy research.

Xanthosine: This amino acid is high in ESRD, together with most other purines from the nucleotide metabolism (106, 107). Ganda et al measured plasma metabolites in patients with hypertensive nephropathy (n=16), diabetic nephropathy (n=34), other CKD etiologies (n=10), and controls (n=30) using LC-MS, all diagnoses set by a nephrologist (108). Xanthosine was increased in hypertensive arterionephrosclerosis: 1.9-fold and 2.4-fold higher than in diabetic nephropathy and other CKD etiologies, respectively. In a median follow up of 2.6 years, xanthosine was significantly associated with first occurrence of a cardiovascular event in CKD patients. The role of xanthosine in the pathophysiology of hypertensive nephropathy and CKD is uncertain, but in line with this, a Framingham Heart Study work found that baseline xanthosine levels were significantly associated with incident CKD (85).

Citric acid cycle: Reduced citric acid cycle function is associated with non-diabetic CKD (109). Liu et al carried out gene ontology, pathway enrichment and network analysis on a gene expression dataset of biopsy-verified hypertensive nephrosclerosis (110). The most differentially expressed genes belonged to metabolic pathways, especially the TCA cycle, glycolysis/gluconeogenesis, MAPK signaling, and pyruvate metabolism. The most differentially expressed TCA cycle genes were *PCK1*and *PCK2*, which code for the cytosolic and mitochondrial isozyme of phosphoenolpyruvate carboxykinase (PEPCK), respectively. PEPCK is a gluconeogenesis regulator enzyme in the kidneys, and underexpressed in a rat model of CKD (111). Furthermore, perturbations were found in the gene coding for SORD, an enzyme involved in glycolysis by converting sorbitol to fructose in the polyol pathway in the kidney (112). Microvascular disease is associated with accumulation and toxicity of sorbitol (113).

Oxidative stress: Oxidative stress working through reactive oxygen species (ROS) is believed to be central in the pathogenesis of renal damage and hypertensive nephropathy from hypertension (105, 114). The mitochondria are the major intracellular source of ROS, which

are formed in the respiratory chain (115). In one study, high-salt fed Dahl/Rapp salt-sensitive rats developed human nephrosclerosis-like histologic kidney lesions, which were associated with mitochondrial dysfunction and promotion of apoptosis (116). Also, high-salt fed stroke-prone spontaneously hypertensive rats (SHRsp) develop severe renal damage at blood pressures where the stroke-resistant SHR model does not (117). This has been attributed to the underexpression of the uncoupling protein-2 (UCP2) gene in the SHRsp model. UCP2 is a mitochondrial protein that protects agains ROS, and its underexpression is associated with increased oxidative stress, inflammation, and histological changes (117).

7.3.2 Metabolomics in general CKD

In early metabolomics studies in nephrology, particular interest was shown to the analysis of uremic toxins, which are small and middle molecules in plasma or dialysates of patients with advanced CKD and end stage renal disease (ESRD) (118, 119). Pathophysiology studies have examined the effects of uremic metabolites in promoting oxidative stress, inhibiting wound repair, and promoting coagulation (120-122). Also, efforts have been done to find metabolomics biomarkers for diagnosing early stage chronic kidney disease (123, 124), and which metabolites associate with *de novo* CKD development in disease-free individuals (85). The cross-sectional associations between serum metabolites and eGFR in population-based cohorts have been examined in several studies, such as the Chronic Renal Insufficiency Cohort, Atherosclerosis Risk in Communities, the TwinsUK Registry, the Cooperative Health Research in the Region of Augsburg Study, and the Framingham Heart Study (85, 125-128).

Here is a short resume of where metabolomics stand in chronic kidney disease research.

7.3.2.1 Descriptive metabolomics in general CKD

ADMA/SDMA, arginine metabolism: Assymetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are increasingly elevated in plasma with more advanced CKD stages (123, 129). The biological function of SDMA is unclear. ADMA is an inhibitor of nitric oxide synthetase. High ADMA levels could contribute to inhibition of nitric oxide (NO) production and thus to endothelial dysfunction and impaired relaxation. Ornithine and citrulline, both arginine metabolites, are low in more advanced CKD.

Oxidative stress markers: Oxidative stress is induced when free oxygen radical levels are increased (130). Oxidative stress is increased in CKD, and contributes to the cardiovascular morbidity seen in this condition (131). Already in CKD stages 1-2, changes in free radical scavengers carnosine and hypotaurine have been found, indicating higher renal oxidative stress (132). In CKD 2-4, impaired carboxylate anion transport, with higher levels of mono- and di-carboxylate anions (e.g. γ-glutamyl leucine), are thought to reflect increased oxidative stress because of glutathione depletion (123).

Steroids: Lower levels of metabolites of adrenal steroid hormones are found with advancing CKD, suggesting decreased production of adrenal hormones in CKD (123). Steroids are involved in lipid metabolism, immunomodulation, and stress response, and perturbations in steroid metabolism are thought to participate in hypertension (133, 134).

Urea cycle: Urea is converted to uric acid in the urea cycle in the kidneys and the liver. Citrulline, an amino acid produced in the liver as part of the urea cycle, was higher in urine and plasma in hemodialysis compared to advanced CKD (129). The authors hypothesized that the increased urinary levels were caused by tubular dysfunction or a metabolic mechanism. They claimed support to this by a mouse study where partial nephrectomy induced citrullinuria (135). Ornithine is urea cycle intermediary whose catabolism toward citrulline and possibly proline synthesis is increased in advanced CKD, leading to accumulation (136). Citrulline-to-arginine and ornithine-to-arginine ratios are increased in advanced CKD (129).

Nitric oxide: Nitric oxide (NO) regulation is disturbed in CKD (129). NO is a free radical that causes vasodilatation. It is produced in the endothelium by arginine breakdown catalyzed by NO synthase (NOS), which produces citrulline and NO. The conversion of citrulline, a precursor of nitric oxide via arginine, is reduced in CKD, which likely impacts on endothelial function and reduces NO-mediated vascular relaxation (72, 137). ADMA, which increases in advanced CKD, inhibits NO synthase and is associated with hypertension, glomerulosclerosis, and possibly CKD progression (136).

Tryptophan: Tryptophan and tryptophan intermediate levels, especially indoxyl sulfate and the kynurenines, are high in advanced CKD (138, 139). Tryptophan and its intermediates seem to play a role in blood pressure regulation, hypertension, dyslipidemia, and atherosclerosis (140, 141).

Acid-base, glutamate: Glutamate is an intermediate in glutamine metabolism that produces ammonia and is central to acid-base balance. 5-oxoproline, part of a catabolic pathway from glutathione via 5-oxoproline to glutamate, is its precursor. In CKD stages 1-2 and 3-5 urinary glutamate is higher (78, 132), and urinary 5-oxoproline is lower (78), pointing to amino acid-related disturbances in acid-base balance in CKD.

Hypoxanthine, nucleic acid metabolism: Higher serum hypoxanthine has been found in CKD stage 1-2 (132). Hypoxanthine is found in nucleic acid metabolism, and is implicated in the progression of renal interstitial fibrosis in a mouse model (142).

Tricarboxylic acid cycle: CKD induces systemic changes in carbohydrate metabolism. Reduced urinary levels of most TCA cycle metabolites is a feature of both diabetic and non-diabetic chronic kidney disease (109, 143). CKD patients have increased risk of hypoglycemia due to impaired gluconeogenesis (144), which is linked to underexpression of the gene for phosphoenolpyruvate carboxykinase (PEPCK), a gluconeogenesis regulator. This has been demonstrated in a rat CKD model (111). Even in early stage (1-2) CKD, lower urine citrate and fumarate, and higher serum lactate, have been found (132). Serum citrate has been shown to be elevated across CKD stages 3a to 5 (124). Although the literature consistently reports reduced urinary levels of TCA intermediates in CKD, it is interesting that fumarate has also been found to be increased in the urine in a mouse model of diabetic nephropathy (145), in early human diabetes type 2 (146), and in diabetic humans before reduction in kidney function (147). Whether this discrepancy stems from a difference in selection of patients or differences in eGFR, or mechanisms that are not elucidated, is not known.

Tyrosine: The amino acid tyrosine is involved in catecholamine biosynthesis, natriuresis, and blood pressure control through the tyrosine-phenylalanine axis. Low plasma tyrosine and phenylalanine are found in CKD stage 3a to 5 (124). Urine and plasma tyrosine are reduced in CKD stage 3-4, and are lower in CKD with or without diabetes compared to non-CKD diabetics (148). It has been hypothesized that renal phenylalanine-hydroxylase enzyme activity is decreased in CKD, making kidneys retain tyrosine (72). Kidneys are the major organ for uptake of phenylalanine and conversion to tyrosine in humans. The splanchnic uptake of tyrosine is reduced in CKD, and urinary excretion of phenylalanine and tyrosine impaired, possibly leading to impaired synthesis of tyrosine products dopamine, epinephrine, and norepinephrine (149). This might influence blood pressure regulation.

Acylcarnitines: Physiologically, carnitine is a transporter of fatty acids into mitochondria (150). Higher serum levels of acylcarnitines and of amino acids glycine and phenylalanine have been found with lower eGFRs across a wide specter of eGFR in the cross-sectional KORA F4 Study, and validated in the TwinsUK study (127). The authors hypothesized that this may indicate saturated capacity for mitochondrial beta-oxidation in the setting of lipotoxicity-induced insulin resistance. Concentrations of acylcarnitines have previously been suggested as indicative of the speed of beta-oxidation of fatty acids (151).

7.3.2.2 Predicting eGFR decline in general CKD

In addition to descriptive studies of metabolite levels at different CKD stages, prospective studies have also been carried out in renal metabolomics.

Spermidine, kynurenine-to-tryptophan ratio, and a specific phosphatidylcholine ratio were found to associate with annual eGFR change in an LC-MS study with 1104 KORA participants over 7 years (152). Together with 35 other metabolites they predicted incident CKD. The kynurenine-to-tryptophan ratio was hypothesized to reflect activity of the enzyme indoleamine 2,3-dioxygenase (IDO). IDO is linked to inflammation, obesity, blood pressure, and atherosclerosis (141, 153). The phosphatidylcholine ratio was linked to lipoprotein-associated phospholipase A(2), associated with CKD progression and CV events (154).

Serum pseudouridine, C-mannosyltryptophan, and O-sulfo-L-tyrosine were associated with incident CKD in a GC-MS/LC-MS study of KORA/TwinsUK participants (128). Pseudouridine is a uremic toxin (107). Myo-inositol, N-acetylalanine, N-acetylcarnosine and 49 other metabolites associated with baseline eGFR. High myo-inositol levels are associated with progression to ESRD in diabetes (155). N-acetylalanine and N-acetylcarnosine are results of N-acetylation, a detoxification mechanism whose role in CKD is unclear.

Serum 3-indoxylsulfate, N-acetylalanine, and phenylacetylglutamine and 71 other metabolites associated with eGFR decline in a GC- and LC-MS study on 1921 ARIC participants over 20 years (126). The largest measures of association were in the amino acid pathway. Baseline 5-oxoproline and 1,5-anhydroglucitol were associated with the lowest hazard ratio (HR) for incident CKD. 5-oxoproline is substrate for glutamate and involved in glutathione synthesis and degradation. Glutathione deficiency contributes to oxidative stress (156). High urinary 1,5-anhydroglucitol has been suggested as an indicator of renal toxicity (157).

High plasma levels of kynurenic acid and kynurenine predicted incident CKD in an LC-MS study of 1434 Framingham Heart Study participants over 8 years (85). So did choline derivatives (choline), citric acid cycle intermediates (aconitate, isocitrate), and purine metabolites (xanthosine, adenosine). Kynurenine and kynurenic acid are tryptophan metabolites and implicated in inflammation, vascular tone regulation, and atherosclerosis (153, 158). Proximal tubular cells metabolize citrulline and choline, and are the location for organic anion transporters (OATs) that transport kynurenic acid (159). Perturbed levels of these might reflect an underlying tubulointerstitial dysfunction.

Lower threonine, methionine, phenylalanine, and arginine, and higher uric acid, glucoronate, 4-hydroxymandelate, cytosine, and homogentisate were found in CKD progressors compared to non-progressors in an LC-MS study of 200 participants from the CRIC cohort with CKD (125). Uric acid has been associated with incident CKD and CKD progression (160, 161). The authors hypothesized that depletion of arginine, a substrate for the production of nitric oxide, could have negative vascular effects and potentially contribute to CKD progression.

High plasma acylcarnitines and low urinary collagen associated with eGFR decline in an LC-MS study of CKD over 3 years (162). High plasma acylcarnitines in progressors might indicate impaired fatty acid beta-oxidation and an adjustment to prevent the accumulation of lipotoxic acyl-CoA in CKD. Lower urinary collagen in progressors might reflect fibrosis and reduced extracellular matrix degradation. A 76 metabolite and peptide panel associated with eGFR decline. Low uromodulin has previously been associated with fibrosis, and high protein S100-A9 with inflammation. Again, high ADMA and hydroxykynurenine were implicated in endothelial dysfunction and oxidative stress, respectively.

7.3.3 Proteomics in general CKD

Like metabolomics, proteomics is also a relatively new analytical technique in medicine and biosciences. By 2010, around 5000 unique peptides had been identified and catalogued.

Urinary proteomics-based classifiers have been shown to discriminate biopsy-verified CKD from healthy controls (163), and to discriminate asymptomatic hypertensive patients with left ventricular diastolic dysfunction from healthy controls (164). Among the most studied biomarkers in proteomics is a panel of 273 distinct urinary peptides, the so-called CKD273 classifier (Mosaiques Diagnostics GmbH, Hannover, Germany). This proteomics platform has also been evaluated as a prognostic tool, predicting the progression in diabetics from

normoalbuminuria to macroalbuminuria (97, 98), and progressive eGFR loss in general CKD (99, 165). It has also been shown to predict cardiovascular complications in CKD patients (165) and hard end-points like ESRD or death (100).

The most significant findings in the CKD group have been decreased levels of urinary collagen α1 (III), collagen α1 (I), and uromodulin fragments. Lower urinary collagen have been proposed to indicate disturbance of matrix turnover, with reduced breakdown and excessive accumulation of matrix collagen, possibly contributing to the fibrosis seen in CKD (95). Other peptides associated with CKD are α1-antitrypsin, fragments of albumin and fibrinogen, which are all proteins abundant in plasma. Uromodulin, arising from tubular protein secretion, and plasma-derived polymeric immunoglobulin receptor, clusterin, and α2-HS-glycoprotein are other peptides linked to CKD (166).

7.3.4 Genomics in general CKD

In plasma-based genome-wide association studies (GWAS), certain single nucleotide polymorphisms (SNPs) have been associated to CKD. Some SNP associations with CKD have been found at the gene locus *UMOD* (103). *UMOD* codes for uromodulin, or Tamm-Horsfall protein, which is the most abundantly excreted tubular protein in the urine. Uromodulin has been linked to tubular dysfunction (167). Risk alleles at the *APOL1* locus (coding for Apolipoprotein L1) have been associated with increased risk of focal segmental glomerulosclerosis, progressive CKD, and ESRD, especially in patients of African descent (168). Risk alleles at the *MYH9* locus (Myosin heavy chain) have been shown to associate with ESRD in African Americans (169), and with non-diabetic CDK and diabetic ESRD in European Americans (170, 171). Genes related to angiotensinogen, angiotensin converting enzyme and apolipoprotein E have show association with accelerated renal function decline in Caucasian women (172). and genes related to permeability glycoprotein associated with increased risk of hypertension and CKD in the Chinese population (173).

Another genomics platform are the genome-wide association studies of metabolite concentrations (mGWAS), which bring together genomic and metabolomic data to provide genome-wide association analyses of metabolic compounds (104). Examples are *CKD12/PNMT*, which code for enzymes involved in e.g. tyrosine metabolism, which again is linked to catecholamine biosynthesis. Another example is *ALDH2*, which codes for enzymes in

the kynurenine pathway, which in turn is implicated in CKD-related inflammation and endothelial dysfunction (104).

7.3.5 Hypertension

7.3.5.1 Metabolomics in hypertension

Several metabolite classes have been found in metabolomics studies on hypertension:

Amino acids: Perturbed amino acid metabolism is a frequent finding in metabolomics studies in hypertension. In a study of young hypertensives compared to controls, 12 of 20 significantly different serum metabolites were amino acids, and 6 of the 8 most enriched pathways related to amino acid metabolism (174). In the hypertensives, low ornithine, tyrosine, valine, isoleucine, glycine, threonine, methionine, asparagine, glutamine, citrulline, lysine, tryptophan, and cystine were found, and high s-fumarate, glycerol, and uric acid. Another case-control study found that serum arginine was increased, and serum alanine, pyruvate, methionine, adenine, and uracil were reduced in essential hypertension cases (175). In a cross-sectional study, high urine alanine was associated with hypertension in 4630 INTERMAP participants (176). High baseline serine, glycine, and the acyl-alkyl-phosphatidylcholines C42:4 and C44:3 levels associated with lower incidence of *de novo* hypertension (177). Many different mechanisms are at play. Alanine increases blood pressure and modulates cardiovascular catecholamine response in animal models (178). Serine produces vasodilation in rat vessels (179), and glycine has anti-inflammatory effects on human coronary endothelial cells (180). Uric acid has been shown to play a role in early hypertension (181) and established hypertension (182). Fumarate given intravenously increased salt-induced hypertension and medullary ROS levels in a rat model (183). High dietary tyrosine has been related to lower blood pressure (184). Arginine acts as a vasodilator by conversion to NO via NO synthase (NOS), (185), and perturbations of the arginine/NO pathway have been linked to hypertension. ADMA blocks NOS and reduces NO availability (186), and increased ADMA levels may follow from reduced activity of dimethylarginine dimethylaminohydrolase (DDAH), an enzyme linked to inflammation, hyperhomocysteinemia, and hyperglycemia (175).

Carbohydrate metabolism: Elevated glucose, galactose, sorbose and other sugar species have been found in hypertension, and could be linked to the tendency towards impaired glucose tolerance in hypertension (187). Succinate was found to increase blood pressure in mice through

activation of the RAAS (88). In the same study, a-ketoglutarate and succinate were found to act as G-protein-coupled receptor activators, and to potentially have a signaling function in addition to being intermediate metabolites in the TCA cycle.

Hippurate, gut-related metabolites: An association between serum 4-hydroxyhippurate and incident hypertension was found in the Atherosclerosis Risk in the Communities cohort (188). Four-hydroxyhippurate is a product of microbial degradation of polyphenols, and the authors proposed a link to hypertension via gut microbial fermentation and oxidative stress. Formate and hippurate are products of gut metabolism, and associate with hypertension (176).

Lipid metabolism: Dysregulated lipid metabolism with higher serum free fatty acids has been found in hypertensive elderly (189). This could indicate a metabolic syndrome-associated perturbation of insulin-controlled lipid catabolism. Free fatty acids also increase neurovascular tone and inhibit endothelium-dependent vasodilation (190). Diacylglycerols were associated with baseline systolic and diastolic hypertension in a cross-sectional study (191). Diacylglycerols are increased in G-protein-coupled receptor activation of intracellular phospholipase C seen in vasopressin signaling (192), and activate the TRPC6 channel, a calcium flux regulator in vascular smooth muscle cells (193). The authors suggested there is a biological plausibility for a role of diacylglycerols in hypertension pathophysiology.

Steroids: A positive association between hypertension and a sex steroid pattern of pregnenolone, and estrogen and androgen derivatives has been found. The sex steroids have multiple effects on vascular, renal and heart cells, and also modify aldosterone, renin and aldosterone-to-renin ratio (194).

7.3.5.2 Proteomics in hypertension

In one case-control study of hypertensives vs. normotensives, 27 peptides were found to accurately classify hypertension (195). Some of the significant peptides were fragments of the proteins osteocalcin and humanin, which have been linked to atherogenesis. In another hypertension proteomics study of hypertensives vs. controls, different levels of uromodulin and nephrin 1, a protein involved in the slit diaphragm, were found (196). There is scarce data in this field of proteomics hypertension research.

7.3.5.3 Genomics in hypertension

A large case-control GWAS study of hypertensives and controls found associations between hypertension and a *UMOD* gene variant (197). The authors suggested a possible link with sodium reabsorption and homeostasis, as uromodulin production is chiefly localized in the proximal tubulae, where also sodium reabsorption takes place.

7.4 Analytical methods

There are two main analytical approaches in metabolomic studies in nephrology. Mass spectrometry, in conjunction with a separation technique such as gas chromatography (GC-MS) or liquid chromatography (LC-MS) on one side, and nuclear magnetic resonance spectroscopy (NMR), on the other.

7.4.1 Mass spectrometry

Mass spectrometry is an analytical technique for molecular characterization of substances, most commonly used in chemistry. A mass spectrometer is capable of analyzing gases, liquids and solids. It works by ionizing the molecules in the sample under analysis, accelerating the ions so that they travel in a vacuum through a graded electromagnetic field, and colliding them onto a detector. Based on mass and ion charge the ions bend to different degrees as they pass through the graded electromagnetic field, and hit the detector at different locations. The MS ultimately describes the metabolites by the mass-to-charge ratio (*m/z*) of their ions. In tandem mass spectrometry (MS/MS), which is used in quantitative MS, the molecules are ionized, then selected in a first filter (Q1) that lets through only ions of a certain *m/z*. Then those ions collide with a gas (usually argon) in a collision chamber (Q2) to characteristically fragment into "daughter" ions, and then the "daughter" ions move through a second filter (Q3) that again lets through only ions of a certain *m/z*. Based on known fragmentation patterns, this allows precise identification of ions and hence molecules, at high sensitivity and specificity.

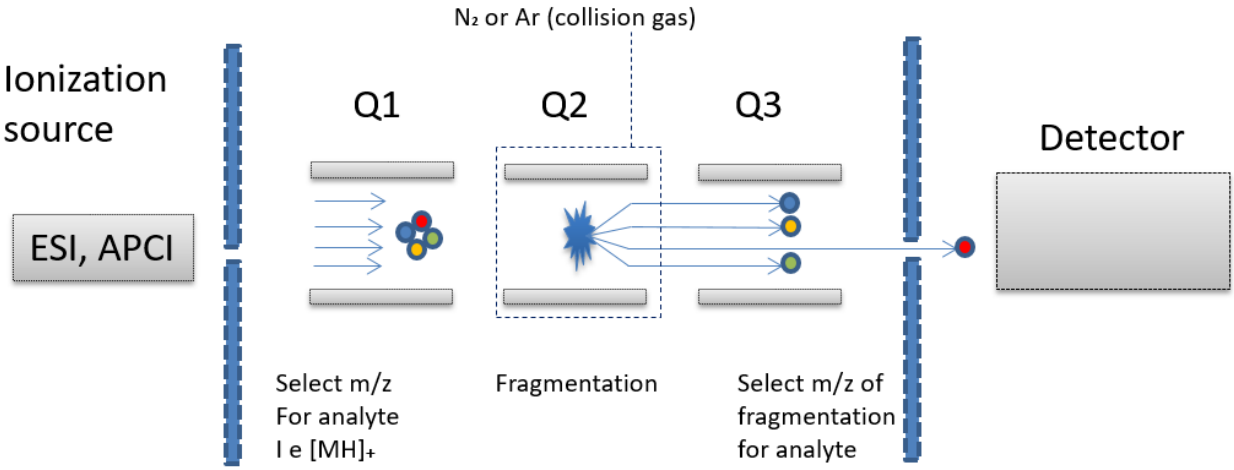

Figure 7. Principle of mass spectrometry. Molecules are ionized, travel through an electromagnetic field, and crash into the detector. (Copyright PK Øvrehus)

There are different analytical methods in mass spectrometry, of which time-of-flight (TOF) MS, triple quadrupole MS, and ion trap MS are the principal (198).

Before the molecules can travel through the spectrometer they have to be ionized. There are several different ionization methods, such as electron ionization (EI) and chemical ionization (CI) in GC-MS, and electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in LC-MS.

Common to most MS analyses is a separation step prior to mass spectrometry. The most common separation steps are gas chromatography (GC) and liquid chromatography (LC).

7.4.2 GC-MS

In gas chromatography, the gas phase of the sample travels with a carrier gas (usually helium) through an analytical column. Compounds interact differently with the carbon lining inside the column, causing sample compounds to elute from the column at different time points (called retention times), and so you have separation, prior to injection into the mass spectrometer. Gas chromatographic retention times are reproducible and robust (199). Samples are converted into the gas phase in part spontaneously, as some metabolites are naturally volatile, and in part through pretreatment with substances to make the metabolites in the sample more volatile, through a process called derivatization. Trimethylsilylation (TMS) (sugars), and deritivization by ethyl chloroformate and methylchloroformate (MCF) (amino and organic acids), are commonly used (200). Electron ionization (EI) is the most common ionization method in GC-

MS. Characteristic retention times, together with characteristic MS patterns, allow separation and identification of molecules by the aid of libraries and databases. GC-MS is particularly adept to the analysis of volatile metabolites, and offers high sensitivity and reproducibility (201). One disadvantage of GC-MS is the laboursome sample pretreatment. Also, incomplete derivatization, which may produce different adducts of the same parent substance and at different rates, may disturb accurate quantification. Also, a GC-MS set-up is less stable than NMR, and requires regular calibration to correct for analytical drift over time (200). The major limitation of GC-MS is that it is able to analyse only volatile molecules or molecules that can be made volatile (201).

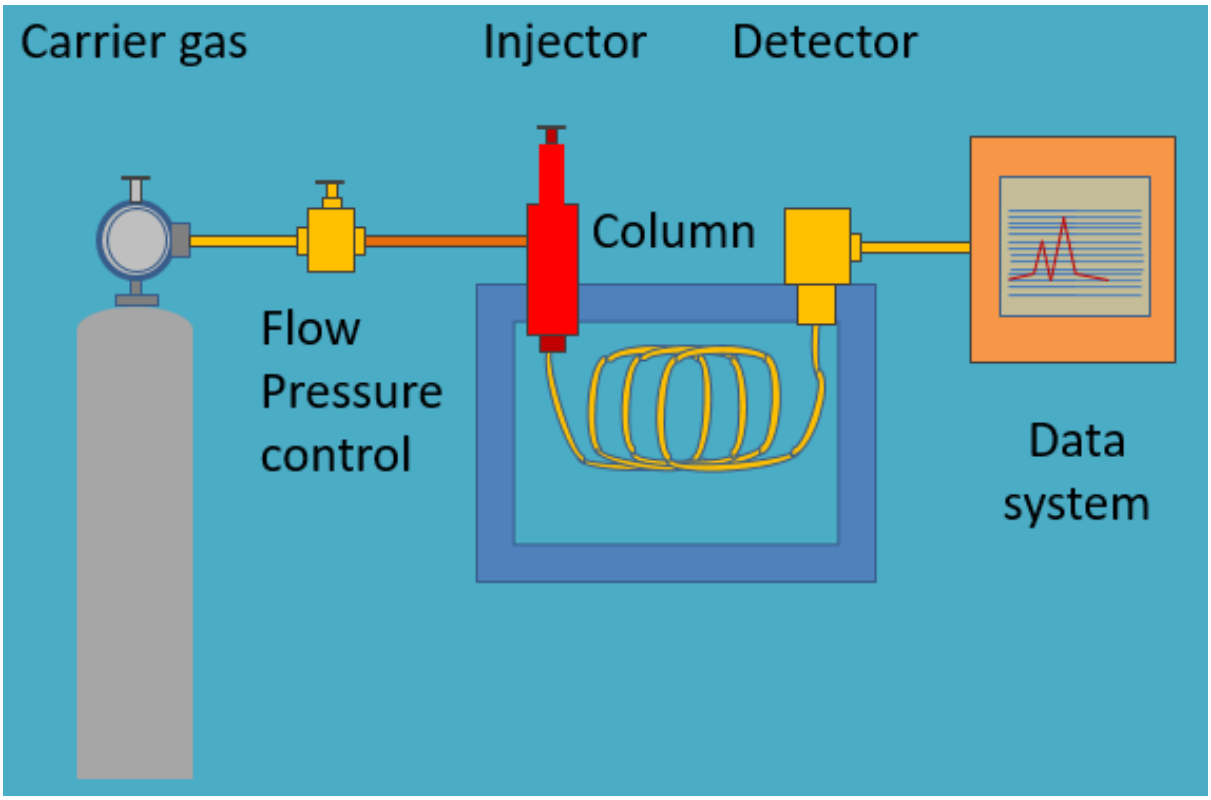

Figure 8. Principle of gas chromatography. The sample travels from the injector through the column to the detector. In GC-MS, the sample is further injected into the mass spectrometer. (Copyright PK Øvrehus)

7.4.3 LC-MS

In liquid chromatography, the liquid phase of the sample travels through an analytical column. Due to different physiochemical interaction between sample and column the compounds elute at different times (retention times), and so you have separation, prior to injection into the mass spectrometer. LC-MS has high sensitivity, in the pico mole range, offering detection of

metabolites that are below the NMR detection limits in some cases. It uses different column chemistries to separate different metabolite groups. For example, hydrophilic interaction chromatography (HILIC) for polar metabolites (e.g. amino acids, sugars), and traditional reverse phase chromatography (RP) for non-polar metabolites (e.g. lipids) (201, 202). This way LC-MS is able to cover a broad range of metabolite classes. LC-MS does not demand derivatization. LC-MS is rapid with run times in certain standard reverse phase set-ups as short as a few minutes. It has advantages over NMR in dealing with biofluids with high salt content, such as urine (90). As for limitations, LC-MS requires that the analytes are ionizable, and so optimization of separation for each class of metabolites is quite important. Another weakness is the gradual change in chromatographic column and MS properties, called analytical drift. Changing physiochemical properties of the column over time, and the mass spectrometer getting dirty, causes gradual analytical differences both within batches and over time. One way of surveilling intra-batch drift is by quality control (QC) samples. Standardized QC samples, made up of internal standards or pooled analytes, are analyzed regularly at the beginning, throughout and at the end of the run. If the drift is small, the QC samples results should cluster well (203). Also, LC-MS is less reproducible between labs, and has more challenges with data standardization and reporting, than GC-MS (204).

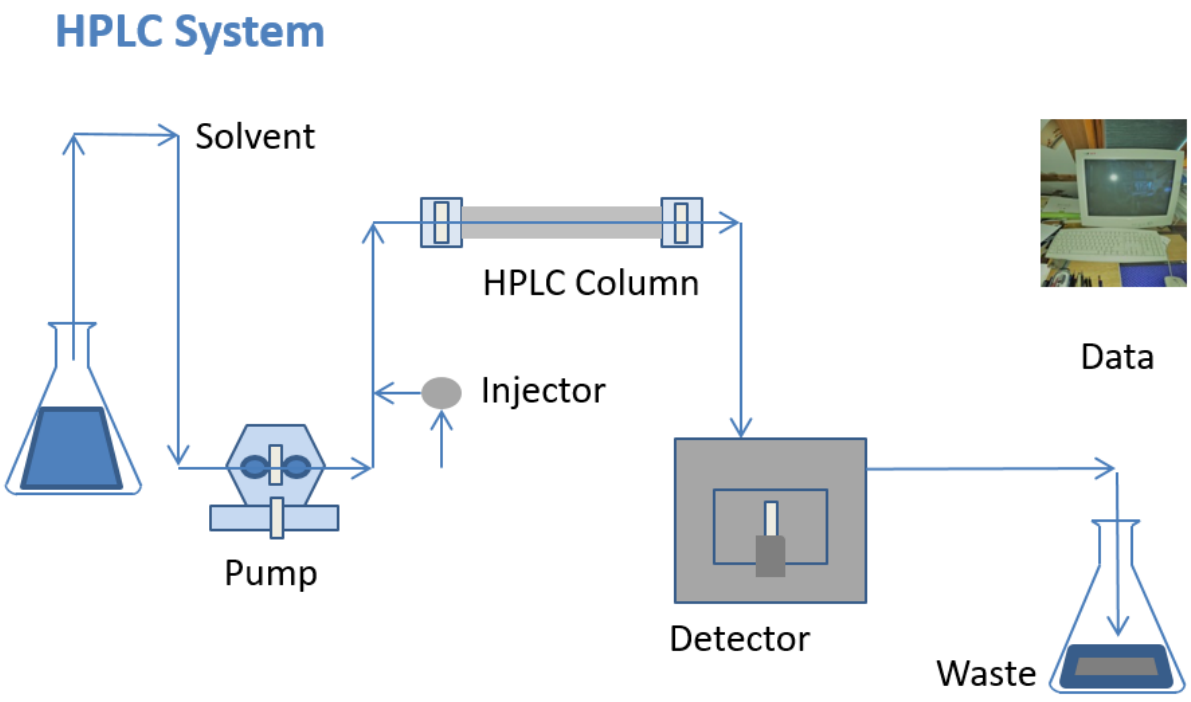

Figure 9. Principle of liquid chromatography. The sample travels from the injector through the column to the detector. In LC-MS, the sample is further injected into the mass spectrometer. (Copyright PK Øvrehus)

7.4.4 CE-MS

Capillary electrophoresis is a separation step that sends the liquid sample through a long capillary rather than through a shorter column, and achieves separation through the interaction between the metabolites and the lining of the capillary. It is a relatively fast technique with good resolution and sensitivity (205).

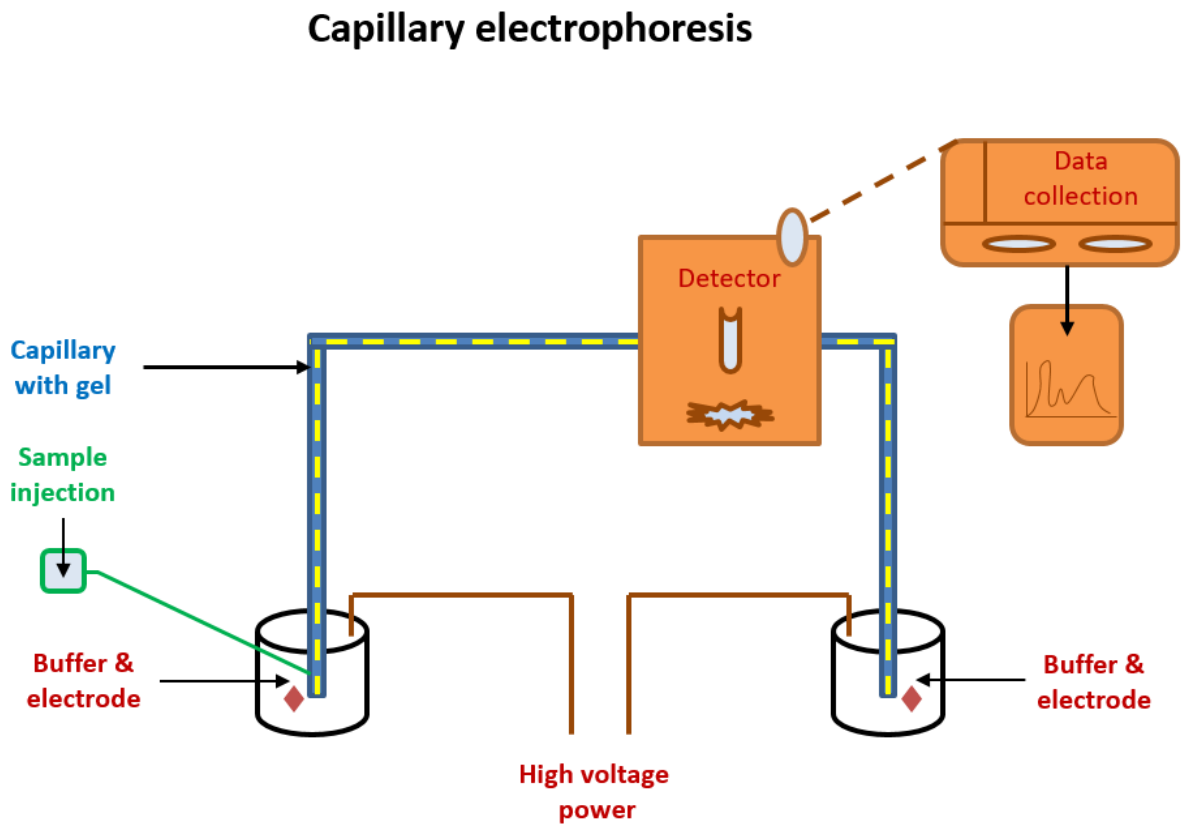

Figure 10. Capillary electrophoresis. The sample travels from the injector through the capillary with a liquid buffer to the detector. In CE-MS, the sample is further injected into the mass spectrometer with its detector. (Copyright PK Øvrehus)

7.4.5 NMR

NMR was the first of these techniques to emerge, and has been around in practical use since the 1970s. NMR involves sending a radiofrequency pulse into a biological sample located in a strong magnetic field, where the magnetic features of a few atomic nuclei (most commonly ^{1}H or ^{13}C) in the sample absorb the pulse, and subsequently re-emit, or resonate, electromagnetic radiation in a manner characteristic of the substance. Using so-called chemical shift patterns in this signal, that are influenced by neighbour atoms, it is possible to elucidate local molecular structure and abundance of compounds, thus making chemical identification and quantitation possible (198). NMR has the advantage of requiring little sample pre-treatment or separation

step, in contrast to GC-MS and LC-MS. It is able to analyze a broad range of samples, from biofluids such as urine and extracts in the liquid form, to tissues such as kidney biopsies and solid organs (90). NMR examination does not destroy or consume or permanently alter the samples, as derivatization in GC does, so samples may easily be carried over from NMR to other platforms. NMR has proven to have high analytical reproducibility, both over time and between labs (206), and sample automation is readily feasible. Furthermore, accurate quantitation is possible in NMR without the use of internal standards.

A major limitation of NMR is its sensitivity, which is relatively low compared to mass spectrometry methods. This has in part to do with the fraction of NMR detectable nuclei in a given substance, which, following the Bolzmann distribution, is grossly outnumbered by nuclei that do not give rise to a detectable signal by NMR. This leads to the detection of only the most abundant metabolites. Another disadvantage is that NMR instruments are relatively expensive. NMR was not used in our studies.

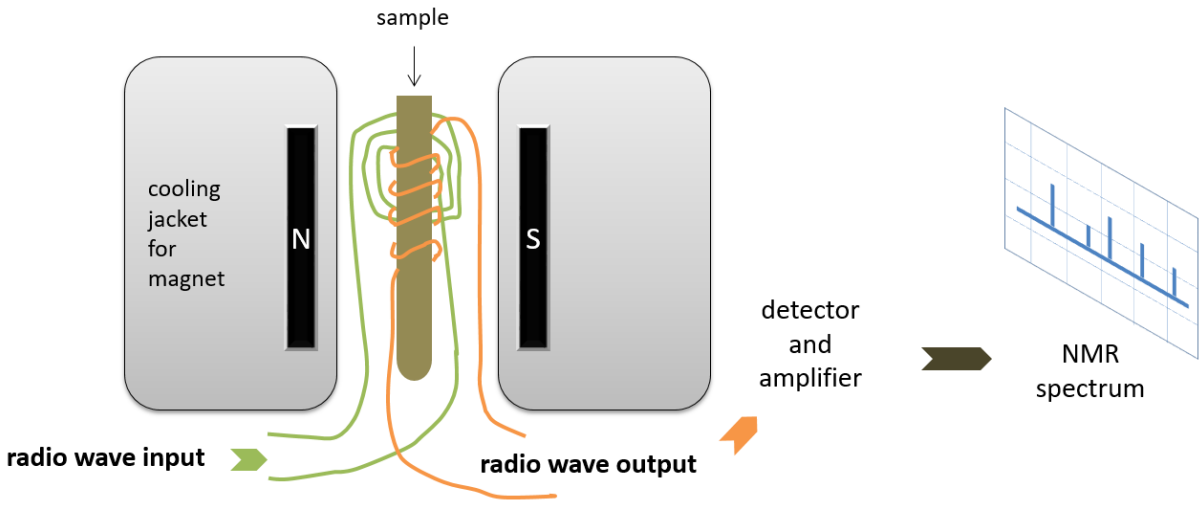

Figure 11. Nuclear magnetic resonance. The sample is inserted inside the electromagnetic coil, radio waves are fired at the sample, and the atoms in the sample resonate, producing radio waves back. (Copyright PK Øvrehus)

7.4.6 Targeted and non-targeted analysis

Targeted studies measure a limited number of compounds with a high degree of quantitative accuracy. This allows robust metabolite identification and quantification for a precise phenotype characterization. This may help the biological interpretation of the findings (207). However, targeted studies will only find perturbations in the metabolites measured, so one must

have an *a priori* hypothesis that the measured metabolites are biologically interesting. Untargeted studies measure all the metabolites visible to your analytical technique. They are suitable for *de novo* biomarker discovery, hypothesis generation and often offering a wider set of compounds than targeted studies. However, untargeted studies are also fraught with a lower degree of definitive compound identification, or at least more steps necessary to ensure identification. This may increase the risk of false positive results, i.e. type I error.

In targeted approaches, both NMR and MS are applicable. NMR, albeit limited by low sensitivity, has good specificity and ability to assign definitive metabolite identities from the peaks that do arise from the sample. MS conversely generates many peaks, but the identification of metabolites is often not complete using only chromatographic retention time and *m/z*. Here, tandem MS is very useful, which makes possible the monitoring of selective "mother"/"daughter" ions corresponding to known metabolite fragmentation patterns from databases and commercial standards (198). A weakness with targeted strategies is that they confer a narrower vision of the metabolism, maybe missing out on significant biological processes that shape the role of the targeted metabolites.

In non-targeted approaches, the strategy is to generate many peaks from the samples, for example using scan mode methods, and then identify qualitative differences in peak distributions between the different groups, for example case vs control. Being more of a descriptive strategy, and good at identifying qualitative differences, the non-targeted approach has a weakness in that it does not necessarily give definitive insight into the biological processes at hand, as the focus is not on metabolite identification.

All in all, NMR and targeted LC-MS and/or GC-MS have been the most used analytical methods in metabolomics studies to date (34).

7.5 Data analysis and statistics

Given the complexity and vastness of data produced in metabolomics methodology, proper data analysis methods are required. This includes data preprocessing, featuring for example baseline correction, normalization, scaling and peak alignment, the object of which is to correct for data variance that is associated with analytical and matrix effects (202).

7.5.1 Data correction

There is often a need for data correction to adjust for analytical variance before data analysis in metabolomics experiments. The aim is to correct for analytical variance related to matrix effects, ion suppression, and drifts in instrument performance within and between batches. One technique is to add known internal standards in known amounts to each sample, and then analyze the internal standard signal within and between batches, and over time. Ideally, if the internal standard signal is reduced by 15% for some reason, the metabolite response should go down by 15% simultaneously in order to avoid a falsely high response. Optimally, one should have one internal standard per analyzed metabolite, but for practical purposes a group of internal standards are often used to evaluate the response of an even larger group of metabolites (208). These internal standards are often deuterated (heavy) versions of metabolites. Another technique is based on quality control (QC) samples for every n sample analyzed. A QC sample often consists of very small aliquots from all the samples added together into a so-called pooled QC sample. By comparing signal from the QC samples across batches, and adjusting metabolite responses to QC sample responses, it is possible to correct for analytical variance (208-210).

7.5.2 Normalization

In contrast to blood, where solute and water concentration are finely regulated, urinary solute concentrations vary greatly from day to day and hour to hour according to water intake. Accordingly, metabolite concentrations will vary with urine concentrations, and normalization is used to correct for these fold changes. There are many urine normalization strategies. Normalization to urine volume, to urine osmolality, to creatinine by either measured creatinine concentration of fixed volumes or dilution of urine samples to the same creatinine concentration, and to total combining injection volume calibration with creatinine and MS signal normalization (211, 212). The optimal method is not clear (213, 214). Although no true universal standard exists, it is fair to say that urinary creatinine and urinary osmolality are the most frequently used normalization factors (212), and they have been recommended in at least one review (214).

7.5.3 Statistics

A combination of univariate and multivariate testing is used, in addition to more elaborate statistical techniques. Univariate testing of non-normally distributed values, such as urine concentrations, often requires non-parametric methods, such as the Mann Whitney *U*-test (two-sample Wilcoxon rank test).

Multivariate statistical analyses are essential to simplify and find patterns in the data. Often the starting point is unsupervised methods, such as principal component analysis (PCA), which identifies the underlying component that explains most of the variance of the data set (principal component 1), then identifies the component that explains most of the residual variance of the data set after principal component 1 is subtracted (principal component 2), and so on. This is useful for giving an overview of clustering in the material, and does not use a training set, so the input is clustered in what is called an unsupervised way (201). Hierarchical clustering analysis (HCA), K-means clustering, and independent component analysis (ICA) are other unsupervised methods. Supervised methods, on the other hand, such as orthogonal partial least squares discriminant analysis (O-PLS-DA), use a training set of the data to build a classification model, and then classifies or clusters the data according to this model, to evaluate overall discrimination between patients and controls. Variable importance in projection (VIP) is used as a measure of the importance of variables from the PLS-DA analysis. The VIP score is based on the sum of variable influence over all model dimensions, looking at the PLS loadings relative to the amount of explained Y-variation (215), and can be used to identify discriminating variables or predictors (216). False Discovery Rate (FDR) is one of several techniques to adjust for multiple testing calculated according to the Benjamini Hochberg ranking procedure (217).

8 AIMS

The overall aim is to improve the treatment and outcomes in patients with hypertensive nephropathy. The initial motivation for the thesis was the lack of knowledge on hypertensive nephropathy, despite its high prevalence in clinical nephrology and in registries. We aimed to describe the extent of hypertensive nephropathy, its clinical characteristics, and how well we diagnose it today. We also aimed to elucidate the pathophysiology of hypertensive nephropathy, as there is scarce biological data, especially from the omics disciplines.

Specific aims:

Paper 1: How prevalent is CKD, and how has the prevalence changed? We aimed to describe the trends in the prevalence of chronic kidney disease, and evaluate the influence of modified cardiovascular risk factors on CKD prevalence.

Paper 2: How prevalent is hypertensive nephropathy, and how do we diagnose it today? We aimed to describe the clinical characteristics of biopsy-verified hypertensive nephropathy patients, and test the accuracy of traditional and tentatively new clinical criteria for the diagnosis**.**

Paper 3: How can we improve diagnosis and prognosis in hypertensive nephropathy? We aimed to describe the diagnostic precision of a urinary proteomics test in advanced chronic kidney disease with a set of hypertensive nephropathy cases, and retrospectively evaluate the association between proteomics and rapid kidney function decline.

Paper 4: What are the pathophysiological mechanisms behind hypertensive nephropathy? We aimed to describe perturbations of gene expression and urinary amino acid excretion in individuals with early stage hypertensive nephrosclerosis**.**

9 MATERIAL

Epidemiologic data was gathered by accessing patients' electronic health records (Paper 3), and through structured questionnaires in combination with central Norwegian health registries (Paper 4 and III). Data was also collected from national Norwegian health registries (Paper 2 and IV).

9.1 The Nord-Trøndelag Health Study (HUNT3)

Nord-Trøndelag county is situated in the central region of Norway, with a population of 128 694 in 2006 (218). The population is ethnically homogenous, with approximately 3% being of non-Caucasian origin (219). Migration out of or into the county is low, at only 0.3% of the population per year (220). The county is fairly representative of Norway in regards to age distribution, geography, economy, morbidity and mortality(219).

The Nord-Trøndelag Health Study III (HUNT3) was a cross-sectional population study performed in the county of Nord-Trøndelag between October 2006 and June 2008 (Paper 4, III and IV). It was preceded by the previous HUNT1 (1984-86) and HUNT2 (1995-97) studies. The HUNT3 study invited all inhabitants aged 20 years or more (n=93860) living in Nord-Trøndelag county, by mailing a questionnaire along with a personal invitation to undergo a physical examination. A total of 50 807 (54.1%) participated, with lowest participation rates in those older than 80 and younger than 40 years (221). Data was collected in all the 24 municipalities at temporary examination sites by nurses and technicians.

A self-report questionnaire (Q1) was filled in at home before attending the physical examination, and included questions on education and profession, diseases such as coronary heart disease, stroke, diabetes, and hypertension, as well as smoking, physical activity, and diseases in the family. After the clinical examination, participants received a second questionnaire with a sex and age specific part (Q2), and a third questionnaire (Q3) to participants with particular diseases such as diabetes or cardiovascular disease or cancer as stated in Q1, with questions on symptoms, disease duration, medication, and complications.

In September 2017 the HUNT4 study started collecting data.

9.2 The Norwegian Kidney Biopsy Registry

The Norwegian Kidney Biopsy Registry (NKBR) collects clinical and histopathological data for all patients who undergo a kidney biopsy in Norway (5.0 million inhabitants, biopsy frequency 150 per million inhabitants per year in 2013) (222, 223) (Paper 2). After its

establishment in 1988, around 14 000 biopsies have been included (until 2015)(18). Only biopsies on native kidneys for non-neoplastic indications are registered. The NKBR registers the biopsy indication, known or suspected systemic diseases, clinical and lab data. All biopsies are examined by light microscopy and standard immunohistochemistry (staining for IgA, IgM, IgG, C1q and C3), and electron microscopy when necessary. Biopsies are examined first locally in one of the six Norwegian nephropathologist centres, and then reviewed by an experienced nephropathologist at Haukeland University Hospital, Bergen, for uniform diagnosis. In 2016, the NKBR was fused with the Norwegian Renal Registry.

10 METHODS

10.1 Clinical measurements

In HUNT3 trained nurses measured clinical data, including blood pressure, which was measured three times with one minute intervals with participants sitting down, by an automatic oscillometric method (Dinamap 845XT, Criticon, Tampa, FL, USA). Furthermore, heart rate, weight, height, and circumferences of hip and waist were measured. Venous blood sampling was done non-fasting for all participants when they attended. Fresh midstream urine samples were collected at the study venue, and first frozen to -20° Celsius, then further to - 80° Celsius within 24 hours, without centrifugation or additives.

10.2 Urine analysis

Dipstick analysis was carried out on an Urisys 1100 apparatus (Roche Diagnostics, Basel, Switzerland). Urine creatinine was measured at Levanger Hospital, Levanger, Norway by the enzymatic method using an ABX Pentra 400 apparatus (Horiba ABX SAS, Montpellier, France).

Urine metabolomics analysis of amino acids was performed using liquid chromatography coupled with mass spectrometry, in our lab at NTNU. The frozen urine samples were thawed at the HUNT Biobank lab, aliquoted into 200 µL tubes, and refrozen. We subsequently thawed 200 µL of urine on ice, vortexed for 5 seconds, removed 80 µL urine and added to 320 µL of MS-grade water and 10 µL of internal standard stock. The internal standard stock consisted of a pool of five deuterated so-called "house-keeping" metabolites: d3-alanine, d4-succinate, d8-valine, d2-tyrosine and d2-fumarate (Cambridge Isotope Laboratories, Tewksbury, MA, USA). The internal standards were all diluted to a concentration of 1 mM. The 400 µL samples were then centrifuged for seven minutes at 14 000 rotations per minute at 4 °C to remove protein using a 3000 Da molecular weight cut-off filter (VWR Centrifugal Filter, modified PES, 3 K, VWR International, West Chester, PA, USA). Forty µL of the resulting urine was pipetted into a well on four parallel 96 well plate duplicates and frozen at -80°C for subsequent analysis. The 96 well plates were then loaded into the autosampler at a temperature of 4°C.

For the targeted amino acid analyses, the urine samples were derivatized using the EZ:faast LC/MS Physiological (Free)Amino Acids Kit (Phenomenex, Inc, Torrance, CA, USA). All samples were spiked with three isotopically labelled internal standards, HARG, HPHE, and d3Met. Standard mixes and internal standards were provided in the EZ:faast kit. In brief, the previously spin-filtered and frozen urine was thawed and gently vortexed, and 30 µL of it was

mixed with 10 µL internal standard solution and 160 µL MS-grade water. Then 200 µL Na2CO3 was added, and the mixture was absorbed into the Phenomenex absorbing tip, and eluted with 200 µL of an eluting agent consisting of NaOH and N-propanol, 3:2 ratio. Then 50 µL chloroform was added, vortexed and allowed to react (alternative: oximation reaction with chloroform?). Then 200 µL iso-octane were added and allowed to react, resulting in a two-layered mixture. From the supernatant 50 µL was taken and desiccated over 5 minutes under a gentle flow of N2, followed by reconstitution in 80 µL H20/formic acid. Thirty µL of this was inserted into an MS vial and capped, and 2 µL was injected into the LC-MS for analysis. A 5 uM standard mix was injected for every 10 to 20 sample as a quality control sample.

Urinary metabolomics analysis was carried out using liquid chromatography coupled with mass spectrometry. Liquid chromatography was performed on an Acquity HSS (High Strength Silica) T3 1.8 µm UPLC 2.1 x 100 mm chromatographic column, running on the ACQUITY ultra-performance liquid chromatography (UPLC) platform (Waters Corporation, Milford, MA, USA). The sample injection volume was 2 µL and UPLC mobile phase flow rate kept at 400 µL/min. Sample and column temperatures were 4°C and 40°C, respectively. Mobile phases: A: 95% acetonitrile and 0.1% formic acid, at a 10mM final concentration. B: 50% acetonitrile, 50% ammonium acetate, at a 10 mM final concentration. With seven minutes run time, the eluent gradients were as follows: A 99% and B 1 at 0.0 minutes; A 99%, B 1%; at 1.0 minutes, The column was primed by five initial injections of high-grade water, then repeated injections of the Standard Mix 1 (QC) sample. Then 12 samples were run, followed by a QC sample. Then 12 new samples were run, followed by a QC sample, and this was repeated until the last sample, which was followed by three QC samples. The liquid chromatography was coupled with the Synapt G2-S High Definition Mass Spectrometer (MS) (Waters Corporation, Milford, MA, USA) for LC-MS/MS analysis. Samples were run in negative mode and positive mode, and in UPC2 mode using the UPC2 (Waters Corporation, Milford, MA, USA). The instrument was set up according to the manufacturer's manual, using leucine-enkefalin (MW 554.2615 Da in negative mode) as lock-mass compound. Calibration was carried out using mM sodium formate solution. Capillary voltage was set to 2.5 kV in negative mode and 3.2 kV in positive mode. Source temperature was 120 °C. Desolvation temperature was 250°C. All amino acids were analyzed as mass spectrometry responses. In addition, calibration curves were constructed for 24 of 47 amino acids, where we had available standard mixes in dilution series.

Urine proteomics analysis was performed at the Mosaiques Diagnostics lab in Hannover, Germany. For CE-MS analysis a 0.7 mL urine aliquot was thawed immediately before use and

diluted with 0.7 mL 2 M urea, 10 mM NH_4OH containing 0.02 % SDS. In order to remove high molecular weight polypeptides, samples were filtered using Centrisart ultracentrifugation filter devices (20 kDa molecular weight cut-off; Sartorius, Goettingen, Germany) at 3,000 g until 1.1 mL of filtrate was obtained. Subsequently, filtrate was desalted using PD-10 column (GE Healthcare, Sweden) equilibrated in 0.01% NH_4OH in HPLC-grade water. Finally, samples were lyophilized and stored at -20°C. This procedure results in an average recovery of sample in the preparation procedure ~85%.(92) Shortly before CE-MS analysis, lyophilisates were resuspended in HPLC-grade water to a final protein concentration of 0.8 µg/µL checked by BCA assay (Interchim, Montlucon, France). CE-MS analysis was performed as previously described.(224, 225) The limit of detection was ~1 fmol, mass resolution was above 8000 enabling resolution of monoisotopic mass signals for $z \leq 6$.

After charge deconvolution, mass deviation was <25 ppm for monoisotopic resolution and <100 ppm for unresolved peaks (z>6). The analytical precision of the platform was assessed by (a) reproducibility achieved for repeated measurement of the same replicate and (b) by the reproducibility achieved for repeated preparation and measurement of the same urine sample; details on analytical precision were reported recently (163). To ensure high data consistency, a minimum of 800 peptides/proteins had to be detected with a minimal MS resolution of 8,000 in a minimal migration time interval of 10 min. Mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using MosaiquesVisu software (226). Both CE-migration time and ion signal intensity (amplitude) showed variability, mostly due to different concentrations of ions in the sample, and were consequently normalized. Reference signals of 1770 urinary polypeptides were used for CE-time calibration by local regression. For normalization of analytical and urine dilution variances, MS signal intensities were normalized relative to 29 internal standard peptides generally present in at least 90% of all urine samples with small relative standard deviation. For calibration, linear regression was performed (227). The obtained peak lists characterized each polypeptide by its molecular mass [Da], normalized CE migration time [min] and normalized signal intensity. All detected peptides were deposited, matched, and annotated in a Microsoft SQL database (Microsoft, California, USA) allowing further statistical analysis.

CE-MS measurement of the urine samples and data processing resulted in a maximum of 5,616 distinct peptides, which described the human urinary low-molecular-weight proteome (228, 229). The CKD273-classifier is a SVM-based classification model (230-232), which allows the

classification of samples in the high dimensional parameter space using MosaCluster software (version 1.7.0) (233).

Applying the CKD273-classifier to CE-MS data of unknown samples, MosaCluster calculated classification scores, based on the amplitudes of the 273 CKD-biomarkers. Classification is performed by determining the Euclidian distance (defined as the SVM classification score) of the 273-dimensional vector to a 272-dimensional maximal margin hyperplane, which was defined previously (163). The cut-off of the classification score was determined with the result of the biomarker discovery cohort in Good et al (163). Patients with urine samples who had classification factors exceeding 0.343 were classified as CKD273-classifier positive cases and patients with urine samples scoring below 0.343 were classified as CKD273-classifier controls. All data were calibrated and annotated to the Mosaiques human urinary database.

10.3 Data analysis and statistics

Data correction

Raw data files were processed using the Mosaiques Visu software (Biomosaiques Software, Hannover, Germany) in Paper 3, and the Transomics software (Waters, Milford, MA, USA) in Paper 4 for peak picking, alignment and deconvolution. Data was checked for analytical variance in Paper 4 by evaluating responses from the kit-provided internal standards homoarginine (HARG), homophenylalanine (HPHE) and methionine-D3 (d3Met), and then adjusted based on responses from QC samples consisting of a standard mix of 24 amino acids for every 20 sample, as explained below.

In Paper 3 quantitation was based on calibration lines calculated by linear regression. In Paper 4 quantitative analyses were done using the MassLynx software application TargetLynx (Waters, Milford, MA, USA). To be included in the further quantitative analyses in Paper 4 metabolites had to show relatively stable intensities across the repeated QC samples, with a predefined criterion of relative standard deviations < 30%. According to QC requirements metabolites were excluded if >20% of measurements were at zero concentration or missing. The MS data was filtered in a stepwise manner outlined by Want (210). For every metabolite, the non-normalized intensity data was exported to Excel, and adjusted to the signal of the different QC samples (as mentioned above) throughout the batch to cater for analytical drift: the samples were first sorted in run order and the sample list then divided into QC blocks. Each

block started with a QC sample, then a QC sample in the middle, and ended with a QC sample. The average of these three QC samples from the same block was divided by the grand average of all the QC samples, to produce a block constant. Intensities in all the samples in the same block were divided by this constant, to adjust the feature intensities according to the feature intensities seen in the QCs along the batch. This model of "feature-based correction algorithm (*using*) the QC intensities in the local neighborhood of each sample to formulate a correction factor" has been described by Kamleh (209). The resultant intensities were processed using Metaboanalyst 3.0 (215).

Normalization

In Paper 3, responses were normalized to 29 internal standard peptides present in ≥90% of all samples with small variance. In Paper 4, responses were filtered using non-parametric relative standard deviation data filtering, as recommended in large data sets (234), normalized to urinary creatinine, log transformed and auto scaled (mean-centered and divided by the standard deviation of each variable) in Metaboanalyst 3.0 (215).

Statistics

Univariate analysis: In Paper 3 mean proteomic score in the two groups were compared with two-sample t-test. The metabolites in Paper 4 were non-normally distributed, so the non-parametric Mann Whitney *U*-test was used for univariate testing of difference in means of amino acids between controls vs hypertensive nephropathy. Data exploration was done using fold change and volcano plots. Adjustment for multiple testing was done using False Discovery Rate (FDR) according to the Benjamini Hochberg ranking procedure (217) in Metaboanalyst 3.0.

Multivariate analysis: In Paper 3 the formerly established support vector machine (SVM)-based classification model CKD273 was applied to CE-MS data of unknown samples (230). The MosaCluster software calculated classification scores based on the amplitudes of the 273 CKD biomarker peptides, and samples were classified as CKD or not CKD relative to a previously validated cut-off value. Linear regression was used for calibration and for computing decline in kidney function over time, and logistic regression to describe the associations of specific urinary proteins to CKD and to rapid kidney function decline. Also in Paper 3 we created Receiver Operator Characteristic (ROC) curves, and tested the diagnostic precision by ROC area equality of different logistic regression based models (e.g. base model + albuminuria vs enhanced model

+ albuminuria + proteomic score). We also used risk reclassification in Paper 3. Significance testing is difficult in risk reclassification, but these are useful for demonstrating how different risk prediction models changes the risk estimates in subjects with and without the outcome (235).

In Paper 4, preliminary data exploration was done using principal component analysis (PCA), an unsupervised clustering analysis that explains the variance of the data set by a small number of factors, called principal components (PCs). In our metabolomics study, every PC is a linear combination of metabolite contributions to variance, and PC1, the first of PCs produced in the analysis, explains the largest amount of variance in the data set. PC2 explains the largest amount of variance that PC1 did not account for, and so on. The PCs are all orthogonal to each other, and together form a new matrix built up of a score matrix and a loadings matrix. The scores matrix has the positions of the linear transformations of the original observations (i.e. individual samples) in the new coordinate system, and the loadings matrix indicates the variables (i.e. metabolites) with the highest weights in transforming the samples from the original data to their positions in the scores matrix (236, 237). This way one can reduce a complex metabolomics matrix of for example 100 patients x 100 metabolites into a three-dimensional coordinate system defined by PC1, PC2 and PC3, for quick visualization of the data, identifying outliers, evaluating analytical drift, and getting a first impression of how the data clusters, and what metabolites and samples drive that separation (236).

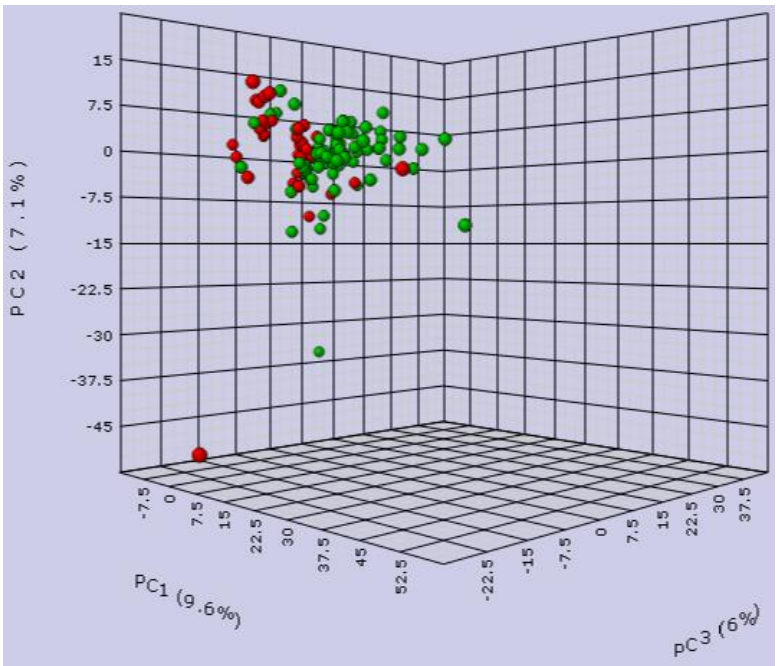

Figure 7. A PCA 3D plot showing clustering in a three-dimensional coordinate system created by PC1, PC2 and PC3 (copyright MA Øvrehus).

Next we used partial least squares discriminant analysis (PLS-DA), a supervised method which employs multiple linear regression techniques to find the direction of maximum covariance between the data set and group label. PLS-DA uses the data to build a training classification model, then clusters the data according to this model to evaluate overall discrimination between patients and controls. Variable importance in projection (VIP) is a frequently used outcome measure in PLS-DA analysis, and is based on the sum of variable influence over all model dimensions, looking at the PLS loadings relative to the amount of explained group variation (215). VIP scores can be used to identify discriminating variables or predictors (216).

Another technique used in Paper 4 was over-representation analysis, which tests if compounds are present more than expected by chance. We also did pathway enrichment analysis, which tests if a group of compounds representing known metabolic pathways is present more than expected by chance. A pathway topology analysis in addition takes into consideration that some compounds are more central than others in a metabolic pathway, and that changes in metabolites that are crucial to a pathway has larger impact than changes in more peripheral compounds.

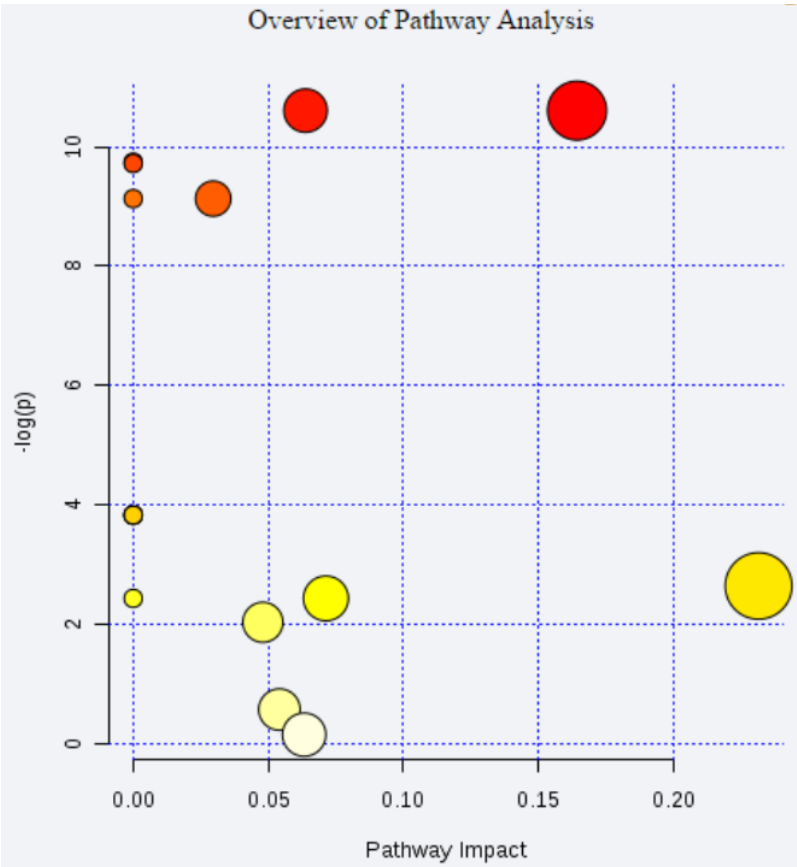

Figure 8. Example of a pathway analysis plot (over-representation analysis) on Metaboanalyst. Each circle represents a different biological pathway; x-axis: pathway impact, y-axis: -log(p). (Copyright MA Øvrehus)

Parallell to this we was also utilized gene ontology (GO) enrichment analysis, to integrate gene expression data to an easier to understand description of overall biological function. (238). Finally we combined gene and metabolite information into a so-called integrated pathway analysis.

Analytic variance was estimated using Excel software (Microsoft, Redmond, WA, USA), and descriptive statistics and some hypothesis testing were done using Stata software (StataCorp LP, College Station, TX, USA).

10.4 Ethical considerations

All participants gave informed consent when included in the Norwegian Kidney Biopsy Registry and the HUNT study, including linkage to central national registries. The studies in

Paper 3, II, III, and IV were approved by the Regional Committee for Medical and Health Research Ethics Central Norway. The studies in Paper 2 and IV were approved by the Norwegian Data Protection Authority. The study in Paper 2 was approved by the Norwegian Ministry of Health. All participants gave written consent.

10.5 Funding

The work was supported by a research grant from the Liaison Committee between the Central Norway Regional Health Authority and the Norwegian University of Science and Technology.

11 RESULTS

We have described trends in CKD development, evaluated the prevalence, diagnosis, and prognosis in hypertensive nephropathy, and carried out urine proteomics and metabolomics analysis for diagnosis in advanced CKD and early hypertensive nephropathy.

11.1 Summary of Paper 1

Surveillance of CKD prevalence over time and information on how changing risk factors influence this trend are needed to evaluate the effects of general practice and public health interventions. However, very few studies have addressed this topic. We studied the total adult population of a demographically stable county representative of Norway using cross-sectional studies 10 years apart (HUNT2 and HUNT3, participation rates 70% and 54%, respectively). Thorough quality-control and comparisons of methods over time excluded analytical drift, and multiple imputations of missing data and attendance-weights contributed to unbiased estimates. CKD prevalence remained stable in Norway from 1995-97 (11.3%, n=65,237) to 2006-08 (11.1%, n=50,586) (p=0.42). The association of survey period with CKD prevalence was modified by a strong blood pressure decline, more physical activity and lower cholesterol; without these improvements 2.8, 0.7, and 0.6 percentage-points higher CKD prevalence could have been expected, respectively. In contrast, prevalence of diabetes and obesity increased moderately, but the proportion of diabetic patients with CKD decreased substantially (33.4%-28.6%, p=0.002) so only a 1.0 percentage-point lower CKD prevalence could have been expected without these changes. In conclusion, CKD prevalence remained stable in Norway over a 10-year period characterized by strong improvements in blood pressure, lipids and physical activity and only modestly increasing diabetes and obesity.

11.2 Summary of Paper 2

Hypertensive nephrosclerosis is assumed to be the second most common cause of kidney failure. The diagnosis is often assumed but not always biopsy-verified in CKD patients with long-standing hypertension, no/low-grade proteinuria, no diabetes, and no hematuria. However, several important aspects of the disease are not well studied, especially in white patients. The aim was to improve our understanding of the nephrosclerosis phenotype, prevalence, prognosis, and diagnostic process.

We analyzed data from representative adults of the Norwegian general population (n=36878) participating in the cross-sectional population-study HUNT-3 (2006-08). We also used data from the Norwegian Kidney Biopsy Register (1988-2012) on all CKD patients referred for kidney biopsy (n=7261), with follow-up until 2017. In addition we included data from random unselected nephrology clinic patients (n=193) for matching. We evaluated the sensitivity/specificity, predictive values, and net benefit in decision curve analysis using current clinical criteria, and built new diagnostic models using logistic regression, decision tree, and other methods for optimizing diagnostic accuracy.

The prevalence of clinical criteria-based nephrosclerosis was 2.6% in the general population, and their risk for rapid GFR decline, kidney-related hospital admissions, and ESRD was significantly increased compared to age and sex matched subjects from the general population and comparable to diabetic kidney disease (DKD) patients. Mortality risk was higher in DKD and in the biopsy cohort, but nephrosclerosis patients had a 50% increased risk after adjusting for age, sex, cohort type and cardiovascular risk factors. Current clinical criteria had very low sensitivity (0.14) but high specificity (0.94) for biopsy-confirmed nephrosclerosis. Many patients with biopsy-verified nephrosclerosis had overt proteinuria (60%) or hematuria (34%). The most common biopsy-verified diagnoses in patients fulfilling clinical criteria were nephrosclerosis (41%), glomerulonephritis (21%) and interstitial nephritis (17%). Decision curve analysis indicated that adding age >50year and increasing proteinuria cutoff to <1.0g/d would slightly improve net benefit of current clinical criteria, but a regression-based continuous model had the highest net benefit, especially for risk-willing patients.

Hypertensive nephrosclerosis is a common, high-risk disease, often with an atypical phenotype compared to current clinical criteria, which have low sensitivity but high specificity. A positive test will reduce the need for kidney biopsy, but the current “no-biopsy” strategy in suspected nephrosclerosis implies a risk for misclassification and under-treatment. Increased biopsy frequency should be considered in selected patients assumed to have hypertensive nephropathy.

11.3 Summary of Paper 3

The contrast between a high prevalence of chronic kidney disease (CKD) and the low incidence of end-stage renal disease highlights the need for new biomarkers of progression beyond

albuminuria testing. Urinary proteomics is a promising method, but more studies focusing on progression rate and patients with hypertensive nephropathy are needed.

We analyzed urine samples with capillary electrophoresis coupled to a mass-spectrometer from 18 well characterized patients with CKD stage 4-5 (including six with hypertensive nephropathy) and 17 healthy controls. Classification scores based on a previously developed panel of 273 urinary peptides were calculated and compared to urine albumin dipstick results. Urinary proteomics classified CKD with a sensitivity of 0.95 and specificity of 1.00. Overall diagnostic accuracy (area under ROC curve) was 0.98, which was better than for albuminuria (0.85, p=0.02). Results for hypertensive nephropathy were similar to other CKD diagnoses, including diabetic nephropathy, glomerulonephritis, and other CKD. Adding the proteomic score to an albuminuria model improved detection of rapid kidney function decline (>4ml/min/1.73m2 per year) substantially: area under ROC curve increased from 0.762 to 0.909 (p=0.042), and 38% of rapid progressors were correctly reclassified to a higher risk and 55% of slow progressors were correctly reclassified to a lower risk category. Reduced excretion of collagen types I-III, uromodulin, and other indicators of interstitial inflammation, fibrosis and tubular dysfunction were associated with CKD diagnosis and rapid progression. Patients with hypertensive nephropathy displayed the same findings as other types of CKD.

Urinary proteomic analyses had a high diagnostic accuracy for CKD, and strongly improved identification of patients with rapid kidney function decline beyond albuminuria testing. The method performed equally well in hypertensive nephropathy, and our results indicate that hypertensive nephropathy shares many of the pathophysiological pathways and mechanisms found in other CKD diagnoses.

11.4 Summary of Paper 4

Hypertensive nephrosclerosis is among the leading causes of end-stage renal disease globally, but its pathophysiology is poorly understood, and metabolomic data is scarce. We wanted to describe both gene expression in renal tissue, and urine metabolic changes in early hypertensive nephrosclerosis, compared to healthy controls.

We used gene expression data from the European Renal cDNA Bank and included 15 patients with biopsy-verified hypertensive nephrosclerosis and 21 healthy living kidney donors. We compared urinary amino acid levels measured by LC-MS in 95 participants from a Norwegian

cross-sectional population study, 62 participants with assumed hypertensive nephrosclerosis that fulfilled traditional clinical criteria, and 33 healthy controls.

The main finding from the gene expression data was substantial underexpression of genes related to amino acid catabolism and synthesis in hypertensive nephrosclerosis (15- and 8-fold, respectively). Also, gene expression was decreased for fatty acid oxidation (13-fold), and increased in interferon gamma and cellular defense response (both 8-fold). Urinary metabolomics analysis revealed significantly lower excretion of eleven amino acids in hypertensive nephrosclerosis, among them tyrosine, phenylalanine, dopamine, homocysteine and serine. Pathway analysis showed perturbations of catecholamine biosynthesis (tyrosine, phenylalanine), homocysteine/methionine homeostasis (homocysteine) and the serine pathway.

A combined genomic and metabolomic analysis in hypertensive nephrosclerosis showed several changes relevant to the pathophysiology of nephrosclerosis. The main findings were perturbations of catecholamine biosynthesis, which is linked to natriuresis and blood pressure; homocysteine/methionine homeostasis, which is linked to cardiovascular risk, atherosclerosis and fibrosis; and the serine pathway, which is linked to endothelial dysfunction and oxidative stress.

12 DISCUSSION

12.1 Methodological considerations

12.1.1 Epidemiological aspects

Selection bias: In prospective cohort studies (HUNT), the main selection bias is non-participation. The participation rate in HUNT2 was high (69%), but dropped to 54% in HUNT3, as observed in other more recent population-based studies (221). Non-participation in HUNT3 was most common in ages 20-39 and 80+, and lack of time, no perceived benefit, being too ill to participate were the main causes. Non-participants had lower socioeconomic status, higher prevalence of cardiovascular disease (CVD) and diabetes, and higher mortality (239). Given the association between chronic kidney disease (CKD) and CVD/diabetes, for Paper 4 it could imply that our study missed participants with serious CVD and renal disease, and is biased towards weakening the association between urinary metabolite patterns and early CKD. I.e. the association between urinary metabolite patterns and CKD could in reality be stronger than Paper 4 indicated. In Paper 1 (HUNT2 and HUNT3) one tried to counter the non-participation selection bias altogether by constructing attendance-weights for HUNT3 based on predicted probability of attendance, and using multiple imputation of missing data.

The Norwegian Kidney Biopsy Registry likely has a selection bias towards patients with hematuria and/or albuminuria, as has been postulated earlier (51, 240) and confirmed in an international questionnaire-based query on nephrologists' biopsy indications (241). In suspected hypertensive nephropathy, historical biopsy practices have possibly been restrictive, in line with Luke: "(…) biopsy for the diagnosis of hypertensive nephrosclerosis is indicated (…) only when there is substantial doubt based on the clinical evidence" (240). It is therefore possible, as the authors stated, that the biopsied cases of hypertensive nephropathy/ nephrosclerosis "(…) represent cases with more advanced hypertensive nephrosclerosis" (51). Paper 2 is therefore most likely biased towards high proteinuria levels that could reflect advanced disease.

Information/misclassification bias: Information on diseases such as diabetes, treated hypertension etc was self-reported by questionnaire. A validation study in HUNT however showed high concordance between reported information on hypertensive medication and

medical records in diabetics (242), so there is probably little information bias in Paper 4 and Paper 1. Urine sample handling may not have been uniform, with delayed freezing likely reducing the concentrations of metabolites, possibly at different rates. This may have introduced a bias in Paper 2, possibly showing associations between early CKD and metabolites where in reality there are none. However, the majority of urines were collected at the study site and immediately frozen, ensuring uniform sample handling. Also, it is likely that possible metabolite degradation in storage would affect all urine samples equally. Urinary proteins are relatively stable, and storage procedures probably had little impact on protein/peptide findings in Paper 3.

Confounders and effect modifiers: Confounders are associated with both exposure and effect, so that a measured association between exposure and outcome may not represent a causal effect (243). Factors representing intermediate steps in the causal chain are not confounders but effect modifiers. Stratification and multivariable analyses are techniques to reduce the effect of confounding (Paper 4**,** III**,** IV). We employed stratification by sex and age group in intervals (20-50, 50-70, 70+ years) of cases and controls in Paper 4, and used several multivariate and regression-based analysis techniques, such as principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). In regression analysis in Paper 2 we age stratified and matched biopsy-verified and clinical nephrosclerosis cases to find which clinical features were most strongly associated with nephrosclerosis. These techniques reduce the extent of confounding, but there will probably always be residual confounding due to non-measured confounding variables.

In Paper 1 we performed sensitivity analysis and generalized estimation equation (GEE). Sensitivity analysis is an analytical technique where you first do a statistical analysis, then exclude one (or a number of) variables from the original analysis and re-do the analysis, to see if the result is unchanged. Generalized estimation equation is a statistical technique that allows for use of both multiple imputation and non-participation adjusted data in the analysis.

External validity: It strengthens the external validity of HUNT that it is a population-based study with many participants, high participation, from a county which is fairly representative of Norway as a whole in major demographics. The study has been found to be comparable to other Western countries as to CKD and cardiovascular mortality (20, 221, 244, 245), but the findings are limited to Caucasian populations.

Missing data: As briefly mentioned we used multiple imputation in Paper 1 for missing data. Missing data may reduce power and/or introduce bias. Multiple imputation is a technique that allows prediction of missing values based on observed data from other participants by multiple regression analysis, and has become an accepted strategy to avoid the problems of bias related to missing data (246, 247).

12.1.2 Statistical analyses

In Paper 4**,** Q-Q plots and Skewness-Kurtosis tests showed that the metabolites were non-normally distributed, so the non-parametric Mann Whitney *U*-test was used to test the difference in means of amino acids between controls vs hypertensive nephropathy. Adjustment for multiple testing is necessary when doing multiple significance testing. The more associations you test between cases and controls, the more likely it becomes that one of the tests is falsely positive, i.e. that it shows a statistically significant difference when in fact there is none (type I error). It is especially important adjust for this in modern biostatistics where thousands of features may be tested in thousands of samples. To counteract this several techniques are available, such as the Bonferroni correction, Holm step-down procedure, and controlling for False Discovery Rate (FDR). In Paper 4 we controlled for FDR, which implies controlling the expected *percentage* of falsely positive tests in your test battery. FDR-control is deemed a less stringent multiple testing adjustment than the Bonferroni correction, which controls the probability of *at least one* falsely positive test.

In Paper 3 we used Support Vector Machines (SVM), which is a supervised, machine learning technology developed for classification. Here the software, after having been given a training set with samples belonging to one of two groups, creates a model that classifies new samples into one of the two groups (230). Whereas PLS-DA algorithms model linear relationships between feature and group, SVM and other machine learning techniques are able to model non-linear relationships as well (248). One of the problems with PLS-DA and other supervised learning methods however, is the tendency toward overfitting of the data. Overfitting implies that the learning model is very effective in separating classes within your given data set, but may not be accurate when exposed to new examples or used in another study. There are several methods of counteracting this, most prominently cross validation techniques. In Paper 4 we used pathway and pathway enrichment analyses to put our findings into biological context. When doing pathway analysis on a data set that generated by a specific group of compounds, for example amino acids, it is important to be aware that in the pathway analysis your specific group of compounds is likely to be found important, and that an implicit bias was introduced

by your choice of method (34). This would be true for many types of analyses in translational medicine, and accentuates the importance of an *a priori* hypothesis. Also, most of the compounds central in human metabolism have been characterized and annotated in libraries such as The Human Metabolome Database or METLIN, but probably not all. So it is important to be aware that any pathway analysis is necessarily limited to the known pathways and their known facets (249). Furthermore, in Paper 2 we used discrimination analysis in the form of Receiver Operator Characteristic (ROC) analysis. Here the discriminatory ability is expressed as the Area Under Curve (AUC) of a specific binary diagnostic test, and is a function of the sensitivity and specificity of the test across all of its cut-off values (X-axis: (1-specificity); Y-axis: sensitivity). High AUC values towards 1.0 indicate high discriminatory ability (CKD yes or no, for example), an AUC value of 0.5 indicates that the test is no better than chance, and the values in between are deemed as excellent discrimination (0.80-0.90), acceptable (0.70-0.80) and inadequate (<0.70) (250). We also assessed optimal cut-off values using several variants of ROC analysis (ROC 01, Youden index, specificity >0.90, equal sensitivity and specificity, and cost benefit ratio) (251). We also used decision tree analysis to illustrate these optimal cut-offs. Furthermore we employed decision curve analysis. Full traditional decision analysis is challenging since we must include costs and benefits for different interventions and outcomes. For most scenarios, we have only limited information on these variables, and individual patients also tend to have different valuation of risks and utility of outcomes. Therefore most analyses choose not to do so due to the difficulities mentioned above. Decision curve analysis is a rather new method which omits some of these problems but still manages to include the most important parts of a full medical decision analysis (252). This is a method to include risk/benefit aspects in the evaluation of diagnostic tests without actually measuring these variables (253).

Confounders: Many metabolic processes are influenced by sex, age, body mass, drug use, smoking and alcohol ingestion, shift work and nutrition status, among other things. It is difficult to adjust for all these confounders in the computational models, and this is important to keep in mind especially with regard to study design (254).

12.1.3 Laboratory analyses

Targeted metabolomics studies have strengths and weaknesses. They measure a limited number of compounds with a high degree of quantitative accuracy, which allows a precise phenotype characterization. However, targeted studies will only find perturbations in the metabolites measured, so one must have an *a priori* hypothesis that the measured metabolites are biologically interesting. Untargeted studies measure all the metabolites visible to your

analytical technique. Suitable for *de novo* biomarker discovery, hypothesis generation and often offering a wider set of compounds than targeted studies, untargeted studies are also fraught with a lower degree of definitive compound identification, or at least more steps necessary to ensure identification. This may increase the risk of false positive results, i.e. type I error.

Stability of internal standards: Inter-batch variability was 11.9%, calculated as the relative standard deviation (RSD=standard deviation/mean). The average variance of the internal standard within the same batch (intra-batch) was 2.1% (range 0.5-4.2%). Between all batches, 71% of these amino acids had relative standard deviations of less than 35%. CVs of calibration curves.

Weakness: Calibration curves of targeted compounds were not made in urine matrices, but in water solutions. The exact matrix effects of urine (on for example ion suppression) are therefore unknown.

Interpreting huge datasets is complex, requiring expertise in many fields such as analytical chemistry, biostatistics, computer science, and epidemiology, in addition to basic medicine/biology and medical statistics. The metabolomics field is young. There seem to be many protocols around for sample handling, pre-analysis data cleaning, analytical set-ups and protocols, data management and analysis varieties, and so on. There is a need in the field for more unified experimental protocols, laboratory standard operating procedures, and best practices in data quality control.

12.2 To biopsy or not to biopsy - how do we diagnose hypertensive nephropathy?

As previously mentioned, hypertensive nephropathy has traditionally been suspected in CKD patients with longstanding hypertension and signs of blood pressure-related organ affection, low proteinuria and no signs of other kidney diseases like hematuria, diabetes, glomerulonephritis etc. This assumption based on clinical criteria alone was criticized in the mid-1990s. Schlessinger, evaluating 43 patients referred for kidney transplantation because of assumed hypertensive end-stage renal disease, found that only few had been biopsied, and less than 10% had documented hypertension at the time of normal kidney function (255). In later studies the accuracy of traditional clinical criteria to predict biopsy-verified hypertensive nephrosclerosis was shown to be variable, with positive predictive values ranging from 50% to 85%, across several countries and ethnic backgrounds (45-48). Despite these studies

hypertensive nephropathy is still most often diagnosed on clinical grounds alone, and there is a systematic tendency towards low biopsy rates in patients with a classical suggestive hypertensive nephropathy phenotype, as discussed in Paper 2. Kidney biopsies are widely regarded as safe procedures, with 98% of biopsies having no complications in one study of >9000 kidney biopsies (256). A certain percentage of clinically diagnosed hypertensive nephropathy patients will in fact have another kidney disease such as for example chronic glomerulonephritis, tubulointerstitial nephropathy and lupus nephritis, as described in Paper 2. Conversely, albuminuria and/or hematuria increase the likelihood of kidney biopsy, producing a selection bias (240). The true prevalence of nephrosclerosis in general population-based CKD cohorts is unknown since there has been a general attitude against biopsying this group. An Italian study described a diagnostic algorithm for CKD patients using all available laboratory tests, three experienced nephrologists and extensive use of renal ultrasound, but without an invasive kidney biopsy. They found that hypertensive/ischemic nephropathy encompassed one in four CKD patients which gave a prevalence of 3.4% at the age of 40+ years (257, 258). We found a somewhat lower prevalence in the general population using clinical criteria (2.6%, but a very similar percentage (3.9%) at age 40+. The optimal study would be to systematically biopsy and prospectively follow all incident patients with a clinical phenotype of hypertensive nephropathy, but such a biopsy policy is hardly feasible. The true prevalence of hypertensive nephropathy in the CKD population remains unknown.

12.3 Hypertensive nephropathy – is it all about the pressure?

Also, hypertensive nephropathy is a debated diagnosis. While it is consistently reported as a disease entity in kidney biopsy and ESRD registries around the globe, some also have pointed at the functional and structural resemblance hypertensive nephropathy has with normal aging in absence of CKD. They have questioned whether it is simply an accelerated aging process, as supported by findings in living kidney donors (259) and autopsy studies (260-262). One histopathological finding in arterionephrosclerosis at least, arteriolar hyalinosis, has been proposed to correlate stronger with age than with hypertension (58). Also, as argued by Tracy, there is certain evidence that the first pathogenic change in hypertensive nephropathy is age-related intimal hyperplasia leading to reduced glomerular blood flow and a RAAS activation to increase global blood pressure (263). Recently, important progress has been made in understanding the genetic basis of hypertensive nephropathy. An evolutionary advantage against *Trypanosoma brucei* species causing African sleeping sickness, two variants of the

APOL1 gene (G1 and G2) have recently been found to be highly frequent in populations of African descent, and strongly associate with ESRD, FSGS, HIV-associated nephropathy (HIVAN), and hypertension-attributed CKD (50, 66, 264, 265). On the other hand, many African Americans with hypertension-attributed nephropathy do not have the risk genotype, so it probably does not explain the whole picture (50). Patients with hypertension develop nephrosclerosis across all races, although the frequency of *APOL1* is low in most races except people of sub-Saharan African ancestry (266). This may explain why African Americans develop hypertension-attributed ESRD at an earlier age than European Americans (267), and why adequately treated hypertension seemingly slows CKD progression to a less degree in African Americans than in European Americans (54, 268). The *APOL1* gene encodes the apolipoprotein L1 protein, which has central functions in trypanosome lysis, autophagic cell death, lipid metabolism and certain other vascular processes (269). In addition, claims have been made that hypertension is an immunological disease, supported by signs of continued (micro)inflammation found in both hypertension and hypertensive nephropathy, and activation of immune regulating pathways TGF-β and NF-κB activated in hypertension (270). It is possible that microinflammation contributes to the gradual progression of tubular atrophy and interstitial fibrosis pointing towards ESRD. Kopp proposed that hypertensive kidney disease is not solely a genetic disease, but probably the result of a combination of factors such as aging, obesity, hyperlipidemia, smoking, chronic inflammation, and oxidative stress (50).

Around 30% of incident ESRD in the US was attributed to hypertension by the reporting physicians in 2017, with the inherent uncertainties of registry data (271). But does hypertension cause hypertensive nephropathy/CKD and end-stage renal disease? The association between hypertension and end-stage renal disease was pointed out as early as in 1873 by Professor G Johnson in the UK (272), and repeated by dr Fahr in Germany, who coined the term nephrosclerosis ("Nephrosklerose") in 1919 (42). For many years it has been believed that prolonged non-malignant hypertension could induce hypertensive nephropathy and ESRD. This view was supported by the finding in the MRFIT Study after 16 years of follow-up of 330 000 males of a strong and graded association between hypertension and development of ESRD, independent of the association between ESRD and age, race, diabetes mellitus, myocardial infarction, cholesterol, and smoking (53, 240). Others have seen hypertension as one of many factors contributing to CKD, alongside with traditional cardiovascular risk factors such as hypercholesterolemia, smoking, obesity etc (273, 274). Contrary to this, it has been stated that as long as underlying kidney disease is not definitively excluded by means of kidney biopsy,

the claimed association between hypertension and progressive renal failure cannot be made. Weisstuch pointed this out in his critique of the use of the diagnosis hypertension-attributed ESRD in the USRDS database, and in prospective clinical studies such as the Baltimore Longitudinal Study on Aging (BLSA) or the Hypertension Detection and Follow-Up Program (HDFP) (275). Luft also has pointed to the lack of biopsy data in hypertension-related ESRD, and the progression of renal failure despite optimal treatment of hypertension (276). Also, as Meyrier pointed out, vascular lesions are not unique to hypertensive nephropathy, but have also been found in arteries and arterioles in many patients with glomerulonephritis (277).

If hypertension were causal in inducing nephropathy, one might expect treatment of hypertension to slow or even ameliorate hypertensive nephropathy. On the contrary, Hsu found in 2001, in his meta-analysis of 10 randomized, controlled trials of hypertension drug treatment including 26 000 participants and 114 000 patient-years, that antihypertensive treatment did not reduce the incidence of renal dysfunction in patients with non-malignant hypertension (278). Although only the most recent of these 10 trials included ACE inhibitors (Sys-Eur, 1997), the same finding was done in the overall analysis of the more modern AASK trial, which included ACE inhibitors (279). With recent discoveries of the strong genetic link to a percentage of CKD which is histopathology like hypertensive nephropathy, especially in patients of African ancestry, there is evidence that the condition in some patients is a heritable rather than an acquired disease. All in all, it is likely that prolonged non-malignant hypertension alone can lead to nephropathy and ESRD, but it is not definitively ascertained. The question remains whether observation time in the mentioned studies has been sufficiently long to evaluate the causality between hypertension and reduced kidney function. With most studies having <10 years of follow-up, it uncertain that long-term effects of hypertension are registered. Furthermore, hypertension in assumed hypertensive nephropathy is sometimes indicative of an underlying, unidentified primary nephropathy, where kidney function loss is aggravated by continued hypertension. It is clearly a risk that clinicians clinically diagnose hypertensive nephropathy in patients with “support” in the high numbers of hypertension-attributed ESRD in registries, where kidney biopsy rates are low. There is a risk for a reciprocal dynamic, where registries accept the diagnosis from clinicians in the absence of kidney biopsy, and clinicians use it because it is prevalent in registries.

It is quite possible that other disease-related gene variants may be identified in future studies, and help explain why some cases of hypertensive nephropathy progress rapidly, and have substantial proteinuria, and others have not. The implications of *APOL1* and *MYH9* have

already been mentioned. Lately, several other gene alleles have been identified that confer increased risk of CKD. Studies have shown that genes related to angiotensinogen, angiotensin converting enzyme and apolipoprotein E associated with accelerated renal function decline in Caucasian women (172), genes related to uromodulin associated with increased risk of CKD in individuals of European descent (103), and genes related to permeability glycoprotein associated with increased risk of hypertension and CKD in the Chinese population (173). Whether some of the variations in phenotype could also be related to differences in other factors, such as epigenetics, nephron endowment, infection sequelae, metabolism disturbances, and autoimmunity, is not known.

12.4 Metabolomics and genomics in the context of the current literature

We found that urinary metabolomics combined with gene expression in kidney biopsies displayed perturbations in several, potentially related, pathways relevant to the pathophysiology of hypertensive nephropathy, such as serine metabolism (endothelial dysfunction and oxidative stress), methionine metabolism (cardiovascular risk and fibrosis), and tyrosine metabolism (catecholamine biosynthesis and natriuresis). These could contribute substantially to the major hallmarks of nephrosclerosis; hypertension, atherosclerosis and interstitial fibrosis.

12.4.1 The tryptophane – phenylalanine – tyrosine axis

In addition to low urinary tyrosine we found several perturbations in tyrosine metabolism, which is involved in catecholamine biosynthesis, natriuresis, and blood pressure control. Urine tyrosine is reduced in early CKD (148, 280) and ESRD (149, 281), likely because of low phenylalanine-hydroxylase (PAH) activity (38), also found here. It has been proposed that the mechanism for the reduced PAH activity is an oxidative stress-induced shortage of the necessary cofactor tetrahydrobiopterin due to oxidation (38). A precursor of dopamine, tyrosine is converted to L-dihydroxyphenylalanine (L-DOPA), and L-DOPA to dopamine by L-DOPA-decarboxylase (DDC). Dopamine is abundant in the kidneys, and is important in blood pressure regulation, regulating more than 50% of the kidney salt excretion ability and interacting with the RAAS in sodium homeostasis and blood pressure regulation in normotensives (282, 283). Disturbed renal dopamine is linked to hypertension, both by reduced renal L-DOPA uptake or conversion to dopamine (140, 284), and disturbed dopamine function (285). DDC expression for conversion of L-DOPA to dopamine was strongly down regulated in our nephrosclerosis patients, and has also been shown to induce lower renal and urine dopamine concentrations and reduced salt and water excretion, activation of renin-angiotensin system and increased blood

pressure in mice (286). A defective tubular D_1 receptor function has been shown in salt-sensitive hypertension in humans (287). A role on dopamine-dependent natriuresis of renalase, which is central to dopamine breakdown, has been found in animal studies, but its role in human disease is uncertain (288). Taken together, our data suggests that enzymatic downregulation and metabolite deficiency in the phenylalanine-tyrosine-dopamine axis is found in hypertensive nephropathy, and are linked to impairment of blood pressure control. Further to this, perturbations in the phenylalanine-tyrosine-tryptophan biosynthesis pathway were found in our gene and metabolite combining integrated pathway analysis. Tryptophan and tryptophan intermediates, especially indoxyl sulfate and the kynurenines, show high plasma levels in advanced CKD (122, 129, 138, 139, 141, 289), and are higher in diabetic nephropathy (DN) with proteinuria than without proteinuria (290), and in DN with proteinuric progressors compared to non-progressors (291). Prognostic studies of large cohorts show that changes in tryptophan metabolism, with higher kynurenine-to-tryptophan ratio at baseline, associate with incident CKD defined as eGFR<60 (OR 1.36 per SD) (152). Also, high baseline kynurenine and kynurenic acid both associate with incident CKD (OR 1.49 and 1.53, respectively) (85). It has been hypothesized that high kynurenine/tryptophan ratios reflect disturbed tryptophan degradation and activity of the enzyme indoleamine 2,3-dioxygenase (IDO), which is induced by inflammation and has been linked to dyslipidemia and atherosclerosis in CKD (141) and has a role in blood pressure in inflammation (153). Kynurenine has also been linked to atherosclerosis in several studies (158). IDO is rate-limiting step of breakdown of tryptophan to kynurenine, and is *in vitro* proapoptotic in renal tubular epithelial cells in response to IFN-ɣ and TNF-α. In a mouse model, IDO knockout mice did not experience renal reperfusion injury, whereas blocking IDO in wild-type mice protected against renal reperfusion injury (292). Also, kynurenine may bind to G-protein-coupled receptor 35 on leukocytes and itself promote inflammation (293). There is a plausible link between inflammation-induced tryptophan/kynurenine-associated IDO activity and renal damage in CKD. It is not certain whether this relation is causal or the result of more fundamental processes. In the cross-sectional KORA study, serum phenylalanine was negatively correlated with eGFR (-2.36 mL/min/1.73m2 per SD) (127). Further to this, in an analysis of the CRIC cohort, plasma phenylalanine was lower in fast progressors (<-3 mL/min/1.73m2/year) than in non-progressors (±1 mL/min/1.73m2/year) in a nested case-control study (125). Over a mean follow up of 9 years in the Framingham Offspring study, baseline phenylalanine was associated with a lower risk of incident CKD (OR 0.71 (95% CI 0.55-0.92 per 1SD increase in phenylalanine concentration, p=0.01)) (294). Urinary tyrosine levels were lower in type 2 diabetics with

microalbuminuria than in type 2 diabetics with no albuminuria, and was put together with two other amino acids to improve risk prediction of macroalbuminuria (281). Serum tyrosine levels were higher in CKD cases with type 2 diabetes than in CKD cases without type 2 diabetes (295). Also, in a nested case-control study of diabetes in the Framingham Offspring study, baseline plasma tyrosine was associated with incident diabetes (OR 1.85 (95% CI 1.35-2.55), p=0.0001), and the finding was replicated in the Malmö Diet and Cancer study (296). Kidney function being normal, the authors suggested that the perturbations of serum amino acids, notably high levels of some and low levels of tyrosine, could be an early sign of insulin resistance. In the same Framingham Offspring study, baseline tyrosine was associated with lower odds of incident CKD (OR 0.75 (95% CI 0.58-0.97), p=0.027) adjusted for eGFR, diabetes, hypertension and proteinuria, but failed to reach significance in the final multivariable adjusted analyses (294). In the KORA F4 study, baseline serum O-sulfo-L-tyrosine associated with annual eGFR decline and incident CKD in individuals with a near normal baseline kidney function (mean eGFR 81 mL/min/1.73m2) (128). In a study of young hypertensive men, serum tyrosine levels were 2.8-fold lower than in normotensive controls (p=0.0039), and the tyrosine-related tryptophane pathway came up as significantly perturbed in a pathway enrichment analysis (174). In the Joslin Kidney Study of type 2 diabetes, baseline plasma tyrosine levels were lower in non-progressors than in those who progressed to ESRD (155). In human genome wide association studies (GWAS), low urinary tyrosine has been associated with CKD via the genetic locus *CDK12/PNMT*, with *PNMT* coding for phenylethanolamine N-methyltransferase, the enzyme to catalyze the ultimate step of catecholamine synthesis (297). All in all, the tryptophane – phenylalanine – tyrosine axis participates in in blood pressure regulation through dopamine, plays a part in inflammation-induced dyslipidemia and atherosclerosis through IDO, and in associating with progression of diabetic nephropathy and incident CKD, likely plays a role in creating and/or maintaining chronic kidney disease.

12.4.2 The glycine – serine – threonine axis

We found that serine was lower in the urine of hypertensive nephropathy cases than in controls in Paper 4. The kidneys are the main site of serine production (298), supplying 75% of the body total from *de novo* synthesis (299), the majority of which comes from TCA cycle intermediates converted into phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (PEPCK) (299). Serine is a major methyl group provider in the one carbon metabolism central to methylation of proteins and DNA (300), and has direct blood pressure lowering effects on vessels with intact endothelium in a rat model (179). Serine blood levels have previously been shown to be

inversely associated with reduced eGFR (annual eGFR change -0.12, p=0.016, Suppl.) (152), to be elevated in membranous nephropathy cases with higher proteinuria levels (301), and to induce apoptosis and pro-fibrotic reactions in a human proximal tubular cell line (302). In human genome wide association studies (GWAS), serine has been associated with CKD via the genetic locus *CPS1* (*Carbamoyl-Phosphate Synthetase 1*) (303). *CPS1* encodes a mitochondrial enzyme in the first and rate-limiting step of the urea cycle that converts ammonia to carbamoyl phosphate, which enters the urea cycle and is converted to citrulline. In a gene expression analysis of biopsy-verified hypertensive nephrosclerosis, the glycine-serine-threonine metabolism was on the top 10 list of pathways with the highest number of differentially expressed genes, and the *serine hydroxymethyltransferase 1* on the top 10 list of the most central genes in a gene network analysis of hypertensive nephropathy (110). It is unclear whether serine is causative of CKD or merely the product of CKD, but its proximity to mitochondrial and urea metabolism, as well as a role in methylation control and blood pressure regulation, certainly open up for a role in CKD pathophysiology. Furthermore, we found that urinary glycine was lower in hypertensive nephropathy cases in Paper 4. In support of this, higher urinary glycine has been associated with lower odds of incident CKD in one observational study (OR 0.59 (95% CI 0.43-0.80)) found in the Framingham Offspring cohort and replicated in the ARIC cohort (294). Glycine has been shown to inhibit production of pro-inflammatory cytokines *in vitro* (304), improve ischemia-reperfusion kidney damage when supplemented orally in rats (305), and induce nitric oxide-mediated vasodilation via the NMDA receptor to increase single-nephron GFR in rats (306). Also, serum glycine associated with reduced risk of coronary artery disease in women, but not men, in a plasma-based metabolomics GWAS (307). A human genome wide association study (GWAS) found low serum glycine levels to be associated with CKD, but curiously also high urinary glycine. Gene analyses conferred this association to the genetic locus *CPS1* (297, 308). The mechanistic explanation of the association between glycine and CKD is uncertain, but could be related to inflammation and endothelial function as mentioned above. Also, we found low urinary threonine in our hypertensive nephropathy cases, but were not able to reproduce this finding in the SUGAR cohort, where in fact threonine was slightly higher in cases (+12.8%, p=0.21). Threonine was recently found to be lower in the serum of fast CKD progressors (<-3 mL/min/1.73m2/year) compared to stable CKD patients (eGFR decline ±1 mL/min/1.73m2/year) in a nested case-control study (n=400) from the CRIC cohort (125). Threonine being net released by the kidney (85), the authors hypothesized that low serum threonine may reflect reduced renal metabolic function (mean eGFR 43.3mL/min/1.73m2/year at the start), in parallel with the reduced hemoglobin and 1,25-

hydroxyvitamin D levels seen in CKD. Threonine in plasma was also inversely associated with progression to ESRD in type 2 diabetes in a nested case-control study from the Joslin Kidney Study cohort with follow up of 7 years, but here kidney function was near normal (mean eGFR 79mL/min/1.73m2 ±19) (155). Also, in young hypertensive men with normal kidney function, serum threonine was recently shown to be lower than in normotensive controls (174). Taken together, there is evidence that reduced kidney (metabolic) function is not the only cause of low serum threonine. Also, it seems to be an early marker of CKD, and may interfere with kidney metabolism in early CKD in an unknown fashion. In a GWAS, lower serum levels of threonine were associated with lower GFR via the genetic locus *GCKR*, which codes for the glucokinase regulatory protein, a regulator of glucokinase which is a key enzyme in glucose metabolism (104, 309).

12.4.3 The methionine – homocysteine axis

We found that urinary methionine and homocysteine were lower in hypertensive nephropathy cases than in controls in Paper 4, although nominally significant only for homocysteine. Many previous studies have shown that plasma homocysteine is inversely correlated with eGFR and that high serum homocysteine levels are found in ESRD (310, 311). Hyperhomocysteinemia has long been studied as a risk factor of cardiovascular disease (312, 313), and has also been suggested as a risk factor for hypertension (314) and incident CKD (315-319). Methionine and homocysteine are central in protein synthesis, transmethylation reactions, and the tetrahydrofolate-associated one-carbon metabolism. Methionine is converted into homocysteine via demethylation of s-adenosyl-methionine (SAM) to s-adenosyl-homocysteine (SAH) (320). This step is the major provider of methyl groups used for DNA methylation to regulate transcription (epigenetics) and for proteins methylation. Homocysteine remethylation via *the remethylation pathway* to methionine depends on 5-methyl-tetrahydrofolate and serine or betaine. Alternatively, homocysteine can be metabolized with serine to the end product cysteine via *the transsulfuration pathway*. We find that these patients have pathophysiological perturbations in both serine and methionine/homocysteine metabolism which are closely connected, possibly through the mentioned DNA methylation disturbances. One mouse model has shown that hyperhomocysteinemia-induced DNA hypermethylation and imbalance between important extracellular matrix regulatory proteins contributed to abnormal renal extracellular matrix remodeling and fibrosis in CKD, which was ameliorated by pharmacological reversal of the DNA hypermethylation (321). Furthermore, several studies have shown an association between hyperhomocysteinemia and DNA *hypo*methylation in CKD

(322). One likely mechanism behind this is S-adenosylhomocysteine (SAH), a strong inhibitor of most methylation reactions (323), which increases in CKD, often more strongly than homocysteine levels with reduced kidney function (324, 325). Furthermore, we have previously found that TCA cycle activity is downregulated in non-diabetic CKD (109), and in the current study we find reduced renal expression of PEPCK in nephrosclerosis. These two perturbations could lead to reduced serine production and a state of reduced substrate for the tetrahydrofolate cycle, which is important in methylation. We also demonstrate reduced renal expression of 5,10-methylene-tetrahydrofolate reductase (MTHFR), methionine synthase (MS) and betaine-homocysteine methyl-transferase (BHMT), which are key enzymes for the remethylation of methionine, and reduced methionine levels. Although global hypomethylation has been found in blood cells and vascular lesions of patients with atherosclerosis (326, 327), and is associated with aging (328), several studies show that both *hyper*methylation and *hypo*methylation coexist in various disease (329, 330). Whether this is a direct facilitator of harmful effects or merely a marker of a generalized epigenetic dysregulation is not well studied. Oxidative stress (331, 332) and upregulated inflammation (333, 334) may be induced by high homocysteine levels. CKD experimental models have demonstrated that uremic toxins or other causes of inflammation and oxidative stress lead to hypermethylation of Klotho via upregulation of DNA methyl transferases (DNMT) (335). This leads to Klotho protein suppression and removes the Klotho-mediated inhibition of pro-fibrotic signaling, resulting in increased renal fibrosis, shown both in animals and humans (336, 337). In our study, upregulation of *DNMT1* in nephrosclerosis kidneys and the potential for global hypomethylation could contribute to renal fibrosis and general atherosclerosis through these pathways, respectively. In human genome wide association studies, low serum methionine has been associated with CKD via the genetic locus *CDK12/PNMT* (297), and homocysteine and low serum methionine sulfone with CKD via the genetic locus *DPEP1* (338). *PNMT* codes for phenylethanolamine N-methyltransferase which catalyzes the ultimate step of catecholamine biosynthesis, and several SNPs from this locus have been associated with acute kidney injury, possibly through mechanisms related to catecholamine breakdown (339). *DPEP1* codes for the enzyme dipeptidase 1 in the kidneys, which hydrolyzes many dipeptides. The interaction between *DPEP1* and CKD pathophysiology is unknown. In another GWAS focusing on coronary artery disease (CAD), however, there was no association between the most common gene variants that determine serum homocysteine and risk of CAD (338).

12.5 Proteomics

In Paper 3, we found that a urinary proteomic score had significantly better diagnostic accuracy for CKD than albuminuria alone. When adding urinary proteomics to albuminuria, which is currently among our best predictors of CKD prognosis, it substantially improved the identification of rapidly progressing patients.

Among the most studied biomarkers in proteomics is a panel of 273 distinct urinary peptides. This so-called CKD273 classifier (Mosaiques Diagnostics GmbH, Hannover, Germany) has been shown to accurately distinguish CKD of various etiologies from non-CKD (163). This was replicated in our study in Paper 3. The most significant findings in the CKD group have been decreased levels of urinary collagen α1 (III), collagen α1 (I), and uromodulin fragments. This proteomics platform has also been evaluated as a prognostic tool, predicting the progression in diabetics from normoalbuminuria to macroalbuminuria (97, 98), and progressive eGFR loss in general CKD (99, 165). It has also been shown to predict hard end-points like ESRD or death (100).

Reduced urinary excretion of collagen I-IV fragments was our main proteomics finding. This has been found in many other proteomics studies on CKD, as reported above, both in diabetic and non-diabetic CKD. Urinary collagen fragments have been hypothesized to be a marker of extracellular matrix metabolism (340). It has been suggested that lower urinary collagen might indicate disturbance of matrix turnover, with reduced breakdown and excessive accumulation of matrix collagen, and that this imbalance may contribute to the fibrosis seen in CKD (95). Excessive accumulation of extracellular matrix and subsequent fibrosis is a general pathophysiological characteristic of advanced kidney disease. If the initial trigger event is not cleared, epithelial tubular cells will transition to a more mesenchymal-like cell type starting a chronic interstitial process with increased production of collagen type I and type III (341). Both experimental and human studies have suggested an initial phase with increased extracellular matrix production followed by an imbalance between collagen degradation enzymes (matrix-metallo-proteinases, MMPs) and their tissue inhibitors (tissue inhibitors of metalloproteinases, TIMPs). This leads to reduced degradation and development of tubulointerstitial fibrosis (98, 341, 342). Reduced urinary collagen has been found consistently in CKD patients by use of CE-MS, in line with our findings (100, 166, 343, 344). Lower levels of urinary MMP activity has also been found in progressive compared to stable patients with diabetic nephropathy (345). However, one fragment of the collagen α-5 chain precursor was increased in the urine of CKD and diabetic nephropathy cases in another study (346). It is difficult to explain why almost every

other collagen fragment except this one is low in CKD. The authors propose that this fragment is a component of the basal membrane in addition to the extracellular matrix, and that it might be low because of increased basal membrane breakdown. Furthermore, high urinary levels of procollagen III amino-/N-terminal propeptide (PIIINP) have been studied by radioimmunoassay (RIA) technique, and found to associate with CKD progression and incident ESRD (347). This might seem to be in conflict with our finding of reduced urinary collagen fragments in CKD progressors. However, PIIINP has been shown to correlate with the degree of interstitial fibrosis (348), and it could be argued that PIIINP reflects collagen production, whereas collagen fragments in the urine may be seen as markers of collagen degradation. Whichever way one sees it, collagen fragments could be useful for the development of future biomarkers of rapid CKD progression, and our data indicate that this could hold for hypertensive nephropathy patients as well.

We found lower urinary levels of uromodulin fragments in early hypertensive nephropathy. This is in agreement with earlier reports in both diabetic and non-diabetic CKD (81, 346). Uromodulin, or Tamm-Horsfall protein, is a glycoprotein excreted into the urine from the thick ascending limb of the loop of Henle (TAL) and the early distal convoluted tubule (DCT). It is the major protein excreted in urine and takes part in the formation of urinary casts (349). Less urinary uromodulin in CKD patients has been interpreted as a sign of reduced production of uromodulin in the tubules, indicating tubular dysfunction (167, 346). Low urinary levels have been found in CKD of varied etiologies (163) and to be associated with progression of albuminuria in diabetic nephropathy (97).

Other proteins and peptides that we found were significantly different in cases were osteopontin and CD99 antigen. Osteopontin is a glycoprotein found in several organs, but most abundantly in bone and kidney. The main physiological function of osteopontin in the kidneys is thought to be the inhibition of calcium oxalate crystal formation and blocking adhesion of calcium oxalate to renal tubular cells (350, 351). However, it also seems to play a role in kidney injury, inflammation and tissue remodeling. For example, osteopontin gene and protein expression has been shown to be increased in an acute kidney injury model in rats (350), and is produced in increased amounts in hyperoxaluria. In hyperoxaluria it seems to have a double edge: on one side it may prevent adhesion of crystals in the lumen/tubular interface, and on the other side it may contribute to fibrosis in the interstitium (351). The exact interactions here have not been fully elucidated. Interestingly, osteopontin deficient mice are relatively protected against the lipid accumulation and glomerulosclerosis induced by hypercholesterolemia (352). We found

low levels of urinary osteopontin in CKD, which is in line with earlier studies on CKD (166). Whether this reflects a state of tubular dysfunction with secondary reduced osteopontin production, or reflects perturbed osteopontin metabolism associated with advanced interstitial kidney fibrosis, is uncertain.

We found higher urinary levels of α1-antitrypsin in hypertensive nephropathy, consistent with earlier CKD studies (166, 353). α1-antitrypsin is a protease inhibitor that is abundant in plasma. It has been shown to protect against protease-induced renal tissue injury and fibrosis in a rat model (354). α1-antitrypsin and other highly abundant plasma proteins like albumin and fibrinogen are high in CKD urines. It has been proposed that this reflects a tubular dysfunction characteristic of chronic kidney disease (166).

We found high levels of urinary α-2-HS-glycoprotein in CKD. This glycoprotein has been associated with tubular damage and inflammation in diabetic nephropathy (355). It has also shown to associate with progression of albuminuria in diabetic nephropathy (97). One explanation of this is that many plasma proteins in diabetic nephropathy are hyperglycated, and that the increased urinary levels reflect the increased plasma levels (97). Another explanation is that tubular dysfunction leads to reduced reabsorption of abundant plasma proteins, and hence increased urinary levels (166).

In the original publication, a cut-off level of 0.343 of the CKD273 classifier was found to discriminate optimally between healthy controls and established CKD, those with scores >0.343 having CKD (163). We found an association with rapid CKD progression already from CKD273 scores above 0.0 in Paper 3. Similarly, an increased risk of progression of proteinuria in type 2 diabetics was seen with CKD273 scores > 0.154 in type 2 diabetics in the DIRECT-2 study (356). Also, in a prognostic study of CKD273, all of the participants that developed end-stage renal disease or died had a CKD273 score of >0.55 (100). In other words, there seems to be a positive association between CKD273 score and CKD. It is possible that refined analyses may utilize this "graded response" of CKD273 to identify CKD at ever earlier stages in future studies. One may hope that this could also help identify progressors with particularly high risk of developing end-stage renal disease.

Furthermore, several single protein biomarkers have been studied in CKD, like Neutrophil gelatinase-associated lipocalin (NGAL), Kidney injury molecule-1 (KIM-1), and N-acetyl-β-D-glucosaminidase (NAG). Although not "originating from proteomic studies in a strict sense", as H Mischak put it (357), they certainly are protein biomarkers, and have perhaps come closer

to application in the clinic than many of the multi-protein panels from the proteomics field. KIM-1 is seen as a marker of tubular injury. It has been shown to associate with albuminuria progression in type 1 diabetes in one study, where low urinary levels at baseline associated with regression of microalbuminuria (358). NAG, also seen as a marker of tubular damage, has been shown to be elevated in the urine of microalbuminuric patients. It is also elevated in diabetic patients with normal albuminuria levels compared to non-diabetics (359). NGAL is elevated in both plasma and urine in various forms of CKD. NGAL has been shown to predict future acute kidney injury, and to associate with future risk of developing CKD independent of albuminuria (360, 361). A role in the pathophysiology and progression of CKD itself has also been proposed (361).

12.6 Clinical epidemiology

We found that total CKD prevalence was stable in Norway over a 10-year period. Improved treatment of hypertension, hypercholesterolemia and higher physical activity might have contributed to this in spite of increasing diabetes and obesity prevalence.

12.6.1 Changes in CKD prevalence

How has the prevalence of CKD changed over the last few decades internationally? In the US, the CKD prevalence increased from 10.0% in 1988-94 to 13.1% in 1999-2004 (362), and then stabilized during the 2000s and 2010s (363). In Japan the CKD prevalence grew from 1974 to 2002, with CKD stages 3-5 increasing threefold in men (4.8% to 15.7%) and twofold in women (5.8% to 11.7%) (364). In Scotland, however, a study reported stable CKD prevalence between 2004 and 2009 (365), and in Korea CKD prevalences went down between 2001 and 2009 in men (7.9% to 4.5%) and in women (11.3% to 6.3%) (366). In agreement with this, an English study found that the prevalence of eGFR <60 ml/min/1.73m^2 declined from 5.7% to 5.2% from 2003 to 2010 (367). The Global Burden of Disease project has estimated that the impact of CKD has increased steadily from 1990 to 2010 in both high income and developing countries (459-549 and 339-438 DALYs/100.000, respectively) (368).

Also, the CKD prevalence varies greatly between countries. In a survey of cross-sectional population studies of individuals aged 20-74 years from 13 European countries, CKD prevalences varied from 3.3% in Norway to 17.3% in a region in Germany (369). This variation was stable when comparing both high and low risk populations, suggesting that factors other than hypertension, diabetes and obesity were at least partly responsible for the differences in

CKD prevalences. The authors pointed to variations possibly stemming from biological differences (diet, smoking, physical activity, and genetics), healthcare policy differences, and analytical differences (lab methods and study populations) (369). Large regional variations in CKD prevalence have also been found in the US (4% to 11%) and China (6% to 18%) (370, 371). Different distribution of risk factors, such as hypertension, diabetes, obesity, and cardiovascular disease may naturally also play a role.

12.6.2 The role of hypertension

Hypertension is a central player in chronic kidney disease, and very closely knit to CKD both as cause and effect. Whether or not hypertension causes CKD has been discussed earlier in this thesis (chapter 12.3). Another question is: Can control of hypertension reduce the progression, and thus the prevalence, of CKD? In three randomized controlled trials comparing low vs usual blood pressure (MDRD, AASK, REIN-2), no uniform effect of more intense blood pressure control on slowing CKD progress was found in an aggregate of 2269 patients. In the MDRD trial, low blood pressure (mean arterial pressure (MAP) 92 mmHg) in proteinuric patients (>3g/day) reduced the eGFR decline rate significantly compared to usual blood pressure (MAP 107 mmHg) in patients with moderate CKD (eGFR 22-55 mL/min/1.73m2) (372). It did not, however, reduce the eGFR decline rate significantly when all patients were included in the analysis. Also, the low blood pressure group had more users of ACE inhibitors (48%) than the usual blood pressure group (28%), perhaps pointing to a drug effect rather than a blood pressure effect. In the AASK trial, no difference was seen in the eGFR decline rate between the low and usual blood pressure groups (mean 128/78 mmHg and 141/85mmHg, respectively) in African American patients with moderate hypertension-attributed CKD (eGFR 20-65 mL/min/1.73m2) (55). In the REIN-2 trial of proteinuric non-diabetic moderate CKD (eGFR 34-35 mL/min/1.73m2), there was no difference in kidney outcomes in the intensive vs ordinary blood pressure group (mean 130/80 vs 134/82mmHg, respectively) regarding time to end-stage renal disease (373). Recently, the SPRINT trial of 9361 participants with increased cardiovascular risk, showed that intensive blood pressure control substantially decreased cardiovascular disease and all-cause mortality risk compared to the standard treatment group (mean systolic blood pressure 121 vs 136mmHg) (374). This was achieved at an increased risk of acute kidney injury and of a 30% decline in eGFR that were observed with intensive blood pressure treatment. The sheer size of SPRINT It is noteworthy that compared to the three above mentioned trials, only 28% of the participants in SPRINT had established chronic kidney disease (mean eGFR of 47 mL/min/1.73m2) compared to a mean eGFR of 71 mL/min/1.73m2

for the study as a whole. A possible explanation is that a beneficial cardiovascular effect of intense BP lowering exists in relatively mild CKD (SPRINT), but is overshadowed by the marked CVD risk that follows more advanced CKD (MDRD, AASK, REIN-2). Whether this should translate into accepting a more conventional blood pressure target in more advanced CKD patients, who are also more likely vulnerable to the adverse affects of intense blood pressure lowering, is uncertain. The debate on the most optimal blood pressure levels has been vivid after the SPRINT trial, and will certainly go on.

Can control of hypertension reduce the progression, and thus the prevalence, of CKD? As shown in Paper 1, blood pressure control was improved and more individuals were on antihypertensive medication both in Norway and the UK between 1990 and 2010, whereas the opposite trend was seen in the US (Paper 1, table S4) (375, 376). This disparity of blood pressure trends is mirrored also in the Global Burden of Disease database, where the annual percentual decrease in disability-adjusted life years (DALYs) due to hypertension between 1990 and 2010 was smaller in the US (-1.4%) than in Norway and the UK (-3.4% and -4.1%, respectively). In the same period, prevalences of obesity and diabetes increased across all the three countries (Paper 1, table S4). It is suggestive that the prevalence of low GFR in the period increased in the US while it decreased in Norway and the UK (table S4). It is possible that improved blood pressure control may have contributed to a stabilized CKD prevalence in a period with increasing obesity and diabetes prevalence in Norway and the UK.

Over time, the percentage of hypertensives with so-called controlled hypertension have increased. This is true for both the US, Norway and England (table S4), as well as for Japan and Korea (364, 366). It is noteworthy that in spite of these achievements, more than 50% of hypertensives still have too high blood pressures. This remaining fraction of hypertensives is likely dominated by difficult-to-treat hypertension. Given that hypertension accelerates the progression of established CKD, improvements in hypertension control rates are warranted, and would likely contribute to reduced CKD prevalences.

12.6.3 The role of diabetes

Diabetes is a known risk factor for chronic kidney disease. Increasing diabetes prevalences were seen in the US, Norway and England between the 1990s and the 2000s, with a parallel increase in CKD numbers, at least in the US and England. It is noteworthy that while the prevalence of diabetic kidney disease (DKD) increased in the US from 1988 to 2008, there was no change in the prevalence of DKD among those with diabetes (377). The authors found a large increase in

the use of antidiabetics, RAAS inhibitors, and statins in this period, producing reduced levels of HbA1c, blood pressure, and LDL cholesterol, but no reduction in DKD prevalence. The effects of these favourable changes may have been annulled by increased obesity in the diabetics in the same period, and the accumulated duration of diabetes over time, the authors hypothesized (377). The incidence of end-stage renal disease due to diabetes in the US, however, have declined from 1990 to 2006 (378). The authors hypothesized that this could be attributable to improved glycemic control, better hypertension care, and increased use of RAAS inhibitors. In Korea, which saw a reduction of CKD prevalences from 1998 to 2010-12, antidiabetic use and diabetes control were also better in the 2000s than in the 1990s and turn of the century (366). Unfortunately, this study did not report on Korean prevalences of DKD specifically, but it illustrates that the relationship between changes in risk factor do not translate into changes in end-points in a simple manner.

12.6.4 The role of physical inactivity and obesity

As mentioned earlier, physical exercise has been shown to slow the decline in kidney function in small randomized controlled trials (379). Low physical activity accounted for up to 5% of new cases or progressive CKD in a large observational cohort study (380), and elderly with higher physical activity have 28% lower risk for future rapid GFR decline after multivariate adjustment (381). Regular exercise reduces insulin resistance, reduces total cholesterol, and increases HDL (382, 383). In Norway over the last four decades, a small increase in leisure time physical activity has been overshadowed by a more sedentary work life, in sum leading to reduced physical activity (384). In the US, physical activity increased for the country as a whole from 1984 to 2015, but in absolute numbers, a large proportion of adults were still physically inactive. In 45 out of 51 states, more than 20% of adults reported no leisure-time physical activity (385). The role of physical exercise in the prevention of CKD is still not well-documented.

Obesity is a growing problem, and increases the risk of hypertension, diabetes, and cardiovascular disease. It has been debated whether obesity also increases the risk of *de novo* CKD. A recent meta-analysis of >600 000 participants in 39 cohorts showed that obesity (BMI >30) was associated with *de novo* CKD, defined as eGFR <60 mL/min/1.73m2 or albuminuria (relative risk 1.36 (95% CI 1.18-1.56)) (386). This association was confirmed when analyzing the association between BMI as a continuous variable and low eGFR, with a significantlyl increased relative risk of 1.02 per unit BMI. Several possible mechanisms behind this association have been postulated, including hyperfiltration, abnormal activation of the renin-

angiotensin-aldosterone system (RAAS), and the possible production of RAAS proteins by adipose tissue (387). With the obesity epidemic in the Western world, and increasingly also in developing countries in Asia and Africa, this could imply increased global CKD prevalences in the future. Obesity should be a target in population-scale strategies to prevent CKD.

12.6.5 The role of cholesterol

There is less data available to support our hypothesis that lipid lowering therapy reduces the risk of kidney disease. Several studies show that abnormal lipid levels are associated with accelerated kidney function loss (388, 389) but statin intervention trials have shown conflicting results and are not recommended solely for renal protection (390-392). However, many used hard clinical outcomes like ESRD which are late events requiring long follow-up and large sample sizes. It has therefore been suggested that future studies should test high potency statins before a major GFR decline occurs and to use lesser declines in estimated GFR as an alternative endpoint (393, 394).

12.6.6 The role of smoking

Smoking is a generally accepted risk factor for CKD. A recent meta-analysis with more than 65 000 patients found an increased summary relative risk for incident CKD and end-stage renal disease in active smokers of 1.33 and 1.91, respectively (395). Smoking contributes to CKD by several mechanisms. It produces kidney fibrosis, induces damage to endothelium and epithelium, and promotes inflammation and perturbed DNA methylation (396). We estimated that if smoking patterns had stayed unchanged at HUNT2 levels (1995-97), we could have expected a tendency toward higher CKD prevalence in HUNT3. Daily smoking in Norway was much more prevalent between 1995-97 (at 30%) than in 2006-08 (around 17%) (397).

12.6.7 Future CKD trends and methodology issues

A study on the future burden of CKD in the US estimates that the prevalence will increase from 13% currently to 16% in 2030 (398), and projections also indicate that CKD will move up four places in the global mortality rankings (399). A European study predicted a continued rise in the prevalence of CKD stage 5 in diabetic patients from 2012 to 2025 at 3.2% per year for 12 European countries as a whole (400).

Several methodology issues stand in the way of a precise surveillance of CKD prevalence. A lack of standardization of creatinine and albumin assays, use of different GFR estimating equations, different population sample selections, and lack of national registries are some of the foremost (401, 402).

12.7 Limitations and challenges

Our studies have some limitations that need to be addressed. First, combined arterial and venous measurements across the kidney would be the optimal design to fully describe the renal handling of amino acids, and we do not have blood values from the HUNT study. Second, we have defined hypertensive nephrosclerosis from a set of clinical and laboratory criteria known to be rather unspecific, rather than by histological definition based on renal biopsy findings. The ideal study would be to have a set of cases with biopsy-verified hypertensive nephropathy for the metabolomics and genomics analyses. Alternatively, to find the true prevalence of hypertensive nephropathy by biopsying all with suspected chronic kidney disease would be scientifically interesting, but not warranted from a medico-ethical standpoint, and not feasible. Third, we could not ensure that fasting was uniform, or that amino acid or protein intake was uniform before urine analysis. Although this is seldom the case in metabolomics studies, some studies have managed to accomplish this. Fourth, for definite mechanism elucidation, additional cell line and/or animal experiments with a more focused view is needed.

Briefly, other themes relevant in the field which this discussion has not elaborated on, are oxidative damage, mitochondrial dysfunction, renal nitric oxide metabolism, endothelial dysfunction, the role of trimethylamine N-oxide, and endothelial-to-mesenchymal transition. These are covered in several reviews (34, 254).

So is there a common denominator for all these metabolomic changes? In the metabolomics field today, there is no Grand Unified Theory, with one pathway or one substance to explain all disease conditions. Nor is there one dominant analytical platform, or globally accepted guidelines on research practice such as how to perform data analysis. However, there is ongoing interest in metabolomics, with an increasing body of publications, use of online data analysis tools like Metaboanalyst, and cooperation between groups.

A challenge in the omics field is the multitude of protocols in use, the vast amounts of data generated, and the diverse data management and analysis alternatives available. Also, it may take weeks or months from the time of analysis until a proper conclusion or presentation of results can be made. Today it is fair to say that metabolomics is at the lab bench, rather than bedside. Metabolomics is, however, a valuable tool for both hypothesis generation and mechanistic studies alike. Like other research platforms, the value of metabolomics pends on having a scientific question, and putting the findings into a biological context with physiological meaning.

13 CONCLUSIONS

We found that CKD prevalence remained stable in Norway over a 10-year period characterized by strong improvements in blood pressure, lipids and physical activity, and only modestly increasing diabetes and obesity.

We found that hypertensive nephrosclerosis is a common, high-risk disease, often with an atypical phenotype compared to current clinical criteria, which have low sensitivity but high specificity. A positive test will reduce the need for kidney biopsy, but the current "no-biopsy" strategy in suspected nephrosclerosis implies a risk for misclassification and under-treatment. Increased biopsy frequency should be considered in selected patients assumed to have hypertensive nephropathy.

We found that urinary proteomic analyses had a high diagnostic accuracy for CKD, including hypertensive nephropathy, and strongly improved identification of patients with rapid kidney function decline beyond albuminuria testing.

We found perturbed gene expression and metabolic pathway patterns in our combined genomic and metabolomic analysis of early stage hypertensive nephrosclerosis. Renal gene expression analysis showed reduced amino acid catabolism and synthesis in nephrosclerosis patients. Metabolomics analysis revealed downregulation of the phenylalanine-tyrosine-dopamine axis, which regulates natriuresis and blood pressure. We also found disturbances in methionine/homocysteine and serine metabolism, involved in methylation status, endothelial dysfunction, inflammation and atherosclerosis.

13.1 Future research

Future research should probably concentrate on specific diagnoses of renal disease, rather than studies of the rather large and diverse entity of CKD. Different diagnoses have different pathophysiologies that may be of interest for early diagnosis and prognosis, and studying advanced-stage CKD does not have the potential to do that.

Prospective and larger-sized studies which evaluate the prognostic value of pre-disease and early-stage disease metabolite patterns are interesting future research prospects.

Integration of genomics and metabolomics, and possibly with addition of proteomics, would make more potent use of scarce biological material.

In the future, metabolomic analysis coupled with well-characterized cohorts, and in conjunction with biobanks, has the potential to elucidate associations between metabolite patterns and more classical epidemiology data, such as demographic and clinical data. This has ultimately the promise to identify biomarkers, point towards metabolite pathways that are active in disease, aid earlier diagnosis and more accurate prognosis, and possibly guide future therapies.

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Long-term trends in the prevalence of chronic kidney disease and the influence of cardiovascular risk factors in Norway

see commentary on page 482

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Surveillance of chronic kidney disease (CKD) prevalence over time and information on how changing risk factors influence this trend are needed to evaluate the effects of general practice and public health interventions. Because very few studies addressed this, we studied the total adult population of a demographically stable county representative of Norway using cross-sectional studies 10 years apart (Nord-Trøndelag Health Study (HUNT)2 and Nord-Trøndelag Health Study (HUNT)3, 65,237 and 50,586 participants, respectively). Thorough quality-control procedures and comparisons of methods over time excluded analytical drift, and multiple imputations of missing data combined with nonattendance weights contributed to unbiased estimates. CKD prevalence remained stable in Norway from 1995 through 1997 (11.3%) to 2006 through 2008 (11.1%). The association of survey period with CKD prevalence was modified by a strong decrease in blood pressure, more physical activity, and lower cholesterol levels. Without these improvements, a 2.8, 0.7, and 0.6 percentage points higher CKD prevalence could have been expected, respectively. In contrast, the prevalence of diabetes and obesity increased moderately, but the proportion of diabetic patients with CKD decreased significantly (from 33.4% to 28.6%). A CKD prevalence of 1 percentage point lower would have been expected without these changes. Thus, CKD prevalence remained stable in Norway for more than a decade in association with marked improvements in blood pressure, lipid levels, and physical activity and despite modest increases in diabetes and obesity.

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KEYWORDS: chronic kidney disease; epidemiology; general population; prevalence; risk factors; trend

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Chronic kidney disease (CKD) and end-stage renal disease (ESRD) are inextricably intertwined, and their development over the past decades has sometimes been characterized as an "epidemic."[1] ESRD has increased 3-fold since the 1980s,[2] but CKD is more difficult to define and follow over time. The CKD prevalence is high worldwide (10%–13%),[3–7] but few have studied prevalence trends over time. The topic therefore remains uncertain and is intensely debated because the increased ESRD incidence could be caused by an increased progression rate or improved delivery and availability of ESRD treatment in addition to an increased number of CKD patients. It is also unclear how management of risk factors affects CKD prevalence, but there are some encouraging results from intervention trials as well as public health initiatives.[8–11]

The National Health and Nutrition Examination Survey showed that total CKD prevalence increased by 30% in the US population between 1991 and 2001.[4] Later studies from Europe and Asia have shown both increasing[12,13] and stable[14,15] trends. Furthermore, recent data from England have shown decreasing CKD prevalence from 2003 through 2010, a period with a high priority for preventive kidney medicine.[16] These conflicting results may represent true regional differences, perhaps due to different risk profiles, or may be artifacts caused by technical and analytical challenges in measuring CKD prevalence over time. Many developed countries have achieved substantial blood pressure improvements,[17–20] but the prevalence of hypertension is increasing in developing countries and is now the leading risk factor for global disease burden.[21–23] Furthermore, recent guidelines and randomized clinical trials disagree on the blood pressure treatment goals,[24,25] and other risk factors such as physical inactivity, obesity, and diabetes mellitus are increasing worldwide.[21,26,27] In sum, this creates a setting that could fuel CKD progression and worsen CKD as a public health problem. The Global Burden of Disease Study

2013 estimated that the global disability–adjusted life-years caused by CKD increased by 17.1% from 2005 to 2013,[27] but the estimates are uncertain because the field has substantial data shortages.

Thus, we analyzed changes in estimated glomerular filtration rate (eGFR), albuminuria, CKD stages, and relevant risk factors over a 10-year period in Norway. We also compared the prevalence results and their relationship to individual risk factors and national-level public health and predialysis care measures with corresponding data from England and the United States.

RESULTS

Survey characteristics

The cross-sectional Nord-Trøndelag Health Study (HUNT)-2 survey (1995–1997) had a 70% participation rate ($N = 65{,}237$), whereas the HUNT-3 survey (2006–2008) had a 54% participation rate ($N = 50{,}586$). Table 1 shows demographics and characteristics of the participants; HUNT-3 participants were older and had a higher body mass index, and more subjects had diabetes mellitus. By contrast, there were fewer current smokers; participants were more physically active and more likely to be receiving antihypertensive treatment. These factors were accompanied by lower systolic blood pressure, lower prevalence of cardiovascular disease, and better self-reported general health. Estimates corrected for nonparticipation describe the total adult population. Selected healthy subgroups with identical risk profiles (Supplementary Table S1) had identical mean eGFRs (105.9 vs. 106.2 ml/min per 1.73 m^2, $P = 0.63$) and mean urinary albumin to creatinine ratio (ACR) (10.0 vs. 10.2 mg/g, $P = 0.92$) in the HUNT-2 and HUNT-3, respectively, indicating no analytical drift of kidney measures between the 2 surveys.

Long-term prevalence trends

Kidney function was shifted toward lower eGFR values, especially within the normal range (Figure 1). The mean eGFR was 99.1 ml/min per 1.73 m^2 in the HUNT-2 and 97.8 ml/min per 1.73 m^2 in the HUNT-3 ($P < 0.001$), and corresponding eGFRs <60 ml/min per 1.73 m^2 were 4.5% and 4.8% ($P = 0.033$). In contrast, the ACR distribution based on 3 urine samples changed minimally, but mean urinary ACR decreased from 15.7 mg/g to 14.1 mg/g ($P < 0.001$) due to fewer participants with increased albuminuria (7.9% in the HUNT-2 and 7.4% in the HUNT-3, $P = 0.034$). Severely increased albuminuria (ACR >300 mg/g, formerly termed macroalbuminuria) was 0.3% versus 0.1% ($P < 0.001$), and moderately increased albuminuria (ACR 30–300 mg/mmol, formerly termed microalbuminuria) was 7.6% versus 7.3% ($P = 0.26$).

CKD was risk stratified by 6 eGFR categories × 3 ACR categories (Figure 2). Total CKD prevalence did not differ across the 2 surveys (11.3% vs. 11.1%, $P = 0.42$), and the majority of cases were classified as CKD with a moderately increased risk of complications (9.3% vs. 9.0%, $P = 0.20$). The prevalence of high-/very high risk CKD was also stable (2.0%–2.1%, $P = 0.35$). eGFR categories of

Table 1 | Demographics and characteristics of the HUNT-2 (1995–1997) and HUNT-3 (2006–2008) surveys

	Participants		General population	
	HUNT-2 ($N = 65{,}252$)	HUNT-3 ($N = 50{,}586$)	HUNT-2 ($N = 94{,}094$)	HUNT-3 ($N = 93{,}482$)
Age, yr	50.3 (0.07)	53.2 (0.06)	48.8 (0.08)	50.2 (0.09)
Sex (% male)	46.8 (0.2)	45.3 (0.2)	49.6 (0.2)	49.5 (0.2)
Education (% attended college/university)	20.1 (0.2)	26.4 (0.2)	21.1 (0.2)	27.3 (0.2)
Living in rural area (%)	68.2 (0.2)	60.7 (0.2)	66.6 (0.2)	61.9 (0.2)
General health (%)				
Poor	1.9 (0.05)	1.4 (0.05)	1.8 (0.05)	1.4 (0.05)
Fair	25.6 (0.2)	24.7 (0.2)	24.6 (0.2)	23.1 (0.2)
Good	57.1 (0.2)	58.1 (0.2)	56.8 (0.2)	58.2 (0.2)
Excellent	15.4 (0.2)	15.8 (0.2)	16.8 (0.2)	17.4 (0.2)
Cardiovascular disease (%)	8.0 (0.1)	7.7 (0.1)	8.0 (0.1)	7.1 (0.1)
Confirmed diabetes mellitus (DM) (%)	3.4 (0.1)	4.7 (0.1)	3.3 (0.1)	4.2 (0.1)
Probable survey discovered DM (%)	3.5 (0.1)	4.7 (0.1)	3.3 (0.1)	4.4 (0.1)
Pre-DM (%)	14.5 (0.1)	17.7 (0.2)	14.0 (0.1)	17.0 (0.2)
High DM risk score (%)	17.6 (0.1)	21.5 (0.2)	16.1 (0.1)	18.5 (0.2)
Former smoker (%)	25.0 (0.2)	31.0 (0.2)	23.6 (0.2)	28.6 (0.2)
Current smoker (%)	29.2 (0.2)	21.0 (0.2)	28.7 (0.2)	21.0 (0.2)
Systolic blood pressure (mm Hg)	137.7 (0.1)	130.4 (0.1)	137.3 (0.1)	129.6 (0.1)
Diastolic blood pressure (mm Hg)	80.1 (0.1)	73.2 (0.1)	79.4 (0.05)	72.7 (0.06)
Antihypertensive medication (%)	13.7 (0.1)	20.9 (0.2)	13.1 (0.1)	18.4 (0.2)
Hypertension (%)	44.3 (0.2)	38.7 (0.2)	43.2 (0.2)	35.5 (0.2)
Body mass index (kg/m^2)	26.4 (0.02)	27.2 (0.02)	26.3 (0.02)	27.0 (0.02)
Obesity (%)	16.4 (0.2)	22.5 (0.2)	16.1 (0.2)	21.6 (0.2)
Physical inactivity (%)	73.1 (0.2)	58.1 (0.2)	72.3 (0.2)	58.9 (0.2)
Total cholesterol (mg/dl)	227.8 (0.2)	212.3 (0.2)	224.7 (0.2)	209.2 (0.2)
HDL cholesterol (mg/dl)	53.4 (0.1)	52.2 (0.001)	52.6 (0.002)	51.4 (0.002)

Data are mean and percentage (SE). General population data were weighted for nonresponse, and missing values were imputed.
DM, diabetes mellitus; HDL, high-density lipoprotein.

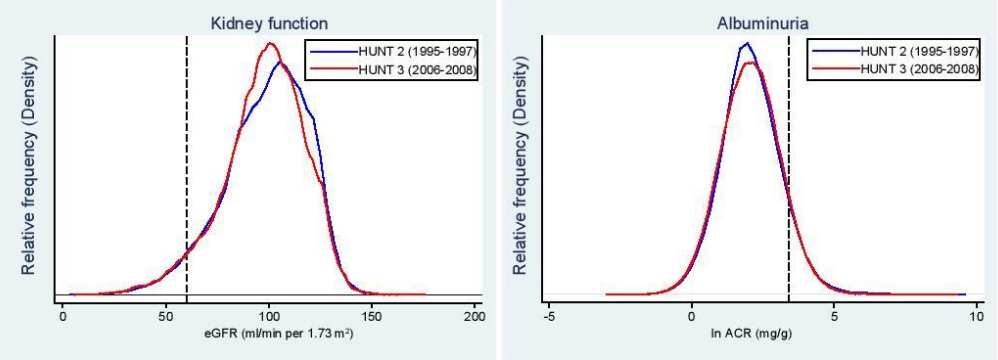

Figure 1 | Distribution of kidney function (estimated glomerular filtration rate [eGFR]) and urinary albumin excretion (mean urinary albumin to creatinine ratio [uACR] of 3 samples) in the adult general population in Norway 10 years apart. The kernel density plots show the local relative frequency on the x-axis. Dotted lines indicate cutoffs used for defining chronic kidney disease (eGFR $<$ 60 ml/min per 1.73 m^2 and uACR $>$ 30 mg/g).

15 to 44 ml/min per 1.73 m^2 with normoalbuminuria were significantly increased, whereas the prevalence of severely increased albuminuria was lower at each eGFR stage.

Among subjects older than 75 years of age, the prevalence of high and very high risk CKD was significantly higher in the HUNT-3 compared with the HUNT-2 (10.2% vs. 11.9%, $P = 0.020$ and 19.6% vs. 24.3%, $P = 0.03$, respectively), whereas moderate-risk CKD was stable (Table 2). For younger subjects, there were no changes in the prevalence of any CKD risk groups. Furthermore, patients with confirmed diabetes mellitus in the HUNT-3 had a substantially lower CKD prevalence compared with those in the HUNT-2 (33.4% vs. 28.6%, $P = 0.002$), whereas a similar pattern was not found in patients with undiagnosed diabetes. Several indicators reflecting intensified preventive treatment over time (e.g., much lower blood pressure, cholesterol, and urine albumin; further data shown in

Albuminuria (mg/g)	A1 (ACR <30)			A2 (ACR 30-300)			A3 (>300)		
eGFR (ml/min/1.73 m^2)	HUNT-2	HUNT-3	*P* value	HUNT-2	HUNT-3	*P* value	HUNT-2	HUNT-3	*P* value
G1 (>90)	65.88%	65.43%	*0.15*	3.80%	3.64%	*0.32*	0.05%	0.04%%	*0.021*
G2a (75–89)	15.87%	16.63%	*0.001*	1.76%	1.69%	*0.50*	0.05%	0.02%	*0.009*
G2b (60–74)	6.94%	6.84%	*0.49*	1.08%	0.96%	*0.12*	0.08%	0.02%	*0.001*
G3a (45–59)	2.64%	2.71%	*0.49*	0.61%	0.62%	*0.78*	0.07%	0.02%	*0.001*
G3b (30–44)	0.64%	0.79%	*0.012*	0.25%	0.32%	*0.11*	0.05%	0.01%	*0.008*
G4 (15–29)	0.10%	0.16%	*0.044*	0.09%	0.11%	*0.29*	0.04%	0.02	*0.057*

	Total CKD	Moderate risk CKD	High risk CKD	Very high risk CKD
HUNT-2 (1995–1997)	**11.30% (SE 0.22)**	9.28% (SE 0.21)	1.42% (SE 0.06)	0.59% (SE 0.03)
HUNT-3 (2006–2008)	**11.11% (SE 0.22)**	9.00% (SE 0.21)	1.47% (SE 0.06)	0.64% (SE 0.04)
	***P* = 0.42**	*P = 0.20*	*P = 0.56*	*P = 0.46*

Figure 2| CKD prevalence trends from 1995 through 1997 to 2006 through 2008 by stages of estimated GFR and albuminuria. Green, no CKD; yellow, moderate risk for complications; orange, high risk for complications; red, very high risk for complications like cardiovascular death, total death, acute kidney injury, and end-stage renal disease. ACR, albumin to creatinine ratio; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate.

Table 2 | Stratified CKD prevalence 1995–1997 compared with 2006–2008

	CKD total (%)			CKD with high/very high risk (%)		
	HUNT-2 (1995–1997)	HUNT-3 (2006–2008)	*P* value	HUNT-2 (1995–1997)	HUNT-3 (2006–2008)	*P* value
Age, yr						
18–44	5.10	4.73	0.24	0.08	0.06	0.50
45–64	8.34	8.16	0.60	0.58	0.50	0.35
65–74	18.64	17.61	0.14	3.54	3.31	0.48
75–84	33.70	35.71	0.08	10.21	11.93	0.020
85+	48.48	53.73	0.05	19.57	24.29	0.030
Overall	11.30	11.11	0.42	2.01	2.11	0.35
Overall, age-adjusted to US 2000 standard population	8.99	8.77	0.11	1.17	1.22	0.33
Diabetes mellitus						
None	9.41	9.05	0.21	1.41	1.41	0.97
Probable undiagnosed	19.64	18.68	0.53	4.34	4.60	0.72
Confirmed	33.41	28.57	0.002	11.28	8.83	0.016
Obesity						
BMI <30	10.36	10.32	0.89	1.67	1.86	0.06
BMI ≥30	16.17	13.94	<0.001	3.80	3.00	0.007

BMI, body mass index; CKD, chronic kidney disease.

Supplementary Table S2) and slightly younger age in the HUNT-3 diabetics could explain this. Obese subjects in the HUNT-3 also had a lower CKD prevalence compared with those in the HUNT-2.

Influence of modifiable risk factors

Possible mediation by risk factors on the association of survey period with CKD prevalence is displayed in Table 3. If we assumed no decrease in systolic blood pressure during the study period, the total CKD prevalence would be 2.8 percentage points higher in the HUNT-3 than observed ($P < 0.001$). Lower cholesterol and higher physical activity also contributed significantly to the stable CKD prevalence. If none of these risk improvements had taken place, the CKD prevalence would have increased by 3.8 percentage points ($P < 0.001$). Correspondingly, without the concurrent increase in prediabetes, diabetes mellitus, and obesity, CKD prevalence could potentially have been 1.0 percentage points lower ($P < 0.001$). Sensitivity analysis using ordinary logistic regression gave very similar results, and analysis of predicted HUNT-3 prevalence substituting various risk factors with mean HUNT-2 values showed less but still significant influence of blood pressure and physical activity improvements (Supplementary Table S3).

Finally we compared Norwegian, US, and British population level data that could contribute to changes in CKD prevalence beyond the individual level risk factors analyzed in previous text. Relevant information from national kidney registries, published studies, governmental and international organizations, and other sources was collected to describe the

Table 3 | Predicted changes in CKD prevalence from HUNT-2 (1995–1997) to HUNT-3 (2006–2008) if modifiable kidney risk factors remained at 1995–1997 level

	eGFR <60 ml/min	ACR >30 mg/g	CKD total	CKD high/ very high risk
Observed prevalence 1995–1997 (%)	4.49	7.93	11.30	2.01
Improved variables				
Predicted CKD change if no change in	(Absolute prevalence change [percentage points], 2006–2008 vs. 1995–1997)			
Systolic BP	+2.06***	+1.11***	+2.77***	+0.91***
CVD	+0.49**	−0.43	−0.01	+0.18
Cholesterol	+1.01***	−0.23	+0.58*	+0.36**
Physical inactivity	+0.93***	−0.13	+0.69*	+0.37**
Smoking	+0.19	−0.34	−0.15	−0.06
None of these risk factors	+3.41***	+1.36***	+3.78***	+1.43***
Worsened variables				
Predicted CKD change if no change in				
Prediabetes/diabetes	+0.03	-0.83**	−0.73**	−0.06
BMI	+0.04	−0.74**	−0.65*	−0.02
None of these risk factors	−0.12	−0.95***	−1.00***	−0.13

Note: Asterisks indicate that change from HUNT-2 to HUNT-3 is significantly different from 0 (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Data are based on generalized estimation equation analysis, an extension of logistic regression for clustered data. For example, with CKD total as a dependent variable and time period (HUNT-2 or HUNT-3) and systolic BP as independent variables, time period has an odds ratio of 1.245: CKD prevalence in HUNT3 would be 1.245 times higher if systolic blood pressure remained unchanged ($1.245 \times 11.3 - 11.3 = 2.77\%$ [percentage points] higher CKD prevalence could have been expected).
ACR, albumin to creatinine ratio; BMI, body mass index; BP, blood pressure; CKD, chronic kidney disease; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate.

risk factors and clinical setting in which these 3 major CKD prevalence studies were conducted (Supplementary Table S4, Supplementary Figure S1). Because the studies describe different time periods, we also display relevant information estimated by the Global Burden of Disease project for the total 1990 through 2010 period for all 3 countries (Supplementary Table S5, Supplementary Figure S2).

DISCUSSION

Paying special attention to design and analytical problems in prevalence trend studies, we found that total CKD prevalence was stable in Norway over a 10-year period. Improved treatment of hypertension and hypercholesterolemia and higher physical activity might have contributed to this favorable situation despite increasing diabetes and obesity prevalence.

Coresh *et al.*[4] reported that the CKD prevalence in the US adult population increased from 10.0% in the period 1988 through 1994 to 13.1% in the period 1999 through 2004, which was partly explained by trends in diabetes and hypertension. Others have questioned these findings due to a possible drift in serum creatinine measurements across National Health and Nutrition Examination Survey exams.[28,29] A conservative trend analysis, that is, adjusting for the assumption that no eGFR change should take place in healthy young subjects without known CKD risk factors, showed a more modest change in CKD prevalence (10.0% to 11.3%).[4] However, moving past creatinine calibration, a recent analysis based on well-calibrated cystatin C measurements found that the prevalence of eGFR <60 ml/min per 1.73 m^2 had increased significantly.[30] It is therefore most likely that CKD prevalence indeed increased in the United States in the 1990s, whereas more recent National Health and Nutrition Examination Survey reports indicate stabilization thereafter.[31]

Other studies present conflicting results on CKD prevalence trends over time. Nagata *et al.*[13] described a smaller but well-characterized Japanese population followed from 1974 to 2002. CKD stages 3 through 5 increased 3-fold in men (4.8% to 15.7%) and doubled in women (5.8% to 11.7%). In contrast, Kang *et al.*[32] concluded that the CKD prevalence decreased in South Korea between 1998 and 2009, but there were more missing data and no information regarding the potential for analytical drift in serum creatinine values over time. A 1.5-fold higher risk of an eGFR <60 ml/min per 1.73 m^2 was reported in Finland between 2002 and 2007,[12] whereas a laboratory-based study from Scotland reported stable CKD prevalence between 2004 and 2009.[15] Furthermore, in one of the best methodological studies on this topic, Aitken *et al.*[16] reported a significant decrease in CKD stage 3–4 in England from 2003 to 2010. Using nationally representative samples adjusted for nonresponse, the investigators found that an eGFR <60 ml/min per 1.73 m^2 decreased from 5.7% to 5.2% based on well-calibrated serum creatinine values. The total CKD prevalence was 13% in 2010, but, unfortunately, albuminuria data were not available for the 2003 survey.

The Global Burden of Disease project, which is an enormous undertaking with excellent opportunities to describe the ongoing epidemiologic transition, has recently estimated that the impact of CKD increased steadily in both high-income and developing countries during the period 1990 through 2010 (459–549 and 339–438 disability–adjusted life-years/100.000, respectively).[26] A study on the future burden of CKD in the United States estimates that the prevalence will increase from 13.2% currently to 16.7% in 2030,[33] and projections also indicate that CKD will move up 4 places in the global mortality rankings.[34]

In our study, although total CKD prevalence remained stable in Norway, there were some worrisome changes in the eGFR distribution in the adult population. First, a low eGFR (<60 ml/min per 1.73 m^2) showed a small but significant increase, but this was, at least temporarily, offset by a decrease in albuminuria, most likely caused by better blood pressure control and a 3-fold increased prescription of angiotensin-converting enzyme inhibitors. Second, there was a large shift in the normal eGFR range with fewer subjects with an eGFR of 105 to 125 ml/min per 1.73 m^2 and correspondingly more subjects with an eGFR of 85 of 105 ml/min per 1.73 m^2. The future consequences of this trend are unknown but could potentially imply a higher CKD prevalence in Norway in future years.

Norwegian cardiovascular guidelines were conservative relative to other high-income nations during the 1980s through 1990s with high age-dependent blood pressure goals,[35] but later recommendations have become more similar to European and US guidelines. Strong blood pressure improvements were achieved thereafter as observed in the HUNT-3 and other Norwegian studies,[36] but attained blood pressure still remains somewhat higher than other Western countries (Supplementary Table S6). The recent Joint National Committee 8 Hypertension Guidelines suggest that systolic blood pressure goals could be increased by 10 mm Hg in subjects older than 60 years of age, and stricter treatment goals in CKD patients with proteinuria or diabetes are no longer recommended,[24] whereas recent randomized trial data published after the Joint National Committee 8 suggest that intensive blood pressure control to systolic blood pressure targets <120 mm Hg substantially decreases cardiovascular disease and all-cause mortality risk.[25] Notwithstanding the all-cause mortality benefit, however, higher risks of acute kidney injury and a 30% decrease in eGFR were observed with intensive blood pressure treatment. This has created an intense debate about the appropriate blood pressure targets.[37,38] Furthermore, British survey data also demonstrated tighter blood pressure control with increased prescription of antihypertensive drugs and an increased proportion of angiotensin-converting enzyme inhibitors/angiotensin receptor blockers.[39] This corresponds to the period when Aitken *et al.*[16] described a reduced prevalence of CKD stage 3–4. Therefore, improved blood pressure control may have contributed to a stabilized CKD prevalence in a period with increasing obesity and diabetes prevalence.

There are fewer data available to support our hypothesis that lipid-lowering therapy and more physical exercise reduce the risk of kidney disease. Several studies show that abnormal lipid levels are associated with accelerated kidney function loss,[40,41] but statin intervention trials have shown conflicting results and are not recommended solely for renal protection.[42–44] However, many studies used hard clinical outcomes such as ESRD, which are late events requiring long follow-up and large sample sizes. It has therefore been suggested that future studies should test high-potency statins before a major GFR decrease occurs and to use lesser decreases in eGFR as an alternative endpoint.[45,46] Physical exercise improves quality of life, muscle strength and muscle mass, and cardiorespiratory fitness in all stages of CKD. Less is known about kidney disease progression. Physical exercise has been shown to slow the decline in kidney function in small randomized, controlled trials.[47] Low physical activity was attributable for as many as 5% of new cases or progressive CKD in a large observational cohort study,[48] and elderly individuals with higher physical activity have a 28% lower risk of future rapid GFR decline after multivariate adjustment.[49] Potential mechanisms could be the anti-inflammatory effects and improved mitochondrial function found after exercise.[50,51] Therefore, lipids and physical exercise are still not well documented in the prevention of CKD, but our findings give support to such a hypothesis.

Comparison of national level data from Norway, the United States, and the United Kingdom illustrate other potential contributors to the differing CKD prevalence and ESRD incidence trends in these high-income countries (Supplementary Tables S4 and S5 and Supplementary Figures S1 and S2). One of the major relevant differences is the obesity epidemic with high diabetes numbers, which started earlier in the United States and has grown faster due to higher energy intake and less physical activity. Improvements will require individual efforts as well as political and food industry involvement, for example, reduction of serving sizes and sugared beverages could be important public health interventions that could decrease obesity and diabetes and downstream CKD at the population level. Access to effective primary preventive care as well as sufficiently early predialysis care by specialists could be important advantages in Norway and the United Kingdom. Several studies show that despite high health expenditure per capita, US health improvements have not kept pace with advances in population health in other higher income nations.[52] This also holds for CKD where years-of-life-lost estimates have increased by 86% and years-lived-with-disability have increased by 44% in the United States from 1990 to 2010.

The current study has several strengths. The 2 HUNT surveys had an identical design with high participation rates, missing data were imputed, and weights for nonattendance were applied to reduce the potential of bias. Both urine and blood samples were analyzed consecutively to avoid potential problems with frozen samples. Avoiding analytical drift over a 10-year period is crucial for a prevalence trend study. An external quality-control program and thorough calibration assessment when changing methods were used to minimize analytical drift over time. Moreover, analyses in young persons without CKD risk factors showed nearly identical eGFR and ACR measurements at both time points. We had a large number of relevant covariates on both the individual and group levels. Three urine samples enabled us to define albuminuria with greater precision. However, the study also has limitations. The observational data presented here support a hypothesis that differences in risk factors may have accounted for the stable CKD prevalence, but this study is not a randomized clinical trial. Although we hypothesize that trends in hypertension treatment, physical activity, diabetes prevalence, and lipid therapy over time may help to explain the findings observed in our data, the effects of treatment of each of these risk factors on CKD prevalence is not known definitively and requires evaluation in randomized clinical trials. Missing-by-design albuminuria testing is not optimal but was necessary in these large cohorts, but with 15,000 urine albumin measurements and 20 differently imputed datasets, we believe that this design feature is unlikely to have introduced substantial error in our overall prevalence estimates. However, multiple imputations combined with generalized estimation equations rely on some assumptions, and we acknowledge some uncertainty within the bounds of the 95% confidence intervals and *P* values. Glucose values were nonfasting but corrected for time since the last meal, which could make prevalence estimates of prediabetes and undiagnosed diabetes less accurate. However, this approach was applied similarly to both HUNT visits, and thus should not have systematically biased results. The study was also conducted in a white population in the geographic and geopolitical environment of Norway, so generalization to other races and countries is uncertain. On the other hand, our focus was to describe CKD prevalence in Norway specifically and to compare it with similar data in other countries. Recently, a 5-fold variation in CKD prevalence across the European general population has been demonstrated, potentially caused by differences in human and environmental factors, public health policies, genetics as well as laboratory methods.[53] The ethnicity effect is well studied in African Americans in whom the APOL1 risk variants are associated with a strongly increased risk of kidney disease, but there was no interaction with the effect of treatment regimen.[54] Even low-risk African Americans potentially eligible to serve as kidney donors have a 4-fold increased risk of ESRD.[55] Furthermore, epigenetic effects arising from adverse prenatal and socioeconomic conditions may modify the effect of traditional risk factors. The risk associations found in our study can therefore be somewhat different in other populations, but the harmful effects of hypertension, diabetes, and other risk factors have been shown to be rather consistent over race, region, and other variables.[56–59]

In summary, CKD prevalence remained stable in Norway from 1996 through 2007. We hypothesize that this could be due to strong improvements in hypertension control, cholesterol control, and greater physical activity, which

counteracted modest increases in obesity and diabetes. Recent British data showed a higher prevalence of diabetes, obesity, and inactivity but also of proactive preventive medicine, which resulted in a decline in CKD stage 3–4 prevalence. In contrast, in the United States, there was a substantial increase in CKD prevalence from 1991 through 2001, likely primarily driven by dramatic increases in obesity and diabetes. In concert with recent clinical trial data,[25,60] these observations have implications for interpretation of consensus guidelines suggesting less intensive blood pressure control in persons older than 60 years and in patients with diabetes or CKD. Continued close monitoring of these risk factors and CKD prevalence are indicated moving forward. Our findings also support initiatives focusing on lipid control, physical activity, weight control, healthy food, and general access to health care, which may ultimately improve CKD prevalence.

METHODS

Study population

The HUNT study is a large Norwegian general health study inviting every resident of Nord-Trøndelag County 20 years of age and older every 10 years.[61] Each survey comprised an extensive questionnaire on medical history and risk factors, and a short clinical examination was performed. We used the HUNT-2 (1995–1997) and HUNT-3 (2006–2008) surveys to study CKD prevalence over time in a repeated cross-sectional design. Three consecutive standardized blood pressure measurements were recorded at 1-minute intervals after a minimum of 5 minutes of rest in the sitting position using an automatic oscillometric method (Dinamap 845XT; Criticon, Tampa, FL). The same blood pressure devices were used in both surveys with thorough technical service and calibration. For analysis, mean of the second and third measurements was used. A more comprehensive description of the population, objective, and methods is given in Supplementary Table S7 and elsewhere.[61,62] The study was approved by the Norwegian National Data Inspectorate and the Regional Committee for Medical and Health Research Ethics.

Analysis

We obtained information about pre-existing cardiovascular disease defined as myocardial infarction, angina pectoris, or stroke from questionnaires. The frequency, duration, and intensity of physical activity were reported, and individuals with <1 hour of light exercise per week were classified as inactive.[63] Socioeconomic status was described by level of education (college, yes/no) and residential area's socioeconomic status (rural/urban, level of income, and proportion receiving disability pension). Glucose values were adjusted for time since last meal,[64] leading to similar mean glucose values by 1-hour categories since last meal. Physician-diagnosed diabetes and/or glucose ≥200 mg/dl was classified as confirmed diabetes, a glucose level of 126 to 200 mg/dl as probable undetected diabetes, and a glucose level of 100 to 125 mg/dl as probable prediabetes.[65] A diabetes screening score was calculated to summarize diabetic risk factors (age, sex, body mass index, family history of diabetes, and physical activity).[66] Smoking was classified as current, former, or never. Hypertension was defined as systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg or use of antihypertensive medications.

Albuminuria was defined as normal (A1) if first urine had an ACR <30 mg/g (3.4 mg/mmol), moderately increased (A2) if the first urine was 30 to 300 mg/g and confirmed in a second or third sample (formerly termed microalbuminuria), or severely increased (A3) if first urine was >300 mg/g (formerly termed macroalbuminuria). CKD was defined as an eGFR <60 ml/min per 1.73 m^2 or increased albuminuria (A2-A3).[67] CKD was stratified according to the risk of complications such as acute kidney injury, progression to ESRD, or death, using categories of eGFR versus ACR (Supplementary Table S8).[67]

Laboratory analysis

Fresh blood and urine samples were analyzed consecutively. Serum creatinine was measured by the Jaffé method and calibrated to isotope-dilution mass spectroscopy level. GFR was estimated with the Chronic Kidney Disease Epidemiology consortium (CKD-EPI) formula.[68] Urine albumin was determined by an immunoturbidimetric method. Further details on analytical methods, quality-control systems, correlations between methods, handling of missing data, and testing for analytical drift over time are given in Supplementary Methods.

Statistical analysis

Participation rates can vary substantially by subgroups, so separate logistic regression models for participation in the HUNT-2 and HUNT-3 were built using public registry information (age, sex, marital status, and geographic information) available for all invited subjects. Individual participation weights were calculated as 1/predicted probability of attendance. Multiple imputation of missing data was combined with nonattendance weights for all analyses to give unbiased estimates: means or proportions (±1 SE and 95% confidence intervals adjusted for the use of multiple data sets) for descriptive statistics and kernel density plots for distribution of kidney measures. Due to repeated measures with 61% of HUNT2 subjects also participating in HUNT3, we used a generalized estimation equation, which can be considered an extension of logistic regression for clustered data.[69] Generalized estimation equations with unstructured correlation matrices were used to test for prevalence differences and to assess the relationship between prevalence trends and modifiable kidney risk factors, that is, to predict what the prevalence of CKD would have been had risk factor profiles not improved over the 10-year interval between studies. Ordinary logistic regression with time period as a covariate and analysis restricted to the HUNT3 but substituting various variables with mean HUNT2 risk factor levels were also performed as sensitivity analyses. Stata/SE 13.1 (StataCorp, College Station, TX) was used for all analyses.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary Methods

Supplementary References

Figure S1. ESRD incidence rates (new cases per million inhabitants per year) in the United States, Norway, and England. Data based on

information published by the national renal registries.[15–17] DM, diabetes mellitus; GN, glomerulonephritis; HT, hypertension; PCK, polycystic kidney disease.

Figure S2. Data from the Global Burden of Disease project showing disability-adjusted life-years (DALYs) in Norway, the United Kingdom, and the United States caused by 4 important metabolic risk factors.

Table S1. Healthy subgroups from HUNT-2 and HUNT-3 free of kidney risk factors and thereby supposed to have equal kidney function and urine albumin excretion, that is, a method for testing for analytical drift over time.

Table S2. Prevalence of modifiable risk factors in relevant clinical subgroups, 1995–1997 and 2006–2008 (estimates stratified to age distribution for HUNT-3 study are also displayed).

Table S3. Sensitivity analysis of changes in CKD prevalence from 1995–1997 to 2006–2008 if modifiable kidney risk factors remained at 1996 level. Data represent the difference between observed HUNT-2 prevalence and predicted HUNT-3 prevalence substituting various risk factors with mean HUNT2 values, that is, no change.

Table S4. Comparison of CKD prevalence and population level measures of potential importance for kidney health in the United States, Norway, and England over 10-year periods.

Table S5. Ranking (2010) and annual change of DALYs (%, 1990–2010) caused by various risk factors in Norway, the United Kingdom, and the United States.

Table S6. Attained blood pressure and serum cholesterol levels in Norway and other European countries.[12]

Table S7. Comparison of relevant health characteristics from Statistics Norway for Nord-Trøndelag County (NT) versus all of Norway. Red line indicates average for Norway, diamonds indicate NT County, red = significantly worse, green = significantly better, yellow = nonsignificant.[13]

Table S8. CKD definition and risk classification according to KDIGO 2012 guidelines.[14]

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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CLINICAL PROTEOMICS

RESEARCH Open Access

Urinary proteomics in chronic kidney disease: diagnosis and risk of progression beyond albuminuria

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Abstract

Background: The contrast between a high prevalence of chronic kidney disease (CKD) and the low incidence of end-stage renal disease highlights the need for new biomarkers of progression beyond albuminuria testing. Urinary proteomics is a promising method, but more studies focusing on progression rate and patients with hypertensive nephropathy are needed.

Results: We analyzed urine samples with capillary electrophoresis coupled to a mass-spectrometer from 18 well characterized patients with CKD stage 4–5 (of whom six with hypertensive nephropathy) and 17 healthy controls. Classification scores based on a previously developed panel of 273 urinary peptides were calculated and compared to urine albumin dipstick results. Urinary proteomics classified CKD with a sensitivity of 0.95 and specificity of 1.00. Overall diagnostic accuracy (area under ROC curve) was 0.98, which was better than for albuminuria (0.85, $p = 0.02$). Results for hypertensive nephropathy were similar to other CKD diagnoses. Adding the proteomic score to an albuminuria model improved detection of rapid kidney function decline (>4 ml/min/1.73 m^2 per year) substantially: area under ROC curve increased from 0.762 to 0.909 ($p = 0.042$), and 38% of rapid progressors were correctly reclassified to a higher risk and 55% of slow progressors were correctly reclassified to a lower risk category. Reduced excretion of collagen types I–III, uromodulin, and other indicators of interstitial inflammation, fibrosis and tubular dysfunction were associated with CKD diagnosis and rapid progression. Patients with hypertensive nephropathy displayed the same findings as other types of CKD.

Conclusions: Urinary proteomic analyses had a high diagnostic accuracy for CKD, including hypertensive nephropathy, and strongly improved identification of patients with rapid kidney function decline beyond albuminuria testing.

Keywords: Chronic kidney disease, Hypertensive nephropathy, Urine, Albuminuria, Proteomics, Disease progression

Background

Chronic kidney disease (CKD) has a high prevalence and represents a large burden of morbidity and health care cost [1, 2]. Diagnosis and staging is based on estimated glomerular filtration rate (eGFR) and the degree of albuminuria, which currently is our most reliable marker of rapid kidney function decline [3]. However, the diagnostic accuracy of albuminuria for CKD is only moderate with most studies reporting area under the ROC curve ranging 0.80–0.85 [4–8], so predicting which CKD patients will have a more rapid disease progression remains a major clinical problem.

Emerging gene-based tests typically report relative risks of 1.2–1.4 for polygenetic diseases like hypertension and CKD, which renders them largely useless as diagnostic tools [9]. Other recent technologies enable us to detect large numbers of proteins and metabolites in urine, and these technologies may have a greater potential as they focus on the end products of biological processes. Several studies have used urine proteomics for diagnostic purposes in glomerulonephritis [10], renal cancer [11] and renal transplantation [12], but there is a strong call

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for more clinically relevant studies and better phenotyping [13]. Despite making up 30% of ESRD cases in the US and Europe, hypertensive nephropathy is surprisingly understudied, and proteomic analyses has never been performed [14]. The diagnosis of hypertensive nephropathy is based on unspecific clinical characteristics, and the pathophysiology of this broad clinically-based entity is not well described and could be different from what has been described in experimental and biopsy based studies.

Our study describes phenotype characteristics, progression rates, and outcomes in unselected Norwegian CKD outpatients, and relate them to their urine proteomic findings, with special attention to clinically diagnosed hypertensive nephropathy.

Results

Eighteen CKD stage 4–5 patients with a wide range of kidney diagnoses were included: eight with glomerulonephritis/diabetic nephropathy, six with hypertensive nephropathy and four cases with miscellaneous causes of CKD (lithium nephropathy, Alport disease, chronic interstitial nephritis, and cyclosporine A toxicity). Seventeen healthy controls were also included. Baseline demographics and kidney status are described in Table 1. As expected, diabetes, cardiovascular disease, and kidney related variables were substantially worse in the CKD group, but their levels of blood pressure, hemoglobin, phosphate, parathyroid hormone (PTH) and other markers of uremia indicated that they were reasonably well treated and in an acceptable metabolic state. Mean eGFR at inclusion was 17 ml/min/1.73 m^2 in the CKD cases, and their mean decline in kidney function over the past 1–11 years prior to inclusion was very similar to the decline over the two and a half years following inclusion [−8.1 ml/min/1.73 m^2/year (±8.4) vs −8.8 ml/min/1.73 m^2/year (±7.6), $p = 0.75$, based on a mean of 8.9 and 2.2 measurements per subject]. We therefore used data from the total observation period to indicate their rate of progression. Controls did not show any significant decline in kidney function over the 2 years, and we display baseline characteristics for rapid progressors (eGFR decline >4 ml/min/1.73 m^2 per year) versus slow progressor (eGFR decline ≤4 ml/min/1.73 m^2 per year). Patients with hypertensive nephropathy typically had higher age, less albuminuria, and slightly slower decline in kidney function compared to patients with glomerulonephritis and diabetes nephropathy. By 2012, three CKD patients were on dialysis, four had been transplanted, and six had died.

The urine proteomic analyses detected 4,276 different proteins, and information from 273 of these were converted into a classification score for each subject with values above the predefined 0.343 cutoff indicating

Table 1 Baseline characteristics of participants

	Major groups		Progression rate		CKD diagnosis		
	Healthy (17)	CKD (18)	Rapid (13)	Slow (22)	HN (6)	GN/DN (8)	Other (4)
Age	47.8 (10.6)	63.7 (16.3)	63.8 (18.3)	51.5 (13.0)	77.8 (4.9)	61.6 (12.3)	47.0 (20.3)
Male gender (%)	58.8	72.2	84.6	54.5	100	87.5	0.0
Diabetes Mellitus (%)	0.0	33.3	38.5	4.5	33.3*	37.5	0.0
Cardiovascular disease (%)	0.0	55.6	53.8	13.6	83.3	37.5	50.0
eGFR (ml/min/1.73 m^2)	87.2 (5.4)	17.4 (7.2)	16.6 (6.9)	71.0 (30.2)	16.7 (8.6)	19.6 (7.7)	14.0 (1.4)
eGFR (ml/min/1.73 m^2) decline per year	−0.3 (1.4)	−6.7 (5.1)	−8.9 (4.6)	−0.6 (1.4)	−5.8 (1.9)	−6.4 (5.1)	−8.8 (8.4)
Albuminuria (dipstick)							
Trace/+ (%)	17.7	22.2	23.1	18.2	33.3	25.0	0.0
++/+++ (%)	0.0	55.6	61.6	9.1	33.3	62.5	75.0
Systolic BP (mmHg)	131.8 (14.3)	144.2 (24.6)	144.1 (28.5)	134.8 (15.5)	142.2 (21.1)	146.6 (32.5)	142.3 (20.0)
Hgb (g/dl)	14.2 (1.3)	11.6 (1.5)	11.5 (1.3)	13.6 (1.7)	11.2 (1.7)	11.9 (1.6)	11.8 (1.1)
K (mmol/l)	4.1 (0.3)	4.5 (0.6)	4.6 (0.6)	4.2 (0.4)	4.6 (0.6)	4.7 (0.5)	4.2 (0.7)
Ca (mmol/l)	2.3 (0.1)	2.3 (0.1)	2.3 (0.2)	2.3 (0.1)	2.3 (0.2)	2.3 (0.1)	2.2 (0.02)
P (mmol/l)	1.1 (0.1)	1.4 (0.5)	1.5 (0.5)	1.1 (0.2)	1.6 (0.7)	1.3 (0.4)	1.4 (0.3)
Urea (mmol/l)	6.1 (1.1)	23.5 (8.5)	24.1 (8.3)	10.0 (8.2)	26.0 (10.7)	22.9 (5.8)	21.3 (10.3)
PTH (pmol/l)	3.5 (0.7)	28.2 (23.4)	30.5 (27.0)	7.2 (10.3)	25.3 (17.4)	29.3 (36.0)	30.0 (15.1)
Bicarbonate (mmol/l)	24.9 (2.4)	20.8 (2.3)	21.0 (2.4)	24.0 (3.0)	20.7 (2.9)	21.0 (2.2)	20.5 (1.9)

Data are mean (1SD) or percentages. Rapid progressors: eGFR declined more than 4 ml/min/1.73m2 per year. Slow progressors: eGFR declined less than 4 ml/min/1.73 m^2 per year.

GN glomerulonephritis, *DN* diabetic nephropathy, *HN* hypertensive nephropathy, *Other* other CKD diagnosis.

* One patient fulfilled the diabetes criteria just before study inclusion and another had nephrosclerosis only in his kidney biopsy.

high probability for CKD [10]. The mean score in CKD patients and controls were 0.71 and −0.31, respectively ($p < 0.001$), indicating excellent overall discrimination. The box-and-whisker plots in Fig. 1 show the distribution of the proteomics scores by CKD diagnosis. Classification scores were higher than the cut-off value in all CKD patients, except for one patient with hypertensive nephropathy. The proteomics score had a sensitivity of 95% and a specificity of 100% using the standard cut-off of 0.343, and the overall diagnostic accuracy was also excellent [area under ROC curve 0.977 (95% confidence interval (CI) 0.930–1.000)] (Fig. 2). ROC analysis of the urine dipstick test for albuminuria gave an AUC of 0.850 (95% CI 0.730–0.970), which is a significantly lower diagnostic accuracy ($p = 0.02$).

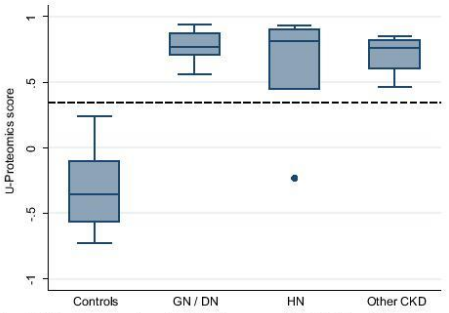

Fig. 1 Urine proteomics classification score (CKD273) by CKD diagnosis. GN/DN: patients with glomerulonephritis or diabetes nephropathy; HN: patients with hypertensive nephropathy. Negative values indicate normal healthy subjects, and 0.343 have been used as cut-off for CKD (*dotted line*) [10].

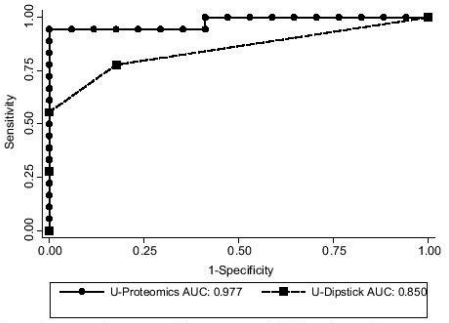

Fig. 2 Receiver Operating Characteristics (ROC) analysis of urine proteomics (CKD273 classifier) and albuminuria (dipstick) for diagnosing patients with CKD. *AUC* area under curve.

Figure 3a shows the continuous relationship between urine proteomics score and kidney function decline. Kidney function deteriorated substantially in the proteomics scores range 0.0–0.5, while the association was rather flat for scores above 0.5 with a decline in eGFR of 7 ml/min/1.73 m^2 per year. The corresponding relationship for albuminuria is shown in Fig. 3b. Kidney function decline per year increased with higher grades of albuminuria, but the figure also demonstrates substantial variation in kidney function decline within each level of albuminuria. Albuminuria had an area under the ROC curve of 0.762 for detecting subjects with rapid kidney function decline (more than 4 ml/min/1.73 m^2 per year). Corresponding results for the urine proteomics score was 0.864. Adding proteomic score to an albuminuria model, which is a clinically relevant evaluation, significantly increased the diagnostic accuracy to AUC 0.909 (p = 0.042 compared to albuminuria alone). Furthermore, reclassification analysis showed that two out of five rapid progressors with intermediate predicted risk were (correctly) reclassified into the high risk group, while one out of five was

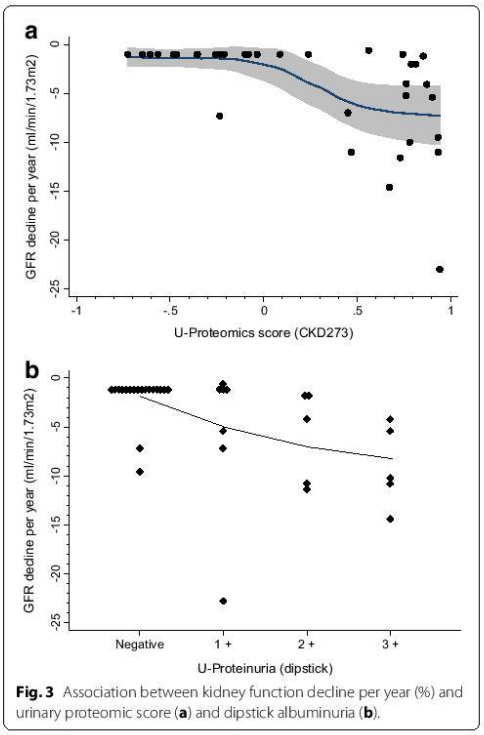

Fig. 3 Association between kidney function decline per year (%) and urinary proteomic score (**a**) and dipstick albuminuria (**b**).

(incorrectly) reclassified to the low risk group. For the slow progressors, three out of four with intermediate risk were (correctly) reclassified into the low risk group (Table 2).

Proteomic intensities of the most important urinary proteins are given in Table 3 for the different diagnostic groups compared to healthy controls. The associations of specific urinary proteins to CKD diagnosis and to rapid kidney function decline are given in Table 4. Data are presented as standardized beta-coefficients, i.e. effect on outcome per 1 SD change to facilitate comparisons, and as area under ROC curves. Fragments from collagen types I, II, and III were strongly reduced and ranked on top with typical ORs of 0.01 (i.e. if collagen concentration decreases with 1 SD the risk increases 100 times) and ROC areas of 0.95 (i.e. the test correctly classifies 95% of pairs with and without the outcome). No association was found to collagen type IV, which is the dominant glomerulus basement membrane type. Urine levels of CD99, uromodulin, sodium/potassium-transporting ATPase gamma chain, and osteopontin were also reduced in CKD patients. Except for osteopontin, these proteins were also strongly associated with a rapid decline in kidney function. The systemic blood-derived proteins were typically lower ranked with ROC area-under-curves below 0.80, and albumin was ranked number 15 for rapid kidney decline.

Table 2 Risk reclassification when adding urine proteomic score to albuminuria for predicting risk of rapid kidney function decline

	Predicted risk for having rapid kidney function decline			
	0–9%	10–49%	50–100%	Total (%)
Subjects with rapid kidney function decline				
Model with albuminuria	0.0%	38.5%	61.6%	100.0
Model with albuminuria + proteomics	7.7	15.4	76.9	100.0
Subjects without rapid kidney function decline				
Model with albuminuria	0.0	90.9	9.1	100.0
Model with albuminuria + proteomics	68.2	22.7	9.1	100.0

Discussion

We found that a urinary proteomics classification score based on 273 different proteins had a significantly better diagnostic accuracy for chronic kidney disease than albuminuria. Adding the proteomic score to albuminuria, our currently best predictor of kidney prognosis, improved detection of patients with rapid progression substantially. Reduced urinary excretion of collagen fragments types I–III was among the most important contributors to these findings.

More than 150 articles have been published on urinary proteomics and the kidney over the last 10 years, a substantial proportion being review articles. Initial studies

Table 3 Amplitudes of most important urinary protein by clinical diagnosis

Peptide information	SwissProt name	Mean amplitude				Fold changes			
Peptide name		Control	HN	GN + DN	Others	Control	HN	GN + DN	Others
Alpha-1-antitrypsin	A1AT_HUMAN	39	11,031	21,119	21,236	1	282.5	540.9	543.9
Serum albumin	ALBU_HUMAN	0	23,348	78,627	13,663				
Apolipoprotein A-I	APOA1_HUMAN	47	127,871	110,906	38,500	1	2,713.9	2,353.8	817.1
Na/K-transp. ATPase gamma chain	ATNG_HUMAN	1,984	396	322	450	1	0.2	0.2	0.2
Beta-2-microglobulin	B2MG_HUMAN	0	239,173	677,860	207,089				
CD99 antigen	CD99_HUMAN	1,357	0	28	129	1	0.0	0.0	0.1
Collagen alpha-1 (I) chain	CO1A1_HUMAN	3,078	1,533	1,123	2,455	1	0.5	0.4	0.8
Collagen alpha-1 (II) chain	CO2A1_HUMAN	3,190	1,344	500	1,367	1	0.4	0.2	0.4
Collagen alpha-1 (III) chain	CO3A1_HUMAN	2,338	1,064	717	1,246	1	0.5	0.3	0.5
Alpha-2-HS-glycoprotein	FETUA_HUMAN	43	12,128	14,441	6,950	1	283.7	337.8	162.5
Fibrinogen alpha chain	FIBA_HUMAN	965	14,993	2,308	6,477	1	15.5	2.4	6.7
Osteopontin	OSTP_HUMAN	410	0	0	39	1	0.0	0.0	0.1
Membrane associated progesterone receptor component 1	PGRC1_HUMAN	536	1,017	8	502	1	1.9	0.0	0.9
Polymeric-immunoglobulin receptor	PIGR_HUMAN	1,624	583	584	740	1	0.4	0.4	0.5
Transthyretin (Prealbumin)	TTHY_HUMAN	15	22,299	53,034	22,292	1	1,471.6	3,499.9	1,471.2
Uromodulin	UROM_HUMAN	2,525	89	136	176	1	0.0	0.1	0.1
Neurosecretory protein VGF	VGF_HUMAN	1,770	9,796	6,140	5,644	1	5.5	3.5	3.2

HN Hypertensive nephropathy, *GN* Glomerulonephritis, *DN* Diabetic nephropathy.

Table 4 Specific urinary proteins listed by association and diagnostic accuracy for CKD and rapid kidney function decline

Protein	Detected peptides	CKD diagnosis				Rapid kidney function decline			
		P value	OR (StdX)	ROC	Rank	P value	OR (StdX)	ROC	Rank
Collagen alpha-1 (I) chain	33	0.002	0.03	0.941	1	0.004	0.101	0.853	3
CD99 antigen	1	0.004	0.001	0.918	2	0.028	0.008	0.832	5
Uromodulin	3	0.007	0.001	0.98	3	0.019	0.025	0.846	4
Sodium/potassium-transporting ATPase gamma chain	1	0.002	0.032	0.954	4	0.016	0.002	0.93	2
Collagen alpha-1 (II) chain	1	0.002	0.04	0.948	5	0.001	0.063	0.93	1
Collagen alpha-1 (III) chain	15	0.002	0.112	0.882	6	0.013	0.195	0.837	6
Neurosecretory protein VGF	1	0.008	37	0.876	7	0.017	4.58	0.825	7
Osteopontin	2	0.016	0.018	0.863	8	0.99		0.5	18
Collagen alpha-2 (I) chain	4	0.012	0.172	0.848	9	0.069	0.35	0.724	13
Transthyretin (Prealbumin)	2	0.086	n.s.	0.892	10	0.062	5.51	0.82	11
Beta-2-microglobulin	1	0.065	n.s.	0.859	11	0.358	1.39	0.811	17
Alpha-2-HS-glycoprotein	2	0.05	n.s.	0.84	12	0.07	2.33	0.86	12
Alpha-1-antitrypsin	3	0.168	n.s.	0.871	13	0.047	11.11	0.811	10
Polymeric-immunoglobulin receptor	1	0.02	0.337	0.77	14	0.097	0.48	0.706	16
Apolipoprotein A-I	1	0.172	n.s.	0.85	15	0.047	9.45	0.822	9
Membrane associated progesterone receptor component 1	1	0.056	0.441	0.801	16	0.103	0.429	0.731	14
Albumin	1	0.397	n.s.	0.845	17	0.17	1.77	0.745	15
Fibrinogen alpha chain	2	0.156	n.s.	0.722	18	0.03	9	0.822	8

Protein names and number of specific peptides detected from this protein are given. Data are given only for the best peptide per protein. Odds ratios are based on unadjusted logistic regression analysis. Peptides with *p* values <0.10 or with special interest (albumin) were included. Data show *p* value and odds ratio for outcome associated with one standard deviation change of protein to improve comparability (logistic regression analysis; OR StdX could not be calculated for all associations). Area under the ROC curve is also given. The separate rankings for CKD diagnosis and rapid kidney function decline (>4 ml/min/1.73 m^2 per year) represent the mean ranks for *p* values, standardized OR and ROC.

n.s. not significant.

focused on acute kidney failure [15], transplantation rejection [12], renal carcinoma [11], obstructive nephropathy [16], and glomerulonephritis [17, 18]. Different biomarkers and panels of biomarkers have demonstrated sensitivities and specificities ranging 0.45–0.98. However, many studies have been based on small numbers, there has been a lack of relevant clinical information, and results have been difficult to reproduce due to difficulties and variations in analytical techniques and sample preparation. Furthermore, many studies have so far been carried out in settings with little clinical relevance, e.g. the patients had already been properly diagnosed with a simpler and cheaper test (s-creatinine, u-dipstick, ultrasound, etc.). Urinary proteomic tests have therefore not come into clinical practice.

However, more recently Good et al. studied chronic kidney disease with capillary electrophoresis coupled to mass spectrometry (CE-MS) [10]. They found a very high diagnostic accuracy (area-under-ROC-curve 0.955) when testing 379 healthy subjects versus 230 CKD patients (the majority having glomerulonephritis and diabetes nephropathy). CE-MS has emerged as a promising technique with stable and reproducible results over time and in different cohorts [17, 19–21]. Our study also find a similarly high diagnostic accuracy (AUC 0.977), and we extend the results to patients with hypertensive nephropathy. This is, as far as we know, the first report on urinary proteomics for diagnostics in the large and increasing group of patients with CKD caused by hypertensive nephropathy.

A central question is whether urinary proteomics can improve clinical handling of patients beyond what is possible with current diagnostic tests. CE-MS based urinary proteomics was recently found to have better ability to predict which patients with diabetes mellitus would progress to diabetes nephropathy over the next 5 years compared to microalbuminuria testing (areas under ROC curves 0.93 and 0.67, respectively) [22]. Using the same analytical methods, we found that urine proteomics testing in combination with albuminuria was able to classify rapid progressors versus slow progressors significantly better than albuminuria alone. We found that urine proteomics contributed important additional information, i.e. it increased the area under ROC curve at the magnitude of 0.15 beyond what was achieved with albuminuria. Typically even major risk factors like HDL cholesterol provide only marginal additional value when evaluated with ROC (delta AUC 0.01) [23]. Furthermore,

a large proportion of the big group of slowly progressing patients having been assigned an intermediate risk of progression were reclassified to the low risk group. Such reduction of the number of false positive cases is important in a potential CKD screening setting. Although we had a few more false negative cases, a stronger increase of true positive cases was seen, and the reclassification lead to an overall improvement of benefit versus risk. Finally, previous studies have used a proteomics score of 0.323 as cut-off for diagnosing CKD [10], but our study showed that the risk for accelerated kidney function loss starts with scores above 0.0.

Excessive accumulation of extracellular matrix and subsequent fibrosis is a general pathophysiological mechanism involved in many if not all types of progressive kidney disease. If the triggering event is not cleared, epithelial tubular cells will transition to a more mesenchymal like cell type starting a chronic interstitial process with increased production of collagen type 1 and type III [24]. Both experimental and human studies have suggested an initial phase with increased extracellular matrix production followed by an imbalance between collagen degradation enzymes (matrix-metallo-proteinases, MMPs) and their tissue inhibitors (tissue inhibitors of metalloproteinases, TIMPs) leading to reduced degradation, favoring the development of tubulointerstitial fibrosis [24, 25]. Previous studies have found correlations between increased urine levels of procollagen III, which probably is a marker of increased production of collagen, and the extent of interstitial fibrosis [26]. This is not necessarily in opposition to our findings in the urine from rapid CKD progressors of reduced amounts of collagen types I–III fragments, which more likely is a marker of reduced collagen breakdown, rather than of increased collagen production. Reduced urinary collagen has been found consistently in CKD patients using the same CE-MS technology as in our study [10, 19–21]. Lower levels of urinary MMP activity has also been found in progressive compared to stable patients with diabetes nephropathy [27]. Potentially this could be useful for the development of future CKD biomarkers of rapid progression, and our data indicate that this could hold for hypertensive nephropathy patients as well. However, there has been conflicting reports on this complex topic [28], and influence of CKD-bone-mineral-disorder has also been proposed.

Several other urinary protein findings in our study also support on-going interstitial inflammation, fibrosis and tubular damage with similar results across CKD diagnosis, including hypertensive nephropathy. Uromodulin is exclusively produced in tubular cells in the thick ascending limb of Henle's loop and increasingly found to be associated with kidney disease [29]. Low urine levels are associated with rapid progression of CKD and have been found in cases with tubular atrophy and fibrosis [30, 31] CD99 antigen is expressed in most tissues, including the kidney, and it is important for the ability of leucocytes to extravasate into the interstitium as part of the inflammatory process [32]. Low urine levels have been associated with kidney disease in other cohorts also, but the pathophysiological reason for this is unclear. Osteopontin, which is excreted when cells in the distal tubules are stressed [33], was also reduced in urine from CKD patients. Osteopontin is involved in remodelling of the extracellular matrix and inhibition of apoptosis [34]. Similar findings have been reported from IgA patients, while patients with membranous glomerulonephritis and minimal change nephropathy who typically have minor tubular and interstitial damage were reported to have normal urine levels [35]. Several of the proteins found in excess in our CKD urine samples are normally reabsorbed in the proximal tubules. Our findings are therefore in line with previous reports and strongly indicate the presence of interstitial and tubular damage in most types of CKD, including non-biopsy verified hypertensive nephropathy. Such patients have often been claimed to only have normal age related reduction of GFR, but at least when eGFR is below 30 ml/min/1.73 m^2 they seem to be suffering from the common pathophysiological pathway found in most types of progressing CKD.

The current study has some limitations. First, the number of participants was rather low, which could lead to loss of precision and risk of type 2 errors. Second, we compared patients with rather advanced CKD stages with healthy subjects, so the differences between the groups were presumably large. Also, we measured albuminuria by dipstick, which is less precise than urinary albumin/creatinine ratio. However, the association of the dipstick test with both mortality, cardiovascular morbidity and CKD progression has been well validated in large international studies [36]. Also, modern dipsticks have a very high diagnostic accuracy for macroalbuminuria (area-under-ROC-curve 0.99) [37], which is often the range of interest for predicting progression rate in clinical practice. Also, the ideal measure of GFR decline would be strictly prospective, i.e. after inclusion. However, long observation time with several creatinine measurements provides a more robust measure of GFR decline, and additional sensitivity analysis based on GFR decline after baseline, albeit with fewer subjects and measurements due to deaths and start of renal replacement therapy, gave similar results (adding the urine proteomics score to dipstick testing increased the area under the ROC curve from 0.91 to 0.97). One of the strengths of this study is that it was based on a clinically relevant and well described patient cohort. The CE-MS

method for urine proteomic analysis has high sensitivity and reproducibility [38]. It is also insensitive to interfering compounds and enables measurement of the relative abundance of the peptides using internal standards (i.e. semi-quantitative measurements with very low coefficients of variation combined with a large human urinary peptidome database enabling identification of significant changes in biomarkers).

In conclusion, a panel of urinary proteins was able to accurately diagnose CKD, and in combination with albuminuria was significantly better to detect patients with rapid kidney function decline than albuminuria alone. Reduced urine levels of collagen types I and III, uromodulin, CD99 antigen and osteopontin, all implicated in fibrosis-promoting processes such as extracellular matrix deposition, inflammation and reduced collagen breakdown, were found in our CKD patients. Urine proteomics performed equally well in patients with hypertensive nephropathy as in patients with other CKD causes. Further studies with prospective designs using this new potentially useful tool are highly required.

Methods

We included consecutive CKD patients with eGFR below 30 ml/min/1.73 m^2 not yet on renal replacement therapy (RRT), from the outpatient clinic at St Olav's Hospital, Trondheim, Norway, in December 2009–February 2010. A convenient sample of healthy persons working in our department not taking any medication and with no history of CKD, cardiovascular disease or diabetes, was included as controls. Age, sex, CKD diagnosis, and blood pressure were recorded in all participants. Blood was drawn for standard evaluation of kidney function. A second morning urine sample was tested with a dipstick, and then immediately frozen to −20 and thereafter to −80°C within 24 h. Creatinine measurements available prior to inclusion and over a 2.5 years follow-up period were recorded in order to calculate eGFR decline per year using the CKD-EPI equation [39]. Individual linear regression analyses of eGFR decline were performed to compare kidney function decline before and after inclusion. Blood pressure was measured as the average of the last two out of three measurements at inclusion.

Urine samples were prepared as previously discussed [10]. Briefly, after dilution with urea, ammoniumhydroxyde, and sodium dodecyl sulfate, the 0.7-ml aliquots of urine were ultrafiltered in order to remove proteins of higher molecular mass (>20 kDa), desalted, lyophilized, and stored at −20°C. The samples were resuspended in HPLC grade water shortly before capillary electrophoresis/mass spectrometry analysis. CE-MS analysis was performed with a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Brea, CA, USA) coupled on line to a micro-TOF–MS instrument (Bruker Daltonics, Bremen, Germany) [40].

For data processing, mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using Mosaiques Visu software [41]. For normalization of analytical and urine dilution variances, MS signal intensities were normalized relative to 29 internal standard peptides generally present in at least 90% of all urine samples with small relative standard deviation. For calibration, linear regression was performed. All detected peptides were deposited, matched, and annotated in a Microsoft SQL database (Microsoft, California).

Previous CE-MS measurements of urine samples have resulted in a maximum of 5,010 distinct peptides, which describes the human urinary low molecular-weight proteome [42]. The CKD273-classifier is a support vector machine (SVM)-based classification model [43–45], which allows the classification of samples in the high dimensional parameter space using MosaCluster software (version 1.7.0) [46]. Applying the CKD273-classifier to CE-MS data of unknown samples, MosaCluster calculated classification scores, based on the amplitudes of the 273 CKD biomarker peptides. Classification was performed by determining the Euclidian distance (defined as the SVM classification score) of the 273-dimensional vector to a 272-dimensional maximal margin hyperplane, which was defined previously [10]. The cut-off of the classification score was previously determined from the result of the biomarker discovery cohort in Good et al. [10]. Patients with urine samples who had classification scores exceeding 0.343 were classified as CKD273 classifier positive cases and patients with urine samples scoring below 0.343 were classified as CKD273-classifier controls [10]. Quantitative differences of individual proteins between cases (glomerulonephritis/diabetes nephropathy, hypertensive nephropathy, or other CKD diagnosis) and control subjects were calculated. Statistical significance was assumed at unadjusted $p < 0.05$ with the Wilcox test. All data were calibrated and annotated to the Mosaiques human urinary database [47].

Statistical analysis were done using Stata 13.1 software (StataCorp, TX, USA). Decline in kidney function over time was compared with linear regression analysis. Mean proteomic score in different groups were compared with two-sample t test. Stata function "roccomp" was used to test for ROC area equality of logistic regression based models (e.g. base model including albuminuria versus enhanced model including albuminuria plus proteomic score). We also used the Stata function "incrisk" from Longton and Pepe, which is a collection of performance improvement measures comparing a base model versus an enhanced model. Significance testing is difficult in

risk reclassification, but these are useful for demonstrating how different risk prediction models changes the risk estimates in subjects with and without the outcome [48]. Predicted risk for rapid kidney function decline below 10% were defined as low, risk above 50% as high, and 10–50% as intermediate.

The study was approved by the Regional Committee for Medical and Research Ethics. It was carried out in accordance with the Declaration of Helsinki. All participants gave written consent.

Authors' contributions

MAØ, SH designed the study, analyzed the data and drafted the manuscript. PZ carried out the CE-MS analysis. BE, PZ revised the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical guidelines

Competing interests

Petra Zürbig is employed by Mosaiques Diagnostics GmbH. Bjørn Egil Vikse has been an adjunct professor at the University of Bergen, a position partly financed by Amgen Inc, since July 2012. The other authors have no competing interests.

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18 Paper 4

Gene expression studies and targeted metabolomics reveal disturbed serine, methionine, and tyrosine metabolism in early hypertensive nephrosclerosis.

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Abstract

Background: Hypertensive nephrosclerosis is among the leading causes of end-stage renal disease, but its pathophysiology is poorly understood. We wanted to explore early metabolic changes using gene expression and targeted metabolomics analysis.

Materials and methods: We included 15 kidney biopsies with nephrosclerosis and 21 normal biopsies to analyze gene expression with an Affymetrix array (17379 genes measured). 31 amino acids were measured by LC-MS in urine samples from 62 patients with clinical hypertensive nephrosclerosis and 33 age- and sex-matched healthy controls, and major findings were confirmed in an independent cohort of 45 cases and 15 controls.

Results: Amino acid catabolism and synthesis was strongly under-expressed in hypertensive nephrosclerosis (15- and 8-fold, respectively), and these patients also showed gene expression patterns indicating decreased fatty acid oxidation (13-fold) and increased interferon gamma and cellular defense response (both 8-fold). Metabolomics analysis revealed significant distribution differences in eleven amino acids in hypertensive nephrosclerosis, among them tyrosine, phenylalanine, dopamine, homocysteine and serine, with 30-70% lower urine excretion. These findings were replicated in the independent cohort. Integrated gene-metabolite pathway analysis showed perturbations of renal dopamine biosynthesis. There were also significant differences in homocysteine/methionine homeostasis and the serine pathway, which have strong influence on one-carbon metabolism. Several of these disturbances could be inter-connected through reduced regeneration of tetrahydrofolate and tetrahydrobiopterin.

Conclusion: Early hypertensive nephrosclerosis showed perturbations of intra-renal biosynthesis of dopamine, which regulates natriuresis and blood pressure. There were also disturbances in serine/glycine and methionine/homocysteine metabolism, which may contribute to endothelial dysfunction, atherosclerosis, and renal fibrosis.

Introduction

Hypertensive nephrosclerosis is one of the leading causes of end-stage renal disease (ESRD) in industrialized countries (1). Renal parenchymal loss is believed to occur as a consequence of antecedent hypertension induced pre-glomerular microvascular alterations. The disease is often diagnosed on clinical criteria only, typically in chronic kidney disease (CKD) patients with longstanding hypertension or signs of blood pressure-related organ affection, low proteinuria and no signs of other kidney diseases like hematuria, diabetes, and glomerulonephritis (2). However, current diagnostic criteria have low accuracy (3-5), the pathogenesis is incompletely understood, and no specific treatment is available. More data on the underlying pathophysiological mechanisms and consequences are needed for this understudied, but still very common, disease.

Human gene expression studies find that chronic hypoxia is a central mechanism contributing to both glomerular and tubulointerstitial damage in hypertensive nephrosclerosis (6)(19). The downstream consequences of ischemia and other risk factors in nephrosclerosis are not well studied. Metabolomics platforms now enable the simultaneous measurement of hundreds of small molecular metabolites describing the downstream effects of genes and proteins. Patients with glomerulonephritis (7-9), kidney transplantation (10, 11), diabetes nephropathy (12), acute kidney injury (13), and general CKD (14-17) have been studied, and important disturbances in amino acid, glucose, and fatty acid metabolism as well as the central energy metabolism have been described (12, 18, 19). Whether this extends to patients with hypertensive nephrosclerosis is not well studied. Although there is no very good animal model for nephrosclerosis, current experimental data indicate decreased TCA-cycle activity (20) and extensive mitochondrial abnormalities and dysfunctions in kidneys exposed to various forms of hypertension (21). Pathway analysis based on gene expression in human nephrosclerosis biopsies indicate that amino acid metabolism (including arginine, serine, and tryptophan metabolisms), TCA-cycle, glycolysis/gluconeogenesis, fatty acid oxidation, PPAR signaling, and others were significantly disturbed (22).

We therefore used kidney gene expression and targeted urinary metabolomic analyses to explore the metabolic consequences in early stages of hypertensive nephrosclerosis compared to age and sex matched controls. The goal of this work was to generate relevant hypothesis to

be further tested in experimental models to elucidate the underlying mechanisms of nephrosclerosis.

Material and methods

Study populations

For our biopsy study, we included patients with biopsy-proven hypertensive nephrosclerosis and healthy kidney transplant donors before kidney transplantation from the European Renal cDNA Bank (23).

For urine metabolomics studies, we included subjects from the Third Nord-Trøndelag Health Study (HUNT3), a cross-sectional population study performed in the Norwegian county of Nord-Trøndelag between 2006 and 2008. Biological sampling, recording of medical and lifestyle information, and simple physical examinations were done as previously described (24). Blood pressure was measured three times in a sitting position by trained nurses at the study venue and recorded as the mean of the last two measurements using the oscillometric method (Dinamap 845XT, Criticon, Tampa, FL, USA). Clinical hypertensive nephrosclerosis was defined as individuals with an estimated glomerular filtration rate (eGFR) less than 60 mL/min/1.73m^2 or eGFR 60-89 ml/min/1.73m^2 with a decline in eGFR larger than 30 mL/min/1.73m^2 over the previous 10 years, combined with self-reported hypertension lasting more than 10 years and absence of diabetes mellitus, hematuria and macroalbuminuria (defined as a urine albumin-to-creatinine ratio (ACR) >30 mg/mmol). Hypertension was defined as systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg or treatment with anti-hypertensive medication. The control group included individuals with estimated glomerular filtration rates above 75 mL/min/1.73m^2, who did not have hypertension, diabetes, hematuria or proteinuria. Controls were matched to nephrosclerosis patients regarding sex and age group in age intervals of 20 to 50 years, 50 to 70 years, and over 70 years.

To replicate the main metabolomics findings of the HUNT study, we also analyzed urine and blood samples from the Study of Glucose and Insulin in Renal Disease (SUGAR) (25). This cohort included non-diabetic CKD patients with eGFR <60ml/min/1.73m^2 as well as age and gender matched controls. Most patients had a clinical diagnoses of hypertensive nephrosclerosis. See Supplementary for further details on study design and analytical methods.

Metabolomic analyses

Fresh urine samples were collected midstream at the venue of the HUNT study, immediately put into a -20° Celsius freezer and transferred to a -80° freezer within 24 hours, without centrifugation or use of additives during storage. Targeted quantitative analysis of amino acids in urine was performed using liquid chromatography coupled with mass spectrometry (LC-MS) using the commercial EZ:faast LC/MS Physiological (Free) Amino Acids Kit (Phenomenex, Inc, Torrance, CA, USA). Urine was diluted, centrifuged, spiked with the internal standard solution and derivatized following the Phenomenex EZ-faast protocol. Samples were separated and measured with a Waters Acquity UPLC – TQ-S tandem mass spectrometer (Waters Corporation, Milford, MA, USA) with cases and controls in a random order with one quality control every 15 sample. Values were normalized to 1 mmol creatinine. See Supplementary methods for details.

Transcriptomic analyses

Total RNA from micro-dissected glomerular and tubulointerstitial compartments had been isolated, reverse-transcribed and amplified, then fragmented and hybridized to the Affymetrix GeneChip Human Genome U133A 2.0 and U133 Plus 2.0 Array (26). Genes with FDR <0.05 and more the 1.5-fold change were considered differentially expressed and included for further analysis. We used gene ontology (GO) enrichment analysis (PANTHER supported by the GO Consortium) to integrate all the gene expression data an easier to understand description of overall biological function. Upregulated and downregulated genes were analyzed separately (27).

Statistics

Central tendency for urine metabolites was evaluated as percent differences in median values (nephrosclerosis patients minus controls), and statistical testing was done with the Mann Whitney *U*-test (two-sample Wilcoxon rank test). We also tested for distribution differences using the two-sample Kolmogorov-Smirnov test. False Discovery Rate (FDR) was calculated according to the Benjamini Hochberg ranking procedure to adjust for multiple testing (28), and FDR 0.10 was considered significant in this exploratory setting. Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) were used to evaluate overall discrimination between patients and controls after normalizing to 1 mmol of urine creatinine, log transformation, and autoscaling (Metaboanalyst 3.0). Variable importance in projection (VIP) was used as a measure of the importance of variables from the PLS-DA analysis. The VIP score is based on the sum of variable influence over all model

dimensions, looking at the PLS loadings relative to the amount of explained Y-variation(29), and can be used to identify discriminating variables or predictors(30).

Bioinformatics

For pathway analysis, we used a combination of enrichment analysis and pathway topology analysis (Metaboanalyst 3.0). Enrichment analysis, rather than evaluating one metabolite at a time for significance, evaluates groups of functionally related metabolites to see if they occur more often than expected or not. Pathway topology analysis takes into consideration that some metabolites are more central and others more peripheral in a metabolic pathway, using so-called relative betweenness centrality and degree centrality measures (29). We also combined gene and metabolite information into the same pathway analysis (integrated pathway analysis, Metaboanalyst 3.0). See Supplementary methods for further details, especially on LC-MS methods.

To further elucidate the differentially expressed genes relevant for our top pathways we also explored the Nephroseq version 5. This public website displays pre-analyzed data on the associations between specific genes of interest and various renal outcomes (eGFR decline, CKD, albuminuria, and others) based on selected datasets. The study was approved by the Regional Committee for Medical and Health Research Ethics, Central Norway. All participants gave written informed consent.

Results

Participants

Baseline characteristics of nephrosclerosis patients and healthy controls with kidney biopsies (n=26), urine metabolomics in the main cohort (HUNT, n=95) and the replication cohort (SUGAR, n=60) are summarized in Table 1. Mean age was close to 60 in all groups except kidney biopsy controls (47 years). By definition, none of the controls had diabetes, hypertension, reduced kidney function or signs of kidney damage. Nephrosclerosis patients had higher blood pressure and BMI combined with lower eGFR (only modestly reduced in the HUNT cohort, mean of 67 ml/min/1.73m^2, with range 40-89).

Kidney biopsy gene expression

Kidney biopsy gene expression analysis displayed substantial differences between nephrosclerosis patients and healthy kidney donors. Among 11936 genes tested, 4113 were significantly over- or under expressed in either the glomerulus or the tubulointerstitial compartment. We carried out gene ontology enrichment analysis to combine this information into main biological functions (Table 2). Amino acid catabolism and synthesis were substantially under expressed in nephrosclerosis patients (15- and 8-fold, respectively). Other highly enriched functions indicated decreased fatty acid oxidation (13-fold) and gluconeogenesis (11-fold), while several immunological and defense functions were increased (interferon gamma response (10-fold), cellular defense response (8-fold), and cytokine signaling (8-fold)).

Urine metabolomics

Table 3 displays median values and distribution of the 31 amino acids included for further analyses. The relative differences indicated lower urine concentrations for many amino acids in nephrosclerosis, but none had a statistically different central tendency (i.e. median) after correcting for multiple testing. For eleven amino acids the distribution of concentrations was statistically different in nephrosclerosis versus controls (serine, tyrosine, leucine, phenylalanine, homocysteine, threonine, ornithine, proline, carnosine, glutamic acid, and dopamine) (Table 3). The distribution difference measure includes information on range and

skewness in addition to central tendency, which can be important for discriminating between groups. Serine, tyrosine, leucine, phenylalanine and homocysteine had the largest distribution differences ($p<0.01$ for all). However, the distribution of these metabolites in the hypertensive nephrosclerosis group and the age and sex matched controls still showed substantial overlap (Figure 1). There was no association between the amino acids and eGFR in multi-adjusted regression analysis, so the amino acids do not vary simply with filtration between the two groups (data not shown).

Metabolomics replication

We were able to replicate the main findings from the HUNT cohort in the SUGAR cohort (Table 3). Regarding the serine metabolism, our top hit, we found that cases had 70% lower urine excretion of serine in this cohort compared to their controls ($p<0.001$), and glycine excretion was 36% lower ($p<0.001$). For the tyrosine metabolism, we found very similar reduction for phenylalanine (-51%, $p=0.004$), tyrosine (-45%, $p=0.04$) and dopamine (-28%, $p=0.05$).

Bioinformatics analyses

Partial Least Squares Discriminant Analysis (PLS-DA) showed that urine amino acid levels moderately separated hypertensive nephrosclerosis patients from controls and explained 72% of the variance (Figure 2). Eighteen of the amino acids (Hcys, Glu, His, Tyr, Kyn, Car, Cys, Met, Gly, Cth, Lys, Phe, Aaa, Orn, Dap, Ser, DA, Ala) had Variable Importance in Projection (VIP) scores >1.0, and these were considered to contribute significantly to the discrimination (see Supplementary Table 1 for details). Top five metabolites were homocysteine (generally known as an independent cardiovascular risk factor), glutamic acid (playing a central role in overall nitrogen homeostasis), histidine (having anti-oxidant, anti-inflammatory and anti-secretory properties), tyrosine (important for protein and catecholamine biosynthesis), and kynurenine (major metabolite in tryptophan metabolism with influence on production of neurotransmitters, inflammation and aging).

We tested the ability to distinguish between a nephrosclerosis CKD patient and a healthy control using ROC analysis. We used Principal Component 1 scores as a measure for all metabolites, and we also tested the most significant individual metabolites from the PLS-DA and the Kolmogorov-Smirnov tests in separate ROC analysis (Table 4). Principal Component 1 had an area-under-the curve (AUC) of 0.63, which indicates a weak but significant ability

(95% confidence interval 0.51-0.75) to correctly diagnose nephrosclerosis versus healthy controls (AUC=0.50 means no information beyond coin tossing). Homocysteine, glutamic acid, and kynurenine also had similar results. When analyzing the various clinical diagnostic criteria of nephrosclerosis separately, the metabolites were only able to correctly diagnose hypertension (PC1, homocysteine, glutamate, and leucine). They contained no relevant information for diagnosing low eGFR, proteinuria or hematuria (Table 4).

Over-representation analysis was used to get insight into the underlying biological mechanisms and functional implications of the statistical significant metabolites discovered. Methionine metabolism was most significantly different from the background set (p=0.00001, FDR= 0.0008) and had a 9.8-fold enrichment (6 significant metabolites found while only 0.612 expected by chance). Catecholamine biosynthesis had the highest enrichment (15.6-fold), but there were also other highly enriched metabolite sets: glycine, serine and threonine metabolism (9.0-fold), ammonia recycling (8.7-fold), glutathione metabolism (11.8-fold), and histidine metabolism (10.6-fold). See Supplementary Table 2 and Supplementary Figure 1 for further details.

Integrated pathway analysis combining gene expression information with the significant urine metabolites showed that several metabolic pathways were substantially disturbed (Table 5). The glycine, serine and threonine metabolism had the highest rank based on enrichment and topology analysis. The enrichment analysis displayed a 2.0-fold enrichment with a p-value of 0.0004, showing that it is very unlikely that so many significantly differing metabolites and genes should occur in this pathway by chance. The topology analysis displayed scores of 2.0, indicating that very central metabolites and genes in this pathway were disturbed compared to the other pathways. The phenylalanine, tyrosine and tryptophan metabolism was also significantly perturbed (3.3-fold enriched with a topology score 2.1), and the methionine metabolism showed similar results (1.8-fold enrichment and topology score 3.3).

Figure 3 gives an overview of biological interesting parts of the top-3 pathways and their interactions. Our metabolite and gene expression data are indicated in the figure, and other well-documented relevant findings from the kidney disease literature are also integrated. Parts of the serine and the methionine metabolisms are strongly connected via the folate cycle into the so-called one-carbon metabolism. Information on metabolites and enzymes indicate that this is down regulated in nephrosclerosis which could lead to disturbances in the methylation of DNA and protein with correspondingly increased renal fibrosis and

atherosclerosis. Finally, the data also show reduced substrate and gene expression consistent with reduced intrarenal dopamine synthesis, which would strongly impair natriuresis and blood pressure control. The activity of main enzymes could even be reduced due to reduced regeneration of their co-enzyme tetrahydrobiopterin, which represents a potential connection with the down regulated folate cycle.

Discussion

Nephrosclerosis patients had lower expression of genes related to amino acid and energy metabolism, while there were increased expression of genes related to immune response. Urinary metabolomics combined with gene expression in kidney biopsies displayed perturbations in several relevant pathophysiology pathways in early hypertensive nephrosclerosis, such as serine metabolism (endothelial dysfunction and oxidative stress), methionine metabolism (cardiovascular risk and fibrosis), and tyrosine metabolism (catecholamine biosynthesis and natriuresis).

High blood pressure is believed to be central in the pathogenesis of nephrosclerosis, but other cardiovascular risk factors are also involved (31) (32, 33). The typical histopathological findings of arteriolar hyalinosis, glomerulosclerosis and tubulointerstitial fibrosis (34) can also be found in kidneys from healthy elderly subjects(5, 35), but the findings are more pronounced in nephrosclerosis (36-38). Furthermore, recent 3-dimentional microscopy shows that nephrosclerosis is a small vessel disease different from the traditional atherosclerotic disease found in arcuate and larger renal arteries (39). Furthermore, our findings point towards several amino acid based dysregulations in central metabolic pathways compared to age and sex matched controls.

Serine is a nutritionally non-essential amino acid, but it is metabolically indispensable and have received increasing attention over the last years. Twenty-five percent of serine comes from protein breakdown, while the remainder 75% is from *de novo* synthesis (40). Both rat and human studies show that the kidney is the main site of serine production (41). Although some serine is produced by regeneration from glycine (15%), the majority (>50%) comes from TCA-cycle intermediates which are converted into phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (PEPCK) (40). Serine is required for cellular and tissue growth in general and for the nervous system in particular (42). It acts as neurotransmitters, is important for the catalytic activity of many enzymes, and is an important building block of many lipids. More recently, kidney relevant properties have also been disclosed; for example, serine is a major methyl group provider(43), and it also have direct blood pressure lowering effects (44).

Methionine is an essential amino acid produced in plants only from TCA cycle intermediates. It plays, in addition to general protein synthesis, critical roles in important human metabolic processes, including transmethylation reactions, the tetrahydrofolate associated one-carbon metabolism, and as a precursor for other sulfur compounds. Methionine is converted into homocysteine via a three-step process where demethylation of s-adenosyl-methionine (SAM) to s-adenosyl-homocysteine (SAH) (45). This step is the major provider of methyl groups in all human cells and are used for methylation of DNA to regulate transcription (epigenetics) and for methylation of proteins to modify their function (e.g. post-translational regulation of the folding of proteins). Homocysteine must then be re-methylated to methionine by combining with 5-methyl-tetrahydrofolate, which depends on serine to be regenerated, or with betaine, or it can be metabolized to cysteine for further degradation or urine excretion.

Tyrosine is a non-essential amino acid which can be synthesized from plant derived phenylalanine and then used for catecholamine synthesis. In general, the first step from tyrosine to L-dihydroxyphenylalanine (L-DOPA), which is catalyzed by tyrosine hydroxylase (TH), is regarded as the rate limited step in this process. However, for the much higher levels of intra-renal dopamine, expression of DDC as well as availability of L-DOPA substrate, could be of additional importance (46). Intra-renal dopamine has been found to account for more than 50% of the kidneys salt excretion ability and is therefore of great importance for blood pressure control (47, 48).

Renal fibrosis and atherosclerosis are often found in nephrosclerosis, although the latter is more likely an associated finding and not a cause of the disease. In early nephrosclerosis, we demonstrate pathophysiological perturbations in both serine and methionine metabolism, which are closely connected and could cause both renal fibrosis and general atherosclerosis. Hyperhomocysteinemia has long been studied as a risk factor of cardiovascular disease (49, 50), and it has also been suggested as a risk factor for hypertension (51) and incident CKD (52-56). Oxidative stress (57, 58) and upregulated inflammation (59) (60) have been suggested as direct effects of the high homocysteine levels. Furthermore, several studies have shown an association between hyperhomocysteinemia and DNA hypomethylation in CKD(61). However, it is more likely that the methylation disturbances are due to the accumulation of S-adenosylhomocysteine (SAH). Plasma SAH levels are often more strongly increased with reduced kidney function than homocysteine levels(62, 63), and SAH acts as a strong inhibitor of most methylation reactions(64). We have previously found that TCA cycle activity is downregulated in non-diabetic CKD (65), and in the current study we find reduced

renal expression of PEPCK in nephrosclerosis. This combination could lead to reduced serine production and a state of reduced substrate for the tetrahydrofolate cycle. We also demonstrate reduced renal expression of 5,10-methylene-tetrahydrofolate reductase (MTHFR), methionine synthase (MS) and betaine-homocysteine methyl-transferase (BHMT), which are key enzymes for the remethylation of methionine, and consequently reduce methionine levels. Unfortunately, we do not have data on SAM and SAH in nephrosclerosis, and the interpretation of the related epigenome literature is also difficult. Several reports indicate that there is a global hypomethylation in CKD (66), and our data shows that nephrosclerosis patients also have the prerequisites for such a epigenetic change. Global hypomethylation has been found in both blood cells and in vascular lesions of patients with atherosclerosis (67, 68), and it is also associated with aging in general (69). However, whether it is a direct facilitator for harmful effects or merely a marker of a generalized epigenetic dysregulation is not well studied. Site-specific and organ-specific information on methylation in various candidate genes is clearly needed, and several studies show that both hyper and hypomethylation coexist in various disease (70, 71).

Blood pressure control could be substantially impaired by the dopamine and serine disturbances described in our study. Urine tyrosine has previously been found to be reduced in early CKD (16, 72) and in ESRD patients(73, 74), and this is caused by reduced phenylalanine-hydroxylase (PAH) enzyme activity (45), also demonstrated in our study. Even though we found no change in renal expression of tyrosine hydroxylase (TH), which is considered the rate limiting enzyme in catecholamine synthesis, production of L-DOPA could still be hampered in CKD. Tetrahydrobiopterin (BH4) is an essential cofactor for all amino acid hydroxylases (e.g. PAH and TH) and nitric oxide synthases. Regeneration of BH4 from dihydrobiopterin (BH2) is reduced in CKD leading to reduced BH4/BH2 ratios (75), which probably is the best functional measure of the tetrahydrobiopterin. We found a significantly reduced expression of dihydropteridine reductase (DHPR), the main enzyme for regeneration, in nephrosclerosis. Furthermore, regeneration is also connected with the folate cycle, which we also found to be suppressed. The expression of L-DOPA-decarboxylase (DDC) for conversion of L-DOPA to dopamine was also strongly down regulated in our nephrosclerosis patients, and mice with reduced renal expression of DDC demonstrate reduced renal and urine dopamine concentrations leading to reduced salt and water excretion, activation of renin-angiotensin system and increased blood pressure (46).

Taken together, our data suggests that enzymatic downregulation and metabolite deficiency in the phenylalanine-tyrosine-dopamine axis is found in early hypertensive nephrosclerosis. Disturbances in renal dopamine is linked to hypertension, both by reduced renal L-DOPA uptake or conversion to dopamine(76, 77), and disturbed dopamine D1-like receptor function(78). In humans, a defect in the tubular D1 receptor has been shown in salt-sensitive hypertension (79). The renal dopamine system has also been shown to interact with the RAAS in sodium homeostasis and blood pressure regulation in normotensives (80).

There are, however, some limitations that needs to be discussed. First, we do not have blood values from the HUNT study, and combined arterial and venous measurements across the kidney would be the optimal design to fully describe the renal handling of amino acids. However, urine metabolite excretion do represent systemic or renal changes in metabolism and is not influenced by reduced GFR until the advanced CKD stages. Second, we have defined hypertensive nephrosclerosis from a set of clinical and laboratory criteria known to be rather unspecific, rather than by histological definition based on renal biopsy findings. Third, we could not ensure that fasting was uniform, or that amino acid or protein intake was uniform before urine analysis. This study was exploratory in its nature, focusing on data-based pathway and gene-metabolite enrichment analysis rather than a predefined hypothesis. As such, its strengths lie in hypothesis generation and elucidation of possible pathophysiological disturbances. For definite mechanism, additional animal experiments with a more focused view is needed.

In conclusion, renal gene expression analysis showed reduced amino acid catabolism and synthesis in nephrosclerosis patients. Urine samples from clinically diagnosed cases with well-preserved eGFR showed significantly reduced excretion of eleven amino acids, among them tyrosine, phenylalanine, dopamine, homocysteine and serine. Metabolite pathway enrichment analysis revealed downregulation of the phenylalanine-tyrosine-dopamine axis, which regulates natriuresis and blood pressure, due to enzymatic downregulation and metabolite deficiency. We also found disturbances in methionine/homocysteine and serine metabolism, involved in methylation status, endothelial dysfunction, inflammation and atherosclerosis. Albeit explorative in nature, our combined genomic and metabolomic analysis highlights pathologically perturbed pathways in early stage hypertensive nephrosclerosis.

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Figure legends

Figure 1: Relative frequency plots of central amino acids. Hypertensive nephrosclerosis (red) vs healthy controls (blue).

Figure 2: Overall ability of urine amino acids to discriminate between early nephrosclerosis patients (CKD stage 2-3) and healthy controls. PLS-DA analysis shows the overall variance between the groups decomposed into three vectors (principal components 1-3). Eighteen amino acids had VIP scores ≥1.0 (in decreasing order; Hcys, Glu, His, Tyr, Kyn, Car, Cys, Met, Gly, Cth, Lys, Phe, Aaa, Orn, Dap, Ser, DA, Ala), which is considered as a significant contribution to discrimination.

Figure 3: Major disturbances connecting the major perturbed pathways in nephrosclerosis: tyrosine, serine and methionine metabolism. Filled figures are data from current study, circles are metabolites, and rectangles are enzymes. Colored but open figures are based on literature findings in nephrosclerosis. Red is increased/up-regulated, green is normal/unchanged, and blue is reduced/down-regulated.

Table 1: Baseline characteristics of participants

	Gene expression studies (European Renal Biopsy Bank)		Metabolomics studies (HUNT study)		Metabolomics studies (SUGAR study)	
	Healthy controls (n=21)	Hypertensive nephrosclerosis (n=15)	Healthy controls (n=33)	Hypertensive nephrosclerosis (n=62)	Healthy controls (n=15)	Hypertensive non-DM CKD (n=45)
Age	47.2	57.1	58.1 (11.3)	59.7 (10.8)	55.6 (11.8)	61.5 (14.3)
Male gender (%)	57	80	64	58	60	53
Systolic BP (mmHg)	<140	146 (22.9)	126.8 (12.2)	141.9 (13.8)	122.4 (14.1)	134.4 (15.9)
Diastolic BP (mmHg)	<80	88 (13.5)	73.1 (8.9)	79.3 (10.9)	77.2 (9.3)	80.6 (9.7)
Cholesterol (mmol/L)	n.a.	6.3 (1.0)	5.9 (1.0)	5.7 (1.0)	5.2 (1.0)	4.7 (1.1)
Current-smoker (%)	n.a.	n.a.	25	11	7	20
Body Mass Index (kg/m^2)	n.a.	29.1 (4.7)	26.4 (3.5)	28.6 (3.6)	27.3 (5.9)	30.3 (6.2)
Cardiovascular disease (%)	0	n.a.	3	18	7	40
Diabetes mellitus (%)	0	0	0	0	0	0
eGFR (mL/min/1.73m^2)	105.4 (30,9)	40.9 (23,8)	94.8 (14.2)	67.1 (10.8)	90.4 (18.0)	36.0 (12.8)
eGFR ≥90 (%)	76	0	57	0	47	0
eGFR 75-89 (%)	10	20	43	44	27	0

eGFR 60-74 (%)	14	0	0	27	27	0
eGFR 45-59 (%)	0	13	0	26	0	31
eGFR 30-44 (%)	0	27	0	3	0	33
eGFR 15-29 (%)	0	40	0	0	0	36
u-ACR (mg/mmol)	<3.0	57 (56)	1.3 (0.5)	2.1 (1.6)	0.8 (0.7)	40.2
ACR 0-2.9 (%)	100	0	100	84	93	34
ACR 3.0-29.9 (%)	0	44	0	16	7	44
ACR ≥30.0 (%)	0	56	0	0	0	22

Note: eGFR calculated using the CKD-EPI equation.

Table 2: Gene ontology analysis showing the top 10 biological processes up or down regulated by fold enrichment in hypertensive nephrosclerosis kidney biopsies versus biopsies from healthy kidney donors.

Biological Process	# total	# found	# expected	Fold Enrichment	+/-	raw P value	FDR
Cellular amino acid catabolic process	53	9	0.62	14.53	-	3.78E-08	4.61E-06
Fatty acid beta-oxidation	20	3	0.23	12.83	-	2.28E-03	3.47E-02
Gluconeogenesis	23	3	0.27	11.16	-	3.26E-03	4.18E-02
Response to interferon-gamma	58	9	0.87	10.30	+	6.27E-07	3.06E-05
Cellular amino acid biosynthetic process	63	6	0.74	8.15	-	1.46E-04	5.09E-03
Cellular defense response	106	13	1.60	8.14	+	2.53E-08	3.09E-06
Cytokine-mediated signaling pathway	74	9	1.11	8.07	+	3.91E-06	1.06E-04
Cellular calcium ion homeostasis	85	9	1.28	7.03	+	1.10E-05	2.44E-04

Steroid metabolic process	100	8	1.17	6.84	+	3.70E-05	1.80E-03
Cell proliferation	60	5	0.90	5.53	+	2.78E-03	2.34E-02

Note: Data from separate GO enrichment analysis of 310 up-regulated and 236 down-regulated genes. Analyzes done with PANTHER application and genes with FDR <0.05 and FC >1.5 were included. Table shows PANTHER GO-Slim Biological Processes. Gene Ontology is a framework for modelling biological function using defined concepts/classes to describe gene function and the relationships between these concepts. GO slims are cut-down versions of the full GO ontologies containing a subset of the most important and instructive terms, i.e. an output particularly useful for giving a summary of the results of when broad classification of gene product function is required.

Table 3: Urinary amino acid excretion in healthy controls versus hypertensive nephropathy patients

	HUNT cohort						**SUGAR cohort**		
	Central location difference (median Nephroscl. vs. median Ctrl.)			Distribution difference (Kolmogorov-Smirnov)		Discrimination (PLS-DA)	Central location difference (median Nephroscl vs. median Controls)		
	Δ %	Raw p-value	FDR <0.10	Raw p-value	FDR <0.10	VIP-score	Δ %	Raw p-value	FDR <0.10
Carnosine (Car)	-71.1	0.15	N.s.	0.03	Sign.	1.25	n.a.	n.a.	n.a.
Glycine (Gly)	-58.1	0.11	N.s.	0.06	N.s.	1.18	-35.8	<0.001	Sign.
Serine (Ser)	-55.4	0.06	N.s.	0.001	Sign.	1.01	-69.6	<0.001	Sign.
Tyrosine (Tyr)	-46.9	0.03	N.s.	0.005	Sign.	1.40	-45.1	0.04	Sign.
Threonine (Thr)	-46.7	0.19	N.s.	0.02	Sign.	<1.0	+12.8	0.21	N.s.
Histidine (His)	-42.9	0.14	N.s.	0.24	N.s.	1.41	n.a.	n.a.	n.a.
2.4-diaminobutyric acid	+35.8	0.4	N.s.	0.7	N.s.	1.02	n.a.	n.a.	n.a.
Dopamine (DA)	-35.4	0.05	N.s.	0.05	Sign.	1.0	-28.2	0.05	Sign.
Ornithine (Orn)	-35.2	0.04	N.s.	0.02	Sign.	1.03	n.a.	n.a.	n.a.
Phenylalanine (Phe)	-33.4	0.07	N.s.	0.009	Sign.	1.08	-51.2	0.004	Sign.
Homocysteine (Hcys)	-32.7	0.02	N.s.	0.009	Sign.	1.82	n.a.	n.a.	n.a.
Leucine (Leu)	-32.6	0.09	N.s.	0.006	Sign.	<1.0	n.a.	n.a.	n.a.
Kynurenine (Kyn)	-31.9	0.06	N.s.	0.1	N.s.	1.38	-21.2	0.31	N.s.
Lysine (Lys)	-31.8	0.11	N.s.	0.08	N.s.	1.08	n.a.	n.a.	n.a.
Alanine (Ala)	-31.8	0.13	N.s.	0.1	N.s.	<1.0	-63.1	<0.001	Sign.

α-aminoadipic acid (Aaa)	-31.5	0.1	N.s.	0.15	N.s.	1.07
Valine (Val)	-30.3	0.18	N.s.	0.11	N.s.	<1.0
Cystathionine (Cth)	-29.2	0.11	N.s.	0.17	N.s.	1.15
Proline (Pro)	-27.8	0.07	N.s.	0.03	Sign.	<1.0
Glutamic acid (Glu)	-26.6	0.04	N.s.	0.05	Sign.	1.43
Glutamyl-Lysine (Glu-Lys)	-26.1	0.17	N.s.	0.27	N.s.	<1.0
5-aminovalerate (5Aval)	-25.5	0.48	N.s.	0.51	N.s.	<1.0
Methionine (Met)	-24.9	0.09	N.s.	0.09	N.s.	1.20
Isoleucine (Ile)	-24.6	0.13	N.s.	0.15	N.s.	<1.0
2,6-Aminopimelat (Dapa)	+22.8	0.21	N.s.	0.2	N.s.	<1.0
Proline-OH-proline (PHP)	-22.1	0.68	N.s.	0.29	N.s.	<1.0
1-Met-Histidine (1Mhis)	-22.1	0.64	N.s.	0.57	N.s.	<1.0
Cystine	-20.9	0.05	N.s.	0.04	Sign.	1.24
Hydroxyproline (Hyp)	-15.6	0.49	N.s.	0.65	N.s.	<1.0
Aspartic acid (Asp)	-9.3	0.51	N.s.	0.69	N.s.	<1.0
3-Met-Histidine (3Mhis)	-3.4	0.85	N.s.	0.45	N.s.	<1.0

Note: We intended to replicate our top 15 metabolites in the SUGAR cohort, but not all metabolites were available and some are therefore marked with "n.a.".

Table 4: Diagnostic accuracy of important amino acids for nephrosclerosis and its clinical criteria.

	PC 1	Homocysteine	Glutamate	Histidine	Kynurenine	Leucine	Phenylalanine	Serine	Tyrosine
Nephrosclerosis	**0.63 (0.51-0.75)**	**0.65 (0.53-0.77)**	**0.63 (0.50-0.75)**	0.59 (0.47-0.72)	**0.62 (0.50-0.74)**	0.61 (0.48-0.73)	0.62 (0.49-0.74)	0.61 (0.48-0.75)	**0.64 (0.51-0.0.77)**
Hypertension	**0.63 (0.50-0.77)**	**0.65 (0.52-0.78)**	**0.63 (0.51-0.76)**	0.58 (0.45-0.72)	0.61 (0.48-0.74)	**0.63 (0.51-0.75)**	0.60 (0.48-0.73)	0.60 (0.47-0.74)	**0.63 (0.50-0.76)**
eGFR	0.50 (0.37-0.62)	0.51 (0.39-0.63)	0.52 (0.40-0.64)	0.58 (0.46-0.69)	0.50 (0.38-0.62)	0.54 (0.42-0.67)	0.53 (0.42-0.65)	0.56 (0.44-0.68)	0.55 (0.42-0.66)
Proteinuria	0.55 (0.36-0.74)	0.55 (0.42-0.76)	0.52 (0.35-0.69)	0.60 (0.30-0.90)	0.54 (0.29-0.79)	0.50 (0.27-0.72)	0.59 (0.44-0.75)	0.51 (0.28-0.75)	0.61 (0.47-0.76)
Hematuria	0.60 (0.41-0.77)	0.56 (0.37-0.76)	0.62 (0.40-0.84)	0.52 (0.32-0.72)	0.58 (0.39-0.79)	0.51 (0.30-0.72)	0.62 (0.45-0.79)	0.68 (0.49-0.86)	0.62 (0.45-0.80)

Note: Principal Component (PC) 1 includes information from all amino acids reduced into one variable describing the variation in the dataset irrespectively of diagnostic group. The specific amino acids displayed are the top five from Table 2 (highest significance for distribution differences) and the top five from Figure 1 (highest VIP score in PLS-DA analysis). Data are area under the ROC curves (95% CI). Negative associations giving values below 0.5, which is the line of indifference, are transformed (1-x) for ease of comparison. Significant data are highlighted in bold.

Table 5: Integrated pathway analysis combining significant genes and metabolites in clinical nephrosclerosis versus healthy controls.

Pathway / metabolism	Enrichment analysis					Topology analysis	Rank
	# Total	# Expected	# Found	P-value	Fold-change		
Glycine, serine and threonine	68	11.36	23	0.0004	2.02	3.70	7.50
Phenylalanine, tyrosine, tryptophan	9	1.50	5	0.0089	3.32	2.06	6.85
Methionine and homocystein	63	10.53	19	0.0053	1.80	3.31	5.98
Glycerolipid	72	12.03	20	0.0116	1.66	2.60	4.31
One-carbon pool by folate	28	4.68	10	0.0116	2.14	1.71	3.65
Arginine and proline	102	17.05	30	0.0008	1.76	2.00	3.52
Glycolysis / gluconeogenesis	91	15.21	23	0.0224	1.51	2.27	3.44
N-Glycan biosynthesis	50	8.36	15	0.0134	1.80	1.40	2.51
Phenylalanine	29	4.85	13	0.0003	2.68	0.88	2.35
Butanoate	47	7.85	17	0.0009	2.16	0.88	1.89
Beta-alanine	50	8.36	19	0.0002	2.27	0.76	1.73
Linoleic acid	34	5.68	15	0.0001	2.64	0.57	1.51

Note: Pathways are ranked according to their combined enrichment and topology using multiplication (81). The analysis is using hypergeometric test for enrichment and betweenness centrality for topology. Enrichment analysis tests if compounds involved in a particular pathway are represented more often than expected by chance, and data is presented as fold-enrichment. Topology analysis take the pathway structure into consideration when determining which pathways are more likely to be involved in the conditions under study with changes in key positions of a network triggering more severe impact on the pathway than changes on marginal or relatively isolated positions. Analyses were done using Metaboanalyst 3.0.

Figure 1.

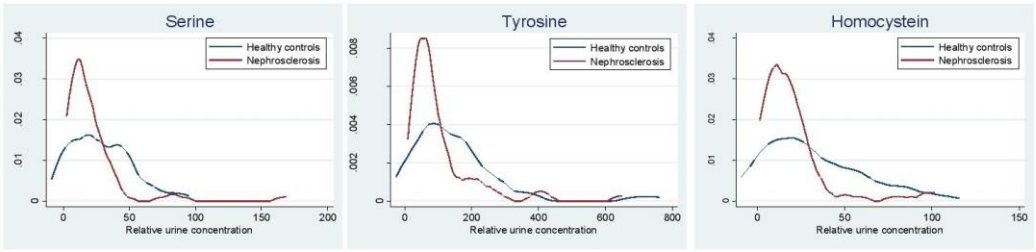


Figure 2:

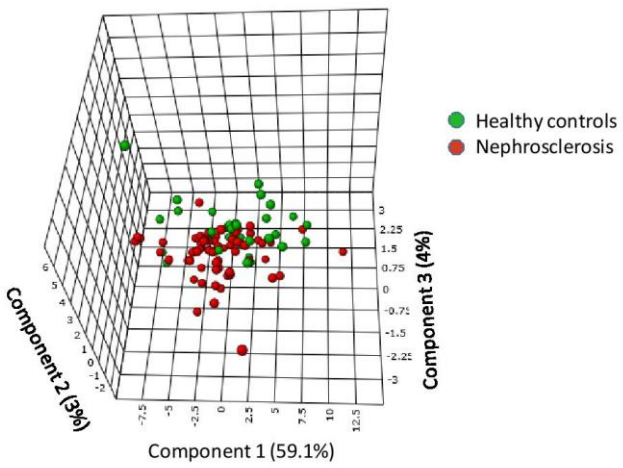
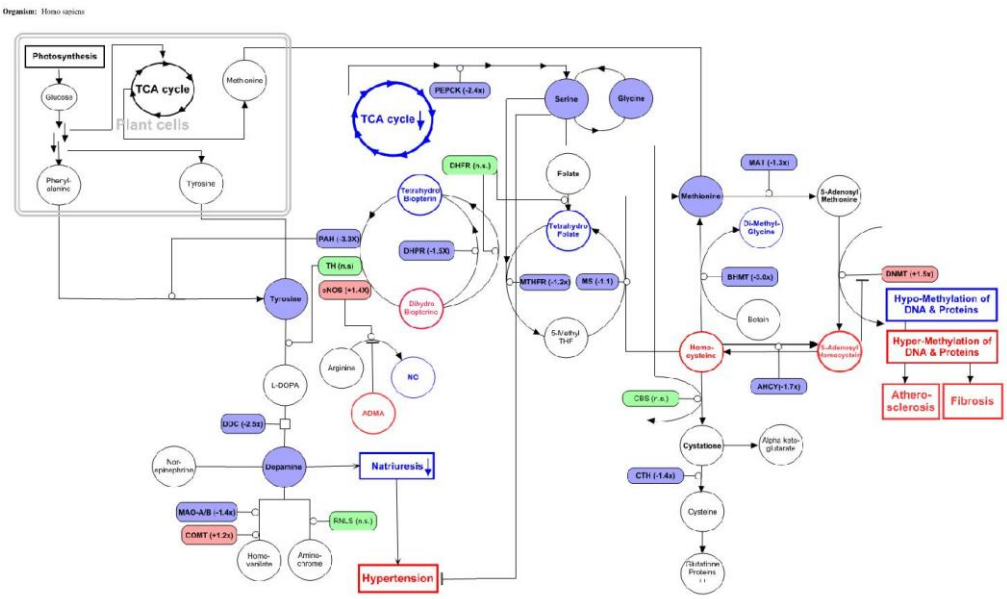


Figure 3:


Organism: Homo sapiens
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Glucose
TCA cycle
Methionine
Plant cells
Phenyl-alanine
Tyrosine
TCA cycle
PEPCK (-2.4x)
Serine
Glycine
DHFR (n.s.)
Folate
Tetrahydro Biopterin
Tetrahydro Folate
PAH (-3.3X)
DHPR (-1.5X)
TH (n.s)
eNOS (+1.4X)
Dihydro Biopterin
MTHFR (-1.2x)
MS (-1.1)
5-Methyl THF
Tyrosine
L-DOPA
Arginine
NC
ADMA
UOC (+2.9x)
Nor-epinephrine
Dopamine
Natriuresis
MAO-A/B (-1.4x)
COMT (+1.2x)
RNLS (n.s.)
Homo-vanillate
Amino-chrome
Hypertension
MAT (-1.3x)
Methionine
S-Adenosyl Methionine
Di-Methyl-Glycine
BHMT (-3.6x)
Betain
DNMT (+1.5x)
Hypo-Methylation of DNA & Proteins
Hyper-Methylation of DNA & Proteins
Athero-sclerosis
Fibrosis
Homo-cysteine
S-Adenosyl Homocystein
AHCY(-1.7x)
CBS (r.s.)
Cystatione
Alpha keto-glutarate
CTH (-1.4x)
Cysteine
Glutathione Proteins