Development of metabolic analytical platforms for the characterization of uremic retention compounds

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LIST OF ABBREVIATIONS

A

AAG	A1-acid glycoprotein
ADMA	Asymmetric dimethylarginine
AER	Albumin excretion rate
ADPKD	Autosomal dominant polycystic kidney disease
AKI	Acute kidney injury
AMDIS	Automated mass spectral deconvolution and identification system
ANN	Artificial neural networks
APCI	Atmospheric pressure chemical ionization
APD	Automated peritoneal dialysis
AUC	Area under the curve

B

β2-M	β2-microglobulin
BA	Bile acid
BEH	Ethylene bridged hybrid
BMI	Body mass index
BP	Blood pressure
BSTFA	Bis(trimethylsilyl)trifluoroacetamide

C

CAD	Coronary artery disease
cAMP	Cyclic 3'-5'-adenosine monophosphate
CAPD	Continuous ambulatory peritoneal dialysis
CE	Capillary electrophoresis
cGMP	Cyclic 3’,5’-guanosine monophosphate
CID	Collision induced dissociation
ciPTEC	Conditionally immortalized human proximal tubule cells
CKD	Chronic kidney disease
CRF	Chronic renal failure
CRP	C-reactive protein
CTN	Creatinine
CVD	Cardiovascular disease
CMPF	3-carboxy-4-methyl-5-propyl-2-furanpropionic acid

D

2D	Two-dimensional
DB	Database
DDA	Data dependent acquisition

DI	Direct injection
DKD	Diabetic kidney disease
DM	Diabetes mellitus
DMA	Dimethylamine
DN	Diabetic nephropathy

E

eGFR	Estimated glomerular filtration rate
EDTA	Ethylenediaminetetraacetate
EFA	Esterified fatty acid
EI	Electron impact
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental protection agency
EPO	Erythropoietin
EPS	Encapsulating peritoneal sclerosis
ERBP	European renal best practice
Ergone	Ergosta-4,6,8(14),22-tetraen-3-one
ESI	Electrospray ionization
ESRD	End stage renal disease
EUTox	European uremic toxin

F

FAB	Fast atom bombardment
FBI	Find by ion
FC	Fold change
FDR	False discovery rate
FLD	Fluorescence detector
FLP	Poria cocos epidermis, Fu-Ling-Pi
FSGS	Focal segmental glomerulosclerosis
FT	Fourier transform
FWHM	Full width at half maximum

G

GABA	Gamma aminobutyric acid
GC	Gas chromatography
GFR	Glomerular filtration rate
GMD	Golm metabolome database
GUDCA	Glycoursodeoxycholic acid

H

HA	Hippuric acid

HCA	Hierarchical cluster analysis
HD	Hemodialysis
HDF	Hemodiafiltration
HDL	High density lipoprotein
HF	Hemofiltration
HILIC	Hydrophilic interaction liquid chromatography
HMDB	Human metabolome database
HPLC	High performance liquid chromatography

I

IAA	Indole-3-acetic acid
IgA	Immunoglobulin A
IL-1β	Interleukin-1 beta
IS	Indoxylsulfate

K

KDOQI	Kidney disease outcomes quality initiative
KDIGO	Kidney disease: improving global outcomes
KEGG	Kyoto encyclopedia of genes and genomes
KIM-1	Kidney injury molecule 1

L

LC	Liquid chromatography
LDA	Linear discriminant analysis
LDL	Low density lipoprotein
L-FABP	Liver-type fatty acid binding protein
LOD	Limit of detection
LOQ	Limit of quantification
LPC	Lysophosphatidylcholines
LPK	Lewis polycystic kidney
LTQ	Linear trap quadrupole

M

MDRD	Modification of diet in renal disease
MFE	Molecular feature extraction
MG	Monoacylglycerol
MN	Membranous nephropathy
MOPEG	3-methoxy-4-hydroxyphenylethyleneglycol sulfate
MPP	Massprofiler professional
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MSC	Molecular structure correlator

MSI	Metabolomics standards initiative
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
MSTUS	MS total useful signal
MW	Molecular weight
MWCO	Molecular weight cut-off

N

ND	Non-dialysis
NEFA	Non-esterified fatty acid
NFK	National kidney foundation
NGAL	Neutrophil gelatinase-associated lipocalin
NIST	National institute of standards and technology
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
NPLC	Normal-phase liquid chromatography

O

oaTOF	Orthogonal acceleration time-of-flight
OATP	Organic anion-transporting polypeptide
OHHA	Hydroxyhippuric acid
OPA	Ortho-phthaldialdehyde
OPLS-DA	Orthogonal projection to latent structures discriminant analysis

P

PC	Phosphatidylcholine
pCG	p-Cresylglucuronide
pCS	p-Cresylsulfate
PCA	Principal component analysis
PD	Peritoneal dialysis
PDA	Photodiode array
PE	Phosphatidylethanolamine
PFTBA	Perfluorotributylamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PKD	Polycystic kidney disease
PLS	Partial least square
PLS-DA	Partial least squares discriminant analysis
PRMTS	Protein arginine methyltransferases
PS	Phosphatidylserine

Q

Q	Quadrupole
QA	Quality assurance
QC	Quality control

R

ROS	Radical oxygen species
RPLC	Reversed-phase liquid chromatography
RRT	Renal replacement therapy
RSD	Relative standard deviation
RTL	Retention time locked

S

SD	Sprague-Dawley
SDMA	Symmetric dimethylarginine
SEB	Staphylococcal enterotoxin B
SLC	Solute carrier
SM	Sphingomyelin
SOAT	Sodium-dependent organic anion transport
SOM	Self organizing maps
SPE	Solid phase extraction
SVM	Support vector machines

T

TCA	Tricarboxylic acid
TD	Thermal desorption
TGF-β1	Transforming growth factor-beta 1
TIC	Total ion chromatogram
TIMP-1	Tissue inhibitor of metalloproteinases 1
TMA	Trimethylamine
TMCS	Trimethylchlorosilane
TMIC	The metabolomics innovation centre
TMNO	Trimethylamine N-oxide
TMS	Trimethylsilylation
TNF-α	Tumor necrosis factor-alpha
TOF	Time-of-flight
TRPM	Melastatin-related transient receptor potential ion channels

U

UA	Uric acid
UCB	Unconjugated bilirubin

U(H)PLC	Ultra high performance liquid chromatography
UV	Ultraviolet

V

VLDL	Very low density lipoprotein

Z

ZDP	Zhibai dihuang pill

"*If I were doing a PhD, I'd be doing it in Metabolomics*" - James Watson (2013)

Chapter 1

General introduction, outline and aims

I.1. Background

The kidneys, a pair of bean-shaped organs located in the retroperitoneal space, have several important functions in the homeostasis of the body. They purify the blood from metabolites and waste products by eliminating them, along with water, as urine. In addition to removal of waste products, the kidneys have a hormonal function. The secretion of erythropoietin (EPO) stimulates the bone marrow to make red blood cells. Renin regulates blood pressure via the renin angiotensin aldosterone axis. The production of 1,25-dihydroxyvitamin D, the active form of vitamin D, regulates the calcium and phosphorus homeostasis and the production of parathyroid hormone, affecting bone metabolism and many other organ systems.

A persistent decrement in kidney function is named “chronic kidney disease (CKD)”, as suggested by the Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines from the US National Kidney Foundation (NFK), to represent the entire spectrum of disease that occurs following the initiation of kidney disease. In 2002, CKD was defined in the guidelines as either kidney damage or estimated glomerular filtration rate (eGFR) below 60 mL/min/1.73m^2 for at least 3 months [1]. Kidney damage is defined as the presence of pathologic abnormalities in blood or urine tests or imaging studies. According to the KDOQI guidelines, CKD was initially classified into 5 stages based only on the level of GFR. In 2009, the Kidney Disease: Improving Global Outcomes (KDIGO) agreed to retain the current definition of CKD based on levels of GFR or presence of kidney damage, but the CKD classification was modified by adding albuminuria stages in addition to GFR stages. Stage 3 was subdivided in subgroups a en b depending on severity of renal failure, and by emphasizing clinical diagnosis [2]. The modified disease classification indicating prognosis is shown in Figure I.1 [3]. Patients with CKD stage 5 (eGFR< 15 mL/min/1.73m^2) require renal replacement therapy (RRT) such as kidney transplantation

or dialysis to survive when their eGFR further declines, or will do so soon (end stage renal disease, ESRD).

CKD is a complex disease that affects multiple organ systems and often coexists with numerous associated conditions, such as cardiovascular disease (CVD), diabetes mellitus, chronic inflammation, lupus [4-8]. These conditions are in many cases independently associated with CVD, implying a vicious circle in which CVD can lead to CKD, which worsens CVD and down the line.

CKD has reached epidemic proportions and continues to increase in incidence at an alarming rate. Many countries have surveillance programmes to monitor kidney failure treated by dialysis and transplantation [9]. Incidence is now as high as 200 cases per million per year in many countries, such as Belgium. In the USA, Taiwan, and some regions in Mexico, incidence is near to 400 cases per million and has risen fastest in older individuals. With average survival of 3–5 years in the USA, prevalence is 1800 cases per million. In Belgium, prevalence is approximately 1000 cases per million. In Japan and Taiwan, high survival and/or low incidence of kidney transplantation translates to high prevalence nearing 2400 cases per million. Diabetes is the main cause of kidney failure in most countries, accounting for 40% or more of new patients [9].

No CKD
Moderate-risk CKD
High-risk CKD
Very high-risk CKD

GFR stages, description, and range (mL/min/1.73m^2)				Albuminuria stages, description, and range (mg/g)				
				A1		A2	A3	
				Optimum and high-normal		High	Very high and nephrotic	
				< 10	10-29	30-299	300-1999	≥ 2000
	G1	High and optimum	> 105					
			90-104					
	G2	Mild	75-89					
			60-74					
	G3a	Mild-moderate	45-59					
	G3b	Moderate-severe	30-44					
	G4	Severe	15-29					
	G5	Kidney failure	< 15					

Figure I.1 Prognosis of chronic kidney disease by GFR and albuminuria. Colours show how adjusted relative risk is ranked for five outcomes from a meta-analysis of general population cohorts: all-cause mortality, cardiovascular mortality, kidney failure treated by dialysis and transplantation, acute kidney injury, and progression of kidney disease. For categories with GFR >15 mL/min per 1.73 m^2 and albuminuria <2000 mg/g, ranks were averaged across outcomes. Mean rank numbers: 1–8=green, 9–14=pink, 15–21=orange, and 22–28=red. For categories with GFR <15 or albuminuria >2000 (corresponding to nephrotic range albuminuria), ranks were extrapolated on the basis of results from a meta-analysis of chronic kidney disease cohorts [3].

The gold standard measurement for CKD is the "true" glomerular filtration rate (GFR) as tracked by 24-hour urine isotope clearance. This method is however costly, labor intensive and cumbersome.

Therefore, GFR is in practice most often estimated (eGFR) by equations that take into account serum creatinine and a combination of age, gender, and sometimes also other factors. Body weight is additionally implemented in the Cockcroft-Gault equation and race in the Modification of Diet in Renal Disease (MDRD) study equation [2,10-11].

I.2. Uremic retention solutes

When renal failure develops, uremic retention solutes are retained which are normally excreted by the healthy kidneys. If these retention solutes exert adverse biochemical/biological effects, they are called uremic toxins. Since the intact glomerular filter clears substances up to 58 kDa, this value is considered the upper cut-off for retention associated with impairment of kidney function.

The uremic retention solutes are preferentially classified according to their physicochemical characteristics, which have an impact on their clearance during dialysis. Traditionally, this classification focuses on 3 types of solutes, i.e. the small water-soluble compounds (MW < 500 Da), the middle molecules (MW > 500 Da) and the protein-bound compounds [12,13]. Table I.1 summarizes the characteristics, toxicity and prototypes of these 3 classes.

Table I.1 Current classification of uremic retention solutes

Classification	Characteristics	Toxicity	Prototypes
Small water-soluble molecules	MW < 500 Da, easily removed by any dialysis strategy	Not necessarily toxic	Urea, creatinine
Middle molecules	MW > 500 Da, removed only through large pore membranes	Large array of biological impacts	β2-M, leptin
Protein-bound molecules	Any MW, difficult to remove with any dialysis strategy	Large array of biological impacts	Phenols, indols

I.2.1. Small water-soluble compounds

Urea and creatinine are protoypes of the small water-soluble compounds. In general, the water-soluble compounds are easily removed by any kind of dialysis strategy. However, guanidino compounds such as creatinine are more difficult to remove because their different kinetic behavior [14].

I.2.2. Middle molecules

The prototype for this group is β2-microglobulin (β2-M). Middle molecules are considered as solutes in the large molecular weight range (> 500 Da). β2-M is cleared more efficiently when the pore size of the dialyzer membrane increases [15], convection is applied [16-18] and dialysis time is prolonged [19]. However, removal of β2-M is not necessarily representative for that of other middle molecules.

Kinetic analysis has only been applied to β2-M up to now. Hence, there is a clear need for thorough studies of the removal pattern of middle molecules at large scale.

I.2.3. Protein bound compounds

Protein bound uremic retention solutes form a heterogeneous group of compounds. Although most of these compounds have a low MW, they are more difficult to remove by dialysis as a result of their binding to albumin or other protein. This large group should not be considered as a homogenous entity, as their retention pattern and the removal during dialysis depend upon the strength and the degree of protein binding, and the number and type of protein binding sites. Prototypes of this group are phenols and indoles, such as p-cresylsulfate and indoxylsulfate.

During the last few years, progress has been made in the identification and quantification of uremic retention solutes. In 2003, the European Uremic Toxin (EUTox) Work Group composed an encyclopedic list of 90 compounds retained in CKD, providing data on normal and pathologic serum concentrations [12]. In 2012, the list was updated with 56 additional uremic retention solutes [20]. Nevertheless, a substantial number of uremic retention solutes remains unknown.

I.3. Therapy in CKD

I.3.1. Pharmacological treatment

Once CKD is diagnosed, patients need to be evaluated to determine the type of kidney disease, the stage of CKD, the complications related to the level of kidney function, the risk for further loss of kidney function and the risk to develop CVD. Based on this information, treatment can be initiated. A pharmacological treatment will initially be started, which will mainly focus on slowing down the progression of kidney failure and preventing and treating CVD and other complications of reduced kidney function. In patients with a GFR <15 mL/min/1.73m^2, renal replacement therapy (RRT) should be considered when there is one or more of the following: symptoms or signs of uremia, inability to control hydration status or blood pressure or a progressive deterioration in nutritional status (European Renal Best Practice, ERBP [21]). The majority of patients will be symptomatic and need to start renal replacement therapy with GFR in the range 9-6 mL/min/1.73m^2. This RRT can either be dialysis or renal transplantation.

I.3.2. Renal replacement therapy

I.3.2.1. Renal transplantation

Although transplantation is the preferred therapy for kidney failure, there is a shortage of donor kidneys, transplants may fail and majority substantial proportion of ESRD patients is not suitable for

transplantation. Consequently, the majority of CKD stage 5 patients will be treated with dialysis, while awaiting transplantation or because they are no valid candidates for a graft.

I.3.2.2. Dialysis

The main strategies that have been used up to now to decrease uremic solute concentration are conventional hemodialysis and peritoneal dialysis. However, dialysis is nonspecific and also removes essential compounds.

Hemodialysis

Hemodialysis (HD) is an extracorporeal technique. The blood is purified through an artificial kidney, which contains 2 chambers separated by a membrane. The blood flows in one compartment and dialysate in the other, in opposite directions (Figure I.2a). Waste products are cleared from the blood by diffusion induced by a concentration gradient across a semi-permeable membrane, and convective clearance and fluid removal are achieved by ultrafiltration induced by hydrostatic transmembrane pressure.

Conventional dialysis uses dialyzer membranes with small pores (low flux), not able to remove middle molecules or protein bound solutes. Several observational and epidemiological studies, however, favoured the high flux membranes with larger pores and more efficient middle molecule removal; subsequent to a better outcome in more recent controlled studies [22,23], low flux membranes are gradually replaced by more open ones [24-27], especially in the Western world.

Removal through large pores can be purely diffusive, but is further enhanced by adding convection, like in hemofiltration (HF, pure convection) or in hemodiafiltration (HDF, combination of diffusion and convection) [28]. The convective strategy includes the removal of substantial quantities of plasma water through high flux membranes by ultrafiltration and its replacement by equivoluminous quantities of buffered saline substitution fluid. Both small and large molecules are dragged through the pores along with the ultrafiltered water.

The fluid volume that could be removed by ultrafiltration and replaced from the patient by convective therapies was previously restricted by the necessity to use pharmacologically prepared sterile substitution fluids, which, for reason of cost, allowed only limited quantities of ultrafiltered volume. With the advent of ultrapure dialysis fluid, the risk of causing a pro-inflammatory response when dialysate was administered as substitution became unlikely [29,30]; hence, convection could be optimized as online H(D)F [31].

HD or HDF therapy is usually applied 3 times a week, generally with a duration of 4 hours or more per session. The increase of the treatment time and more frequent application might result in an improved removal of uremic toxins [32-34].

Peritoneal dialysis

In this form of dialysis, the peritoneum, which surrounds the abdominal cavity and envelopes the intestine, serves as a semi-permeable membrane across which dialysis can occur. In peritoneal dialysis (PD), dialysis fluid is instilled into the peritoneal cavity via a permanent catheter, placed in the lower part of the abdomen (Figure I.2b). At regular intervals, the fluid is drained and replaced.

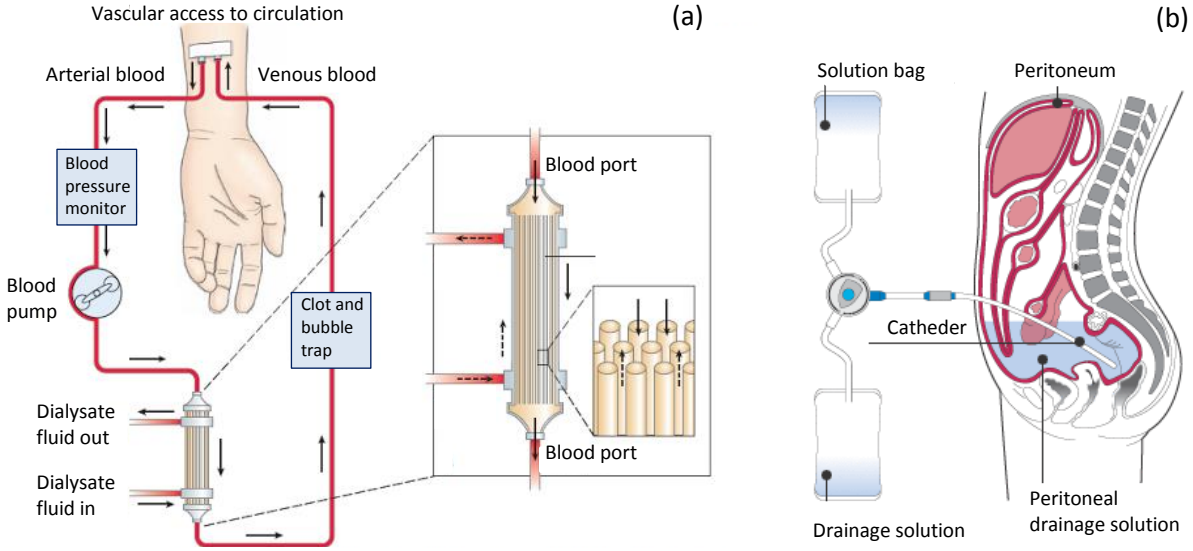

Figure I.2 Schematic overview of hemodialysis (a) and peritoneal dialysis (b)

PD is based on the principles of diffusion and osmosis. Uremic retention solutes and toxins move across the membrane, by diffusion, from the blood stream to the dialysis fluid, or vice versa, depending on the concentration gradient. Fluid removal takes place by osmosis, due to the addition of an osmotic agent, usually glucose, to the dialysate. Other osmotic agents, such as icodextrin or amino acids, can also be added. The pores in the peritoneal membrane are large enough to allow the waste products to pass into the abdominal cavity, but small enough to reject blood cells and larger proteins, or to allow their passage only to a limited extent.

Compared to HD, PD is a more continuous therapy. In continuous ambulatory peritoneal dialysis (CAPD), dialysate is instilled into and drained from the abdominal cavity by gravity, without the need for a machine. The dialysate is continuously present in the peritoneal cavity and every 4 to 6 hours the fluid is changed with a long dwell time overnight. In automated peritoneal dialysis (APD), a cycler performs several exchanges overnight, whereas, most often, in the morning a dwell time starts that lasts the entire day.

I.4. Biomarkers of CKD

A biological marker (biomarker) has been defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" by the NIH Biomarkers Definitions Working Group [35]. The quest for biomarkers is as old as medicine itself; there has always been a search for measurable biological cues that allow insight into the physiological working of the human organism. For most of history, biomarker discovery has relied on intimate knowledge of the pathophysiology of the disease being studied. Known biological substances were related to a disease state and were investigated to see whether they could serve as diagnostic markers, provide a target for therapy or lend further insight into the etiology of the disease. In recent years, the ready availability of powerful tools to scan the genome, the transcriptome, the proteome and the metabolome of an organism has revolutionized and greatly accelerated biomarker discovery. In this way, we have entered an "open loop" or unbiased approach to biomarker discovery, which is in strong contrast to the hypothesis-driven approach of the past [36]. The recent explosion in the omics tools is also believed to generate valuable information of patients which could serve as potential accelerating factors for the development of personalized medicine. Personalized medicine is believed to transform the traditional "one size fits all" model of medicine by applying individual information to better manage a patient's disease or predisposition toward a disease and to tailor strategies for the prevention and treatment of diseases.

Despite the fact that the characteristics of an ideal biomarker highly depend upon the disease under investigation, certain universal characteristics are important for any biomarker [35,37]:

1. a biomarker should be easily measured, inexpensive and produce rapid results
2. a biomarker should be originating from readily available sources, such as blood or urine
3. a biomarker should have a high sensitivity, allowing early detection, and no overlap in values between diseased patients and healthy controls
4. a biomarker should have high specificity, being greatly upregulated (or downregulated) specifically in the diseased samples and unaffected by comorbid conditions
5. biomarker levels should vary rapidly in response to treatment
6. biomarker levels should aid in risk stratification and possess prognostic value in terms of real outcomes
7. a biomarker should be biologically plausible and provide insight into the underlying disease mechanism

The biomarker development process can be divided into 5 phases from biomarker discovery to translation into clinical practice, as shown in Table I.2 [37]. This thesis focuses on phase 1, the preclinical biomarker discovery phase.

Table I.2 Phases of biomarker discovery, validation and translation

Phase	Terminology	Action steps
Phase 1	Preclinical discovery	- Discover biomarkers in tissues or body fluids - Confirm and prioritize promising candidates
Phase 2	Assay development	- Develop and optimize clinically useful assay - Test on existing samples of established disease
Phase 3	Retrospective study	- Test biomarker in completed clinical trial - Test if biomarker detects the disease early - Evaluate sensitivity and specificity
Phase 4	Prospective screening	- Use biomarker to screen population - Identify extent and characteristics of disease - Identify false referral rate
Phase 5	Disease control	- Determine impact of screening on reducing disease burden

Current biomarkers of CKD and its progression that are in widespread clinical use, namely serum creatinine and albuminuria, have limitations [38-42]. Lack of specificity and slow response to alterations in disease severity or treatment are primary reasons why serum creatinine, the clinical surrogate of GFR, is an unsatisfactory biomarker. Firstly, serum creatinine levels change with factors unrelated to renal disease, such as age, gender, diet, muscle mass, muscle metabolism, race, strenuous exercise and hydration status. Moreover, serum creatinine concentrations may not change until approximately 50% of kidney function has been lost, making it a poor diagnostic marker [40]. As mentioned above, CKD is defined by the presence of kidney damage or a glomerular filtration rate less than 60 mL/min/1.73 m^2 for at least 3 months, regardless of cause. However, significant increases in risk for CVD, a major cause of morbidity and mortality in CKD patients, occur at more subtle loss of kidney function (GFR of approximately 75 mL/min/1.73 m^2) [43], so it is inherently important that CKD can be detected in its earliest stages. Additionally, it has recently been shown that eGFR poorly reflects the accumulation of a broad range of uremic retention solutes that have been linked to toxicity [44,45]. This indicates that other factors than GFR such as metabolisation and intestinal generation are important for the development of the uremic status.

Large population studies have shown that albuminuria strongly predicts the risks of CKD progression, cardiovascular disease and all-cause mortality [46-50]. In addition, combining albuminuria measurement with eGFR provides synergistic, complementary risk stratification for both cardiovascular disease and CKD [46,47,50]. An important limitation of assessment of albuminuria as a marker of CKD is potential misclassification of individuals due to variability in levels of albumin over

time and the extent to which conditions at the time of testing may obscure the true level [1]. Excretion of total protein or albumin in the urine is highly variable in individuals with or without kidney disease. Transient albuminuria in the primary care setting can be influenced by a number of pre-existing factors such as urinary tract infection, high dietary protein intake, congestive cardiac failure, acute febrile illness, heavy exercise within 24 hours, menstruation or vaginal discharge, and drugs [51,52].

When CKD is not detected until the onset of symptomatic kidney failure, as is often the case, it is typically too late to prevent many adverse outcomes [53]. Therefore, new biomarkers that can aid in diagnosing, distinguishing subtypes and prognosticating the severity of CKD are urgently needed. Due to the complexity of CKD and the co-existence with other conditions, it is very unlikely that one marker can be found possessing all the ideal characteristics of a biomarker. Ongoing research has produced some biomarker candidates, including neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule 1 (KIM-1), cystatin C, asymmetric dimethylarginine (ADMA) and liver-type fatty acid binding protein (L-FABP), for possible inclusion in a panel of biomarkers for CKD, some of which are performing double duty as markers for both CKD and acute kidney injury (AKI) [39-41].
Several studies indicate that NGAL is emerging as a promising biomarker for the early detection and staging of CKD, for predicting progression, and for monitoring the response to interventions [54-68]. However, several limitations should be recognized. One of the limitations is that these studies represent single center studies with relatively small numbers of cases, and the results need to be confirmed in larger multi-center trials. Another limitation is that increases in NGAL levels are not specific to CKD. It is well known that NGAL is an early responder to AKI, and clinical situations where AKI is superimposed on CKD are common [69]. Moreover, plasma NGAL measurements may be influenced by a number of coexisting variables such as chronic hypertension, systemic infections, inflammatory conditions, anemia, hypoxia, and malignancies [70-72]. In addition, NGAL has been showed to be expressed in human atherosclerotic plaques [73], which may also effect plasma NGAL measurements. Urine NGAL levels may be increased in urinary tract infections [74]. Future studies should aim at addressing these limitations, taking into account these potential confounding variables. Currently, the routine measurement of NGAL in patients with CKD cannot be recommended.
Preliminary studies have reported on the potential utility of KIM-1 as a CKD biomarker [75-78]. Many of the limitations mentioned above for NGAL as a CKD biomarker also pertain to KIM-1, and additional large long-term studies are required to confirm the utility of KIM-1 in CKD. A combination of biomarkers such as NGAL and KIM-1 may provide complementary information, with NGAL reflecting acute inflammatory events and KIM-1 indicating the more chronic fibrotic changes.

Cystatin C is considered as marker of GFR in both AKI and CKD. However, serum cystatin C is influenced by factors other than renal function alone such as age, weight, gender, and smoking [79,80]. In addition, there is no consensus in literature whether serum cystatin C-based estimates of GFR are superior to serum creatinine-based estimates [81].

Studies indicate that plasma ADMA is a promising biomarker for the early detection and staging of CKD, and for predicting progression [82-85]. However, specificity for CKD is a major issue, since increased plasma concentrations of ADMA are encountered in a variety of clinical settings characterized by endothelial dysfunction, including hypertension, hypercholesterolemia, diabetes mellitus, and congestive heart failure.

L-FABP is suggested to predict progression in CKD [86-88]. Additional longitudinal studies are needed to demonstrate L-FABP's ability to predict CKD and its progression. Standardized clinical platforms for the measurement of urinary L-FABP are currently not available.

Thus, despite the progress in CKD biomarker research, the previous clearly indicates that additional studies are required to further validate these biomarkers in larger, more diverse populations before translation into clinical practice and that new contributions to the quest for better disease biomarkers and novel uremic toxins are needed. This is the subject of the current thesis.

I.5. Outline and aims

The general objective of this thesis is to obtain an elaborate insight in the nature and the behavior of uremic compounds involved in CKD.

More specifically the aims are:

1. the set-up and use of metabolic analytical platforms to identify new uremic retention solutes and/or potential biomarkers of CKD
2. the acceleration and the broadening of analytical methods for the determination of uremic toxins

In chapter 2, the workflow in non-targeted metabolomics is discussed in depth. A review of the literature on non-targeted metabolomic studies in CKD is provided.

In chapter 3, a metabolomics platform, based on a combination of reversed-phase liquid chromatography (RPLC) coupled to high-resolution quadrupole time-of-flight mass spectrometry (Q-TOF MS) in positive and negative ionization mode and gas chromatography (GC) coupled to quadrupole MS, has been evaluated as discovery tool for potential uremic toxins and CKD biomarkers. The methodological validity was ensured by use of quality control (QC) samples in the analytical setup, and by thorough data analysis strategy for both GC-MS and LC-MS. The identified

upregulated uremic retention solutes/potential markers surpassing the level of serum creatinine and the downregulated identified metabolites were compared with literature to confirm our findings on the one hand and to reveal new potential biomarkers on the other hand.

Further contributing to the quest for better CKD biomarkers, chapter 4 is devoted to the development of a plasma and urine based hydrophilic interaction liquid chromatography time-of-flight mass spectrometric (HILIC-TOF MS) platform, providing complementary information to RPLC.

Chapter 5 describes the development of a novel ultra-high performance liquid chromatography tandem mass spectrometric (UPLC-MS/MS) method for the simultaneous determination of seven uremic retention toxins with cardiovascular relevance in chronic kidney disease patients, i.e. uric acid (UA), hippuric acid (HA), indoxylsulfate (IS), p-cresylglucuronide (pCG), p-cresylsulfate (pCS), indole-3-acetic acid (IAA) and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF). The method was validated and the applicability was evaluated by analyzing serum samples originating from healthy controls as well as from patients at different stages of CKD. These concentrations were compared with those obtained by commonly used HPLC-PDA-FLD methods.

In chapter 6, the development and validation of a UPLC-MS/MS method for the simultaneous determination of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide (NO) synthesis, and its structural isomer symmetric dimethylarginine (SDMA) is described. The applicability of the method was evaluated by the analysis of serum samples from healthy controls and CKD patients on hemodialysis. Protein binding of ADMA and SDMA was investigated. A comparison was made between the developed UPLC-MS/MS method and the commercially available ELISA assay.

In chapter 7, the general conclusions resulting from this thesis are summarized and discussed. Proposals on how future research could contribute to better CKD biomarkers are made.

I.6. References

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Chapter 2

Metabolomics in chronic kidney disease

II.1. Definitions

The post-genomic era is characterized by an explosion of strategies that provide an integrative view of the molecular regulation of cells and whole organisms [1]. These advances have been driven by the development of novel technologies that can analyze global sets of gene products. Transcriptomics defines the population of mRNA species in a cell at a specific time and set of conditions; proteomics addresses the challenging problem of defining changes in protein expression, protein dynamics and post-translational modifications. Metabolomics is an emerging field that can be regarded as the end point of the "omics" cascade, as shown in Figure II.1 [2].

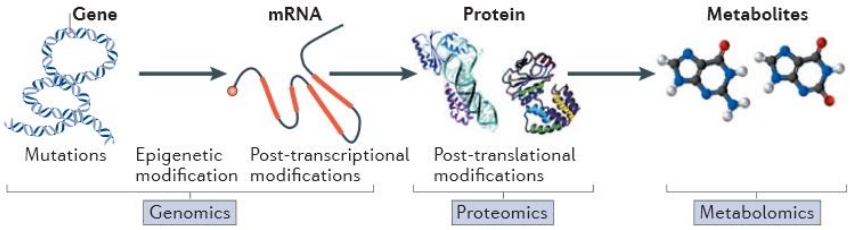

Figure II.1 The "omics" cascade (Adapted from [2])

In 1999, metabonomics was defined as "the quantitative measurement of the multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification" by Nicholson et al [3]. The term metabolomics was introduced by Fiehn in 2001 and defined somewhat differently as "a comprehensive and quantitative analysis of all metabolites" [4]. Although the concepts slightly differ, there is a great deal of overlap in philosophies and methodologies. As a consequence, the two terms are often used interchangeably [5]. Interest in this field has dramatically increased since its introduction in 1999, as illustrated in Figure II.2.

Genomics, transcriptomics, proteomics and metabolomics provide a comprehensive framework, referred to as systems biology. Although '-omic' technologies are complementary, analysis of the metabolome is a particularly useful approach for identifying pathways that are perturbed in a given pathology, when compared with the transcriptome and proteome [6,7]. Changes in mRNA concentrations do not translate directly into corresponding changes in the number of functional proteins and are not necessarily associated with changes in signal transduction and cell biochemistry. Therefore, downstream confirmation by analyzing protein and/or metabolite concentrations is often required. However, changes in protein concentration do not necessarily translate into changes in cell biochemistry and function, for example because of translational modifications. Since metabolites are down-stream of both transcription and translation, they are typically more closely associated with a disease process than proteins, mRNA or genes. The metabolome provides a functional readout of the physiological state of an organism as determined by the sum of its genetic blueprint, regulation, protein abundance, and environmental influences. Next to the metabolome's close biological proximity to the phenotype of the system, metabolomics offers several other advantages such as relatively low price on a per-sample basis, relatively high throughput and possibilities for automation [6]. Furthermore, the metabolome of one species can easily be compared with another. Whereas gene and protein sequences vary between species, many metabolites are conserved between species, and the analytical tools used to detect these in one organism can be applied to each other [6].

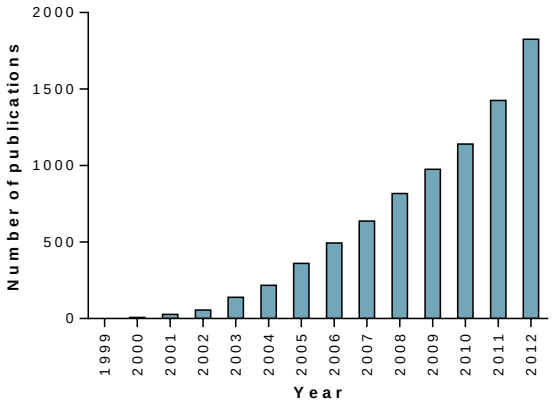

Figure II.2 Publications with "metabolomic*" and "metabonomic*" listed as keywords according to ISI Web of Knowledge from 1999 to 2012

In addition to metabolomics, other -omics fields such as lipidomics and glycomics are emerging. Lipidomics, often regarded as a subset of metabolomics, aims at the comprehensive measurement of the lipids present in a cell, tissue, biological fluid, etc. and the concomitant detection of the lipid responses to various stimuli, e.g. disease and pharmaceutical treatment [8]. Analogously, glycomics refers to studies that attempt to define or quantify the glycome of a cell, tissue, or organism [9].

In the metabolomics field different approaches can be distinguished, depending on the scope of research.

II.1.1. Metabolomics

Non-targeted metabolomics aims to capture as much information as possible. Since the goal is non-biased detection of unknowns, these assays are only semi-quantitative at best and not validated. As there is currently no single technology available that allows the comprehensive analysis of the metabolome, metabolomics is characterized by the use of multiple analytical techniques [10]. Metabolomics uses relative quantification. In general, non-targeted methods are hypothesis-generating strategies whose results will require follow-up with targeted approaches. Figure II.3 illustrates the difference between targeted and non-targeted methods for liquid chromatography-mass spectrometry (LC-MS)-based metabolomics.

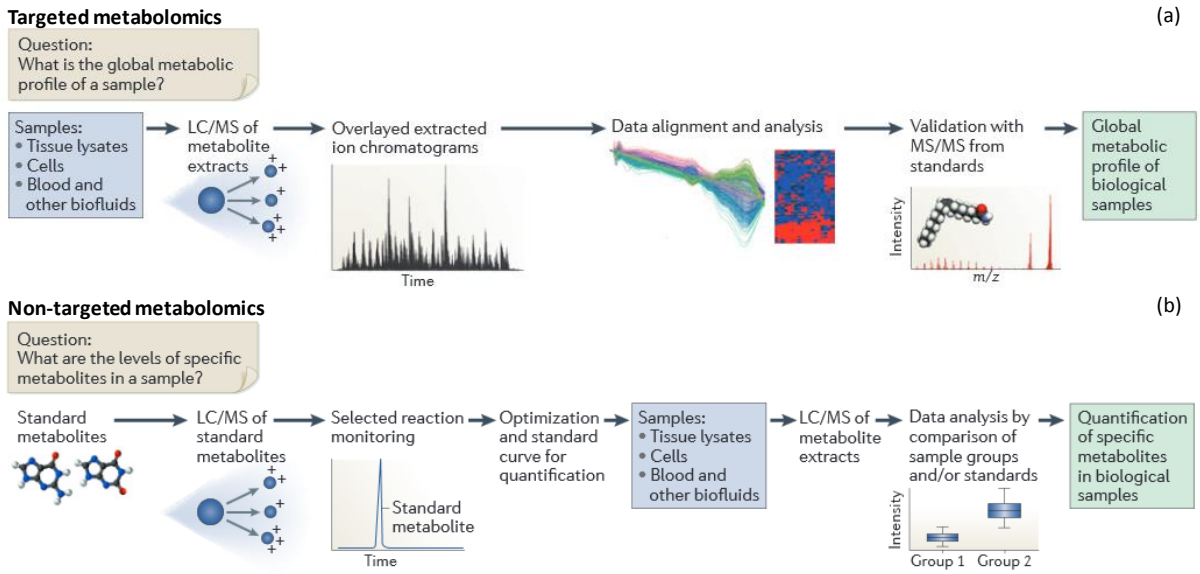

Figure II.3 Targeted and non-targeted LC-MS-based metabolomics (Adapted from [2])
a) In non-targeted metabolomics, metabolites are first isolated from biological samples and subsequently analyzed by LC-MS. After data acquisition, the results are processed by using bioinformatics software such as XCMS to perform nonlinear retention time alignment and identify peaks that are changing between the groups of samples measured. The m/z values for the peaks of interest are searched in metabolite databases to obtain putative identifications. Putative identifications are then confirmed by comparing tandem mass spectrometry (MS/MS) data and retention time data to that of standard compounds.
b) In triple quadrupole-based targeted metabolomics, standard compounds for the metabolites of interest are first used to set up selected or multiple reaction monitoring (MRM) methods. Here, optimal instrument voltages are determined and response curves are generated for absolute quantification. After the targeted methods have been established on the basis of standard metabolites, metabolites are extracted from tissues, biofluids or cell cultures and analyzed. The data output provides quantification only of those metabolites for which standard methods have been developed.

II.1.2. Metabolite profiling

Metabolite profiling restricts itself to a certain range of compounds or to screening a pre-defined number of members of a compound class. Within these constraints, a single analytical platform may be sufficient. Examples of metabolite profiling are the analysis of amino acids by fractionation and gas chromatography coupled to mass spectrometry (GC-MS), or membrane lipid profiling by liquid chromatography with tandem mass spectrometry (LC-MS/MS) [10]. Quantification in metabolite profiling is usually carried out relative to comparator samples, such as controls.

II.1.3. Metabolic fingerprinting

Metabolic fingerprinting is characterized by high-throughput, rapid, global analysis of intracellular metabolites to provide sample classification on the basis of their origin or biological relevance [10]. Quantification and identification are generally not performed. Sample preparation is simple and chromatographic separation is generally absent.

II.1.4. Metabolic footprinting

Metabolic footprinting aims at the study of metabolites in extracellular fluids, also known as exometabolome or secretome [11]. Parallel analysis of metabolic footprinting and fingerprinting can reveal possible mutual interconnections between intracellular and extracellular metabolites, thereby helping in the identification of possible novel cellular metabolic functions.

II.1.5. Target analysis

Targeted methods measure one or several well-defined compounds, and are quantitative and validated. Quantification is usually done in an absolute manner using calibration curves and stable isotopically labeled internal standards. It has been suggested to confine the term metabolomics to non-targeted methods, since omics methods are by definition non-targeted and the term metabolomics therefore better fits methods that involve holistic analysis and advanced statistical tools for data treatment. Chapter 5 and 6 in this thesis are examples of target analysis.

II.2. Workflow

Figure II.4 illustrates the general workflow in non-targeted metabolomic studies, from sampling to biological interpretation. The sampling stage should be preceded by the draw up of a well designed experimental plan.

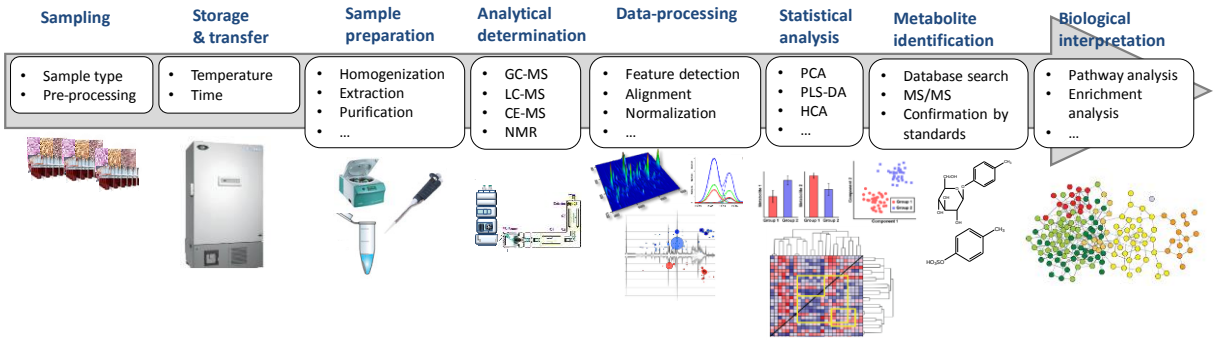

Figure II.4 Schematic representation of the workflow in metabolomics

II.2.1. Sampling

Careful sample collection and storage are vital to ensure the quality of the data generated by the subsequent analysis. It is obvious that the most advanced analytical technology or statistical tool cannot make up for mistakes at the sample collection stage. The study of mammals, particularly humans, can involve a diverse range of sample types, such as blood (serum and plasma) [14-17], urine [18-20], cerebrospinal fluid [21,22], lymph fluid [23], bile [24], feces [25], saliva [26], cells [27,28], and tissues [29,30]. Of these, blood and urine are the most frequently studied sample types for a number of reasons. Sample collection is noninvasive (urine) or minimally invasive (blood) compared with the collection of cerebrospinal fluid and tissues. Blood and urine are integrative biofluids that incorporate the functions and phenotypes of many different parts of the body in a single sample. However, this complexity can dilute small metabolic changes from a specific part of the body, and in these cases, tissues may be appropriate for knowledge discovery. These biofluids also contain many hundreds or thousands of metabolites (the human metabolome database contains to date more than 40,000 metabolites [31]), and hence provide an overview of many areas of metabolism in the body. Since serum, plasma and urine are investigated in this thesis, we focus here on these biofluids.

Blood is preprocessed to obtain serum or plasma. Serum is acquired by allowing the blood sample to clot naturally. The clot is than removed by centrifugation to leave the supernatant. Plasma is prepared by mixing blood with an anticoagulant followed by centrifugation at 4°C to separate the plasma from the formed components of the blood, i.e. red and white blood cells and platelets. A number of anticoagulants are available, including potassium ethylenediaminetetraacetate (EDTA), citrate and lithium heparin. However, there is no general consensus regarding the optimal anticoagulant to use [32-37]. The protein content of serum is lower than that of plasma, because proteins involved in clot formation are removed. To obtain serum, the clotting process takes 30 to 60 minutes, which increases the chance for occurrence of enzymatic conversion and other degradation processes, at least for the more labile compounds, and facilitates possible loss of some metabolites during clot formation/ precipitation. It is therefore important to respect a standardized clotting time

before cooling down. Several recent studies compared metabolite profiles from the 2 fluids. The resulting profiles seem very similar in terms of metabolite coverage and discriminatory ability, with minor differences for a few metabolites [34,38]. It has been shown that both biofluids can successfully be used in metabolomics, but care should be taken in extrapolating results from plasma to serum and vice versa [33].

The metabolic changes, occurring almost instantaneously when collecting tissues, make it difficult to exclude *post hoc* changes and artifacts from the primary process of interest. To that end, efforts have been made to find quenching methods that immediately arrest metabolic processes during sample collection. Common quenching approaches include low temperatures (cold solvent addition, freezing in liquid nitrogen), addition of acid (perchloric or nitric acid), or fast heating [39]. This step is generally omitted for studies of urine, serum and plasma.

II.2.2. Storage and transfer

The time that passes from sampling until freezing is an important parameter for blood and tissues as ongoing biochemical reactions can alter the metabolite profile. As a general rule, biofluids for metabolomics analysis should be stored at -80°C in sterile glass or plastic containers [33,39]. The vials into which the samples are collected and stored should be chosen so as to minimize unwanted variation and to avoid introducing contaminants such as surfactants and plasticizers, which can cause serious interferences in the MS detection [39]. Sodium azide is sometimes added to prevent bacterial growth during storage [20]. Blood serum and plasma samples stored for 1 year and urine samples stored for 9 months under these conditions have been shown to maintain stability [40]. It is recommended to collect multiple aliquots of serum or plasma for each subject and using each aliquot only once in order to avoid multiple freeze/thaw cycles [33].

II.2.3. Sample preparation

Sample preparation (extraction, clean-up, concentration,…) for metabolomic studies depends on the type of sample being analyzed, method of analysis and the metabolites of interest. Sample preparation for holistic analytical approaches should ideally be unselective, simple, rapid and reproducible [39]. The need for an unselective sample preparation procedure is driven by the need to analyze as wide a range of metabolites as possible. Metabolite losses are further reduced by minimizing the overall number of steps.

II.2.3.1. Sample preparation methods for biofluids

For urine, the “dilute and shoot” approach is commonly used. Urine is typically 2 to 10 times diluted with purified water before LC-MS analysis [20,41]. Prior to MS analysis it is also recommended to treat urine with urease to eliminate urea (enzymatic degradation of urea to ammonia and CO_2, which

are lost during drying or degradation) and to reduce the risk of column overloading, peak distortions and ion suppression. The total protein concentration of blood-derived samples is 6-8 g dL^{-1}, in contrast to urine with approximate protein concentrations of 50-100 mg dL^{-1} [42]. Protein removal is therefore necessary and most commonly done by protein precipitation with an organic solvent such as methanol or acetonitrile [43]. Next to protein removal, the addition of organic solvent also disrupts any binding between metabolites and proteins present in solution. Hence, the metabolite concentration represents the total metabolite concentration, which is the sum of bound and unbound (free) metabolite concentration. Specific trends related to the organic solvent are apparent; precipitation with acetonitrile or acetone results in better protein removal [17,44], whereas precipitation with methanol, ethanol or a mixture seems to perform better in terms of metabolite coverage and method precision [17,45]. Heat and acid treatment were also found to enable efficient protein removal, but resulted in much lower metabolite coverage than protein precipitation with an organic solvent [45]. Next to these approaches, ultrafiltration has been shown to be successful [46-50]. The use of 3000 Da filters allows to physically separate low MW species from proteins or other macromolecules present in the sample, which is particularly useful for metabolomic studies. Ultrafiltration enables excellent coverage of polar metabolites and is recommended as the best sample preparation method for use with NMR [51]. However, ultrafiltration suffers from significant loss of hydrophobic species, compared with protein precipitation with an organic solvent. Solid phase extraction (SPE) has also been used for sample preparation in non-targeted metabolomic [52-56]. Next to preconcentration, a major advantage is the excellent sample clean-up, resulting in reduced matrix-effects and an increase in the column lifetime and the overall method robustness. However, the selectivity of the procedure can lead to reduced metabolite coverage, which explains the main use of SPE in targeted bioanalysis. Biofluid sample preparation is more laborious for GC-MS than for LC-MS, CE-MS or NMR. After deproteinization with an organic solvent, blood derived-samples are typically evaporated to dryness and derivatized (see section, II.2.3.2) prior to GC-MS analysis.

II.2.3.2. Derivatization in GC-MS

Since most metabolites are insufficiently volatile or even not, derivatization is required prior to GC-MS analysis, which adds to the number of sample preparation steps. A multitude of chemical derivatization reagents exists [57], but a two-step derivatization consisting of oximation followed by trimethylsilylation (TMS) is most frequently selected (Fiehn method) [58,59]. If sugars are analyzed, the introduction of an oximation step inhibits cyclyzation, resulting in fewer peaks per sugar. In addition, α-ketoacids are protected against decarboxylation and enolizable keto groups are fixed by oximation simplifying the identification of the original molecular structures of the metabolites. Subsequently, the active hydrogens of low molecular weight metabolites are converted by a

silylation step into trimethylsilyl groups. If not masked by derivatization, these active hydrogens allow intra- and inter-molecular hydrogen bonding, which increases the boiling points of the metabolites and makes them unsuitable for GC-MS analysis. N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) has been found superior to alternative chemicals such as bis(trimethylsilyl)trifluoroacetamide (BSTFA) regarding the completeness of the derivatization of amines and amino acids and the reduction of undesired side reactions [58]. Sample stability is a concern. The presence of water can result in the breakdown of TMS esters, although extensive sample drying and the presence of excess silylating reagent can limit this process. However, extensive sample drying can result in the loss of volatile metabolites. An automated system is desirable to ensure maximum sample stability. If on-line automated derivatization is not available, derivatized samples should not be stored at room temperatures for long periods [60].

II.2.4. Analytical determination

It is important to emphasize that the global or non-biased analysis of all metabolites is only a theoretical concept. Metabolites constitute a diverse set of atomic arrangements when compared to the proteome (arrangement of 20 amino acids) and transcriptome (arrangement of four nucleotide bases bonded with sugar and phosphate backbone). Due to the variety of chemical classes and physical properties (from small polar volatiles to large hydrophobic lipids) and the extensive dynamic range (pM - mM), a broad array of analytical techniques has to be used to cover the metabolome [60]. The main analytical techniques in metabolomic studies involve nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS), usually preceded by a chromatographic separation step [60-66]. These hyphenated techniques include gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE) coupled with mass spectrometry (MS). In this thesis MS-based metabolic platforms are developed and implemented and therefore these techniques are discussed in more detail than NMR.

II.2.4.1. GC-MS

GC-MS has long been used for metabolic profiling due to its high sensitivity, reliability and the availability of extensive databases facilitating metabolite identification [60]. Capillary GC-MS provides appropriate chromatographic resolution, with peak widths of 2-5 s and reproducible retention times [60]. GC-MS is not suitable for non-volatile, thermolabile, and/or highly polar compounds; therefore, derivatization of metabolites is often needed to yield volatile and thermostable analytes as discussed in section II.2.3.2. A range of stationary phases can be used for metabolome analysis in GC-MS, although methyl-phenyl columns are typically applied [67,68]. GC-MS provides separation of low molecular weight metabolites and includes a range of relatively polar metabolite classes, such as

amino acids, organic acids, amines, amides and sugars. Time-of-flight (TOF) and quadrupole mass analyzers are most frequently applied in metabolic profiling with GC-MS. TOF analyzers are recommended, as they offer high sensitivity, high acquisition rates, and accurate mass. The mechanism of electron ionization (EI) provides highly reproducible and characteristic fragmentation patterns, enabling the resulting mass spectra to be used for determination of chemical structure. The coupling of highly reproducible GC retention times with EI mass spectra allows the construction of mass spectral libraries, which are transferable between instruments and can be used for definitive metabolite identification. Applications of GC-MS-based metabolomics are widely ranging, especially as GC-MS is a mature analytical technique. The first applications of GC-MS in metabolomics include urinary screening procedures to indicate the presence of diseases related to organic acidemias [63]. More recently, applications in plant metabolomics are becoming widespread. Plants including Arabidopsis [70-72], potatoes [73] and tomatoes [74] have been studied to measure effects of genetic or environmental modifications and stressors, either by analysis of intra-cellular metabolites or volatile metabolites. Other applications, including microbial and clinical metabolomics, are now applying GC-MS to analyze samples such as biofluids or breath [75-78]. Multi-dimensional separations (e.g., GC × GC-TOF-MS) offer much greater peak capacities and are appealing for complex samples such as biofluids [79].

II.2.4.2. LC-MS

Whereas LC is inferior to GC in terms of separation efficiency, technological advancements in LC have resulted in improved performance. The introduction of ultra-high pressure chromatography (U(H)PLC) substantially increased the available chromatographic resolution and the number of metabolites detected compared with traditional LC [80]. Coupling UPLC to mass spectrometers of high mass resolution (typically > 5,000 full width at half maximum (FWHM)) and high mass accuracy (typically < 5ppm) provides a tool for putative metabolite identification. Typical chromatographic peak widths of 1-7s require fast acquisition rates or scan times. TOF or Fourier transform/orbitrap mass spectrometers are most commonly used [14,41,81,82]. The linear dynamic range of these instruments operates over 3 to 5 orders of magnitude. Metabolites at a concentration of < 0.01% of the metabolite of highest intensity can be detected. Electrospray ionization (ESI) or, less typically, atmospheric pressure chemical ionization (APCI) makes up the interface between (UP)LC and MS [63]. The choice of ionization mode plays a major role in the metabolite profile that will be obtained as one methodology cannot cover all types of molecules. Electrospray instrumentation operates in positive and negative ion modes (either as separate experiments or by polarity switching during analyses). ESI in positive mode is the most common mode in LC-MS because it can effectively ionize a wide range of medium polar and polar metabolites. Negative mode ionization provides superior

results for certain analyte classes (e.g. organic acids or carbohydrates). Hence, wider metabolome coverage can be obtained by analysis in both modes [33]. Other interfaces such as APCI would reveal different aspects of the same picture; APCI is preferred for more non-polar metabolites. ESI does not result in fragmentation of molecular ions as observed in EI mass spectrometers, so it does not allow direct metabolite identification by comparison of ESI mass spectra. However, the application of hybrid mass spectrometers (e.g., quadrupole-TOF, linear trap quadrupole (LTQ)-orbitrap and ion trap-TOF) provides both accurate mass and collision induced dissociation (CID) mass spectra, which facilitates metabolite identification. In contrast to GC-MS, retention time and CID mass spectra are not reproducible between different systems because of differences in LC column chemistries and mass spectrometer designs; hence, transferable mass spectral libraries are currently not available.

The majority of the LC-MS-based metabolomic studies employ reversed-phase liquid chromatography (RPLC) [14,19]. The use of reversed-phase columns provides efficient separation and retention of relatively nonpolar metabolites across a large molecular weight range of 50 to > 1,500 Da and includes high molecular weight lipid species and nonpolar amino acids. Polar metabolites, being mainly primary metabolites, elute in the column void or early in the chromatographic run. The latter species represent metabolite classes of high significance, such as amino acids and organic acids, which are directly involved in the normal growth, development, or reproduction of an organism and are thus important for the diagnosis of a number of critical metabolic states. The addition of hydrophilic interaction liquid chromatography (HILIC) can be an useful tool to increase the metabolome coverage [83,84]. Practically speaking, 2 options are currently state-of-the-art. Firstly, the samples can be analyzed twice in separate runs, i.e. with both RPLC and HILIC. This option is straightforward and generates 2 relatively uncomplicated datasets, which are unfortunately difficult to fuse. Secondly, an “orthogonal” combination of RPLC and HILIC can be accomplished with a 2D system, using a column switching approach. However, this requires an excellent repeatability. Applications in LC-MS metabolomics mainly focus on clinical studies, along with NMR as a complementary tool [85]. The technique has been employed in the discovery of biomarkers for a number of diseases or chronic disruptions in metabolism in both rat and human studies [53,86,87].

II.2.4.3. CE-MS

CE is particularly suited for the separation of polar and charged compounds. The orthogonality in separation principle underscores the relevance of CE as a complementary tool to the more established chromatographic techniques –in many cases, samples that cannot be easily resolved by GC or LC can be separated by CE. The use of CE-MS has increased considerably over the last few years, but is still rather limited compared to the other analytical techniques [88-91]. Poor concentration sensitivity, reduced migration time reproducibility, and lack of practical experience

most probably still hamper the wider use of CE-MS for metabolomic studies. Nevertheless, fast, highly efficient separations, without requiring rigorous sample pretreatment, can be obtained. Other advantages of CE include the very low – or even absence of – organic solvent consumption, the small amount of other reagents needed, and the use of simple fused-silica capillaries instead of expensive LC columns. For non-targeted metabolomics, the sample is often analyzed at both low and high pH separation conditions in order to cover as many metabolites as possible. An attractive feature of CE is its small sample requirement, making it particularly well-suited for samples that are volume-limited. Poor concentration sensitivity, which is often cited as a disadvantage, is less a problem when coupled to MS compared to absorbance-related detectors. Additionally, it is possible to perform facile in-capillary sample enrichment to boost sensitivity, without the need for dedicated instrumental modifications, when necessary. Over the past years significant progress has been made in the development of novel interfaces for coupling CE to MS, improving robustness [92,93]. The large number of endogenous metabolites in a biological sample makes multidimensional separation techniques (e.g. LC-CE-MS) very attractive to separate as many metabolites as possible. In conclusion, CE-MS represents a viable platform for metabolomic studies. Recently, various applications have been described in biomedical, clinical, microbial, plant, environmental and food metabolomics [94-101].

II.2.4.4. NMR

NMR spectroscopy provides a rapid, non-destructive, high-throughput method and can offer direct identification and quantification of a range of abundant analytes [61]. The technique is usually more robust and reproducible than LC-MS and can allow the utilization of libraries and interlaboratory validation studies. Its major limitations are the low sensitivity and the high initial instrument acquisition cost. Nevertheless, NMR spectroscopy, especially ^{1}H NMR, is extensively used in clinical and pharmaceutical applications for the analysis of biofluids or tissues [30,102-104].

Table II.1 summarizes the advantages and disadvantages of the different analytical techniques in the field of metabolomics.

Table II.1 Comparison of the main analytical techniques in metabolomics

Analytical technique	Advantage	Disadvantage
GC-MS	Robust, mature technique	Destructive
	Excellent reproducibility	Slow
	Good sensitivity	Requires sample derivatization
	Detects most organic and some inorganic molecules	Novel compound ID is difficult
	Large body of software and databases for metabolite ID	Many analytes thermally unstable or too large for analysis
	Modest sample size need	
	Relatively inexpensive	
LC-MS	Good sensitivity	Destructive
	Flexible technique: many modes of separation available	Slow
	Minimal sample size requirement	Poor separation resolution and reproducibility (vs. GC)
	No derivatization required (usually)	Less robust instrumentation than NMR or GC-MS
	Detects most organic and some inorganic molecules	Limited body of software and databases for metabolite ID
	Has potential for detecting largest portion of metabolome	Novel compound ID is difficult
CE-MS	High separation power	Destructive
	Small sample size requirements	Poor retention time reproducibility
	Rapid	Limited body of software and databases for metabolite ID
	Detects neutrals, anions and cations in single run	Novel compound ID is difficult
	No derivatization required (usually)	
NMR	Robust, mature technology	Low sensitivity
	High resolution	Expensive instrumentation
	No derivatization required	Cannot detect or ID salts and inorganic ions
	Non-destructive	Cannot detect non-protonated compounds
	Detects all organic classes	Requires larger (0.5 mL) samples
	Allows ID of novel chemicals	Libraries of limited use due to complex matrix
	Rapid analysis	
	Compatible with liquids and solids	

II.2.4.5. Quality assurance

In large-scale metabolomic studies, great care in the experimental design is necessary. One of the problems in these studies arises from the fact that not all samples can be analyzed in a single analytical batch because of issues such as medium to long-term instrument reproducibility and necessary preventive maintenance. In any chromatography-MS system, the sample unavoidably interacts directly with the instrument and this results in changes in measured metabolic feature response over time, both in terms of chromatography and MS. Evidently, inclusion of internal standards and mixtures of standards can help in controlling such problems. However, these offer

only limited solutions given the diversity of structures, concentrations and compound-dependent detector responses encountered when analyzing typical biofluid samples. So, along with normal good practice, such as ensuring that the samples are analyzed in a random order, it is a requirement that quality control (QC) samples are periodically analyzed throughout an analytical run in order to provide robust quality assurance (QA) for each metabolic feature detected [33]. One approach is to prepare QC samples by mixing aliquots of the batch of samples under study to generate a pooled sample. This resulting pooled QC sample should be analyzed at the beginning, end and throughout the run. The resulting QC data are examined post-run for evidence of both large changes that would signal a drastic change in the system and also more subtle monitoring (e.g. changes in peak shape, intensity, mass accuracy and retention time).

II.2.5. Data-treatment

After acquiring data by the analysis method of choice, the challenge is to convert the raw, instrumental data into extracted data (e.g., peak tables) that can easily be submitted to statistical tools. For MS-based data the data-processing pipeline usually proceeds through multiple steps, including filtering, feature detection, alignment and normalization, as depicted in Figure II.5 [105,106].

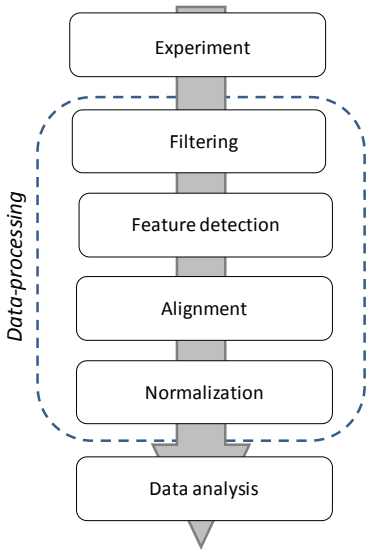

Figure II.5 Data-processing pipeline

II.2.5.1. Raw data pre-processing

Data acquisition is often performed in the centroid mode by combining the multiple m/z values corresponding to a given peak into a single data point characterized by one m/z value, the weighted centre of mass and its associated intensity. The intensity is provided by the normalized area of the peak calculated with the intensities of the associated data points. The main peak of an isotopic cluster can be identified and selected as a monoisotopic peak instead of as profile data. Centroiding

allows a better mass assignment and usually increases the data quality, but it may complicate noise level estimation in LC-MS [107] and limit available strategies for feature reduction.

Binning (data averaging) is crucial for resolution tuning and allows the proper representation and processing of the data. Raw hyphenated data are usually binned in intervals of fixed values to generate regularly sampled data. Ion intensities are summed for a given m/z and retention time interval. The data are therefore converted into a 2D array with one axis describing the retention times values and the other the m/z values. The values within the array are naturally the measured intensities for a given m /z at a given retention time. This data representation allows for easier data handling and treatment, despite a loss in m /z resolution.

II.2.5.2. Filtering

The filtering process suppresses or at least reduces random analytical noise or baseline drift in the data. Signal-processing techniques are usually implemented for noise reduction. During the baseline correction, the shape of the baseline is identified and subsequently subtracted from the data.

II.2.5.3. Feature detection

The aim of the feature detection stage is to identify all signals caused by true ions and avoid detection of false positives. Several strategies exist to distinguish real signals from noise, and the simplest one relies on an intensity threshold, with the selection of all peaks detected above a defined noise level. Either the peak area or the peak height can be chosen as a quantitative measure of the real concentration and can be further associated with the corresponding m/z and retention time information. This threshold can be set independently for both the m/z and the retention time directions using all intensity values along one direction [107,108]. The detection can be refined by constraints on the peak shapes in the chromatographic direction; data points meeting this shape criterion are defined as peaks [109]. The second approach is based on the examination of single ion chromatograms, defined by a given m/z resolution. The chromatograms are processed independently, either with a threshold level or with a Gaussian-filter to find the apex and inflection points for the area integration [110,111]. The third possibility for feature extraction is model fitting against the original raw signal, which involves isotopes detection [112,113]. Comparing the peaks among different samples is another approach for peak detection but it is dependent on a proper alignment of the chromatograms. Consequently, the true peaks are defined by intensity above a given threshold and must be present at least in a given proportion of the samples.

Although single peaks are often considered as single features when soft ionization techniques such as electrospray ionization are used, features should in general combine information from isotopic peaks of an ion, ion adducts, different charge states and also fragment ions of a compound. Isotope pattern

detection can be performed after feature detection by using pattern matching with raw data [114] or grouping detected features with suitable *m*/*z* differences [115]. Alternatively, isotope pattern detection can be already incorporated in the feature detection step by fitting a model of a generic isotope pattern to raw signal [116]. Multiple ions may correspond to different fragments from the same molecule. Deconvolution methods are therefore needed which can assign different ions to the same metabolite. These methods are often applied in GC-MS data processing [117,118]. Deconvolution algorithms commonly rely on the fact that different fragments from the same molecule have the same retention time as well as on assumption that their profiles across multiple samples are highly correlated as they are subject to the same biological variation and systematic error. A major challenge in the use of deconvolution methods is that metabolomic experiments on complex biological matrices lead to a large number of overlapping peaks, with similar retention times and overlapping isotope patterns. Moreover, several metabolites may be subject to the same regulatory mechanisms in a biological system, which makes successful deconvolution even more difficult.

II.2.5.4. Alignment

A one-to-one correspondence between the variables being compared is needed to accurately ascertain the differences in the chemical composition between samples from their chromatograms. Hence, one mandatory requirement is the proper alignment in both the m/z and the retention time dimensions in order to combine results. Shifts along the m/z axis are usually easily corrected using calibration, but changes in retention times are more problematic. Progress in chromatographic techniques has reduced these shifts but variability in the retention time of a given metabolite is still often encountered [41]. These drifts can be triggered by multiple factors, including pH, temperature changes, pump pressure fluctuations or column clogging. An alignment algorithm commonly requires a reference chromatogram to correct these retention time differences, and its choice has a great influence on the results. Moreover, numerous issues need to be tackled when performing such corrections. For example, the method must preserve the chemical selectivity between samples of distinct compositions while minimizing run-to-run shifts [119]. Additionally, corresponding peaks are expected to be shifted by less than the representative distance between adjacent peaks. Three approaches are commonly utilized to align chromatograms [120]. The first solution exists in summing or binning data along the chromatographic dimension. No loss of information is required and the errors are pushed back towards the bin boundaries. A second approach is alignment without peak detection by compressing or stretching the retention time axis of all samples to a common reference. Since there is only a minimal need for manual contribution, this approach is interesting from an automation point of view [121]. The mapping of total ion chromatograms is one of the most

investigated alignment methods, and warping methods are well-known alternatives for that purpose, allowing the stretching and shrinking of segments of the chromatograms [122]. Another way to match the corresponding peaks among samples focuses on the detection of independent signals using curve resolution. The correspondence between the detected peaks of different samples can then be assigned by the use of a time and m/z tolerance to regroup similar peaks. Another solution is to match components with high-spectral similarity in a given time window.

II.2.5.5. Normalization

The goal of normalization is to remove unwanted systematic variations between samples, while retaining the interesting biological variation [123]. Normalization is a hard task due to the great chemical diversity of metabolites, e.g. leading to discrepancies in the different recoveries during extraction or in the obtained response during ionization in MS. Two major normalization strategies for metabolic data can be differentiated. The first strategy uses statistical models to derive optimal scaling factors for each sample based on the complete dataset, such as normalization by unit norm or median of intensities, or the maximum likelihood method. The addition of a single or multiple internal or external standard(s) that cover specific regions of retention time constitutes another way to perform normalization.

Apart from normalization, centering, scaling and transformations can change the pertinence and outcome of the data analysis. Centering converts all the concentrations to fluctuations around zero instead of around the mean of the metabolite concentrations. In this way centering adjusts for differences in the offset between high and low abundant metabolites. It is therefore used to focus on the fluctuating part of the data, and leaves only the relevant variation, i.e. the variation between the samples, for analysis. Scaling methods are data pretreatment approaches that divide each variable by a factor, the scaling factor, which is different for each variable. They aim to adjust for the differences in fold differences between the different metabolites by converting the data into differences in concentration relative to the scaling factor. This often results in the inflation of small values, which can have an undesirable side effect as the influence of the measurement error, that is usually relatively large for small values, is increased as well. There are two subclasses within scaling; the first class uses a measure of the data dispersion as a scaling factor, such as the standard deviation, while the second class uses a size measure, such as the mean. Transformations, such as log or power transformation, are nonlinear conversions of the data. Transformations are generally applied to correct for heteroscedasticity (i.e. the standard deviation of each metabolite determined in replicate samples changes with the mean of the signal), to convert multiplicative relations into additive relations, and to make skewed distributions more symmetric.

II.2.5.6. Software tools for data-processing

The need for powerful data-processing methods gave rise to various commercial or freely available software tools, implementing either one specific part or combining several steps of the data-processing pipeline. Most of these tools include standard procedures of filtering, feature detection, alignment and normalization, while they generate a data matrix containing the peak intensities or areas for each detected ion among all the samples. Table II.2 lists several commercial and freely available software tools with their features. This is by no means a complete overview of the available software. For a more extensive list of metabolomics data-processing software the reader is referred to the webpage of Metabolomics Fiehn Lab [134]. In addition, comparisons of software performance have been published [135-137].

Table II.2 Software tools for metabolomics data-processing

Commercial software		
Name	**Features**	**Vendor**
BlueFuse	Filtering, peak detection, and alignment of MS and NMR data. Univariate and multivariate methods for data analysis	BlueGnome, Cambridge, UK
Genedata Expressionist	Filtering, peak extraction, *m*/*z* and retention time alignment of MS data. Metabolite identification using third-party databases. Includes also analysis and interpretation modules and integrated database	Genedata, Basel, Switzerland
LineUp	Alignment of chromatographic data and spectroscopic data	Infometrix, Bothell, WA, USA
MarkerLynx	Peak detection and alignment of LC-MS data. Principal component analysis (PCA)	Waters, Milford, MA, USA
MarkerView	Peak detection and alignment of LC-MS data. PCA and t-test methods for data analysis. Visualization and reporting	Applied Biosystems, Foster City, CA, USA
MassProfiler Professional	Alignment data and statistical analysis of LC-MS data	Agilent Technologies, Santa Clara, CA, USA
Metabolic Profiler	Bucket raw spectroscopic data into retention time, *m*/*z* table with intensities. Identification using libraries. PCA and PLS for data analysis	Bruker Daltonic & Bruker BioSpin, Billerica, MA, USA
Rosetta Elucidator	Peak detection and alignment, statistical analysis and visualization of LC-MS data	Rosetta Biosoftware, Seattle, WA, USA
Sieve	Differential expression analysis with alignment for LC-MS datasets	Thermo Fisher Scientific, Waltham, MA, USA
Freely available software		
Name	**Features**	**Reference**
apLCMS	Noise filtering, feature identification, retention time correction, m/z feature alignment across multiple spectra, and re-analysis to capture features originally missed because of weak signal relative to the signal to noise filter of LC-MS data	[124]
MathDAMP	Direct comparison of raw LC-MS, GC-MS and CE-MS datasets without peak picking. Includes methods for preprocessing (binning, baseline subtraction, smoothing) and normalization	[125]
MetaboAnalyst	A complete web-based pipeline for processing of GC-MS, LC-MS and NMR data, statistical analysis and functional interpretation	[126]
metAlign	Filtering, baseline correction, peak detection, alignment of GC-MS and LC-MS data	[127]
MET-IDEA	Extracts ion intensity data from LC–MS, GC–MS and CE–MS for listed ion/retention time values from multiple runs	[128]
MSFACTs	Alignment and comparison of raw chromatograms or peak lists generated with a third-party software	[129]
msInspect	Peak detection, alignment, normalization and visualization of LC-MS data	[130]
Mzmine 2	Noise filtering, peak detection, alignment, normalization, visualization, statistical analysis and peak identification of LC-MS data	[131]
XCMS	Noise filtering, peak detection and alignment of LC-MS and GC-MS data	[109]
XCMS Online	Complete metabolomics workflow for LC-MS data including feature detection, retention time correction, alignment, annotation and statistical analysis	[132]
xMSanalyzer	Automated pipeline for improved feature detection and downstream analysis of LC-MS data integrating with existing packages such as apLCMS and XCMS	[133]

II.2.6. Statistical analysis

Once a data matrix has been extracted from the raw data, subsequent steps involve different forms of statistical analysis and data mining to allow the identification of variables (metabolites) that capture the bulk of variation between datasets and that may represent candidates for biologically meaningful variables [138-142]. Data analysis of metabolomic data typically consists of two phases; multivariate analyses such as principal component analysis (PCA) are generally proposed for display and exploratory purposes, whereas univariate statistical tests such as the Student's t-test are used to discern the relevant variables.

II.2.6.1. Univariate analysis

Univariate significance tests such as the Student's t-test and one-way analysis of variance can be used to find statistical differences between samples of distinct classes. The predictive power of each variable is evaluated by finding statistically significant differences between the mean intensity values of a given signal with the calculated p-value as a straightforward indicator. However, the use of these procedures is rather limited when dealing with thousands of highly correlated variables. False positives are likely to occur when performing multiple comparisons. Methods such as the Bonferroni and the Benjamini-Hochberg corrections have been introduced to address this problem [143].

II.2.6.2. Multivariate analysis

As previously mentioned, metabolomic data are essentially multivariate. The multivariate statistical analyses can be divided into unsupervised and supervised methods. The most widely applied unsupervised and supervised methods, i.e. principal component analysis (PCA) and partial least squares (PLS) respectively, are discussed in more detail.

a) Exploratory analysis by unsupervised learning

Unsupervised methods attempt to analyze a set of observations without measuring any related outcome. Since there is no specified class label or response, the dataset is considered as a collection of analogous objects. Unsupervised learning uses procedures that attempt to find the natural partitions of patterns to facilitate the understanding of the relationship between the samples and to highlight the variables that are responsible for these relationships. By providing means for visualization, unsupervised learning is of help in the discovery of unknown but meaningful categories of samples or variables that naturally fall together. The success of such approaches is frequently evaluated subjectively by the interpretability and usefulness of the results with respect to a given problem. Examples of unsupervised methods are principal component analysis (PCA), hierarchical cluster analysis (HCA) and self-organizing maps (SOM).

Principal Component Analysis

PCA is an unsupervised method that can be considered as the starting point of multivariate data analysis. PCA converts high-dimensional data into fewer dimensions, by projecting the data into a reduced dimensional subspace, while maintaining as much variance from the original data as possible. The procedure is repeated until the datasets can be presented usually within two or three dimensions. This simplifies visual inspection of the distributed samples in principal component space, using score plots. The degree of systematic variation in metabolite profiles among samples is reflected by the Euclidian distance between individual samples in score plots. Loading plots show the contribution of individual metabolites to each principal component. The vast majority of metabolomic studies uses PCA as a first exploratory step [53,76,79,95]. The principle of PCA is depicted in Figure II.6.

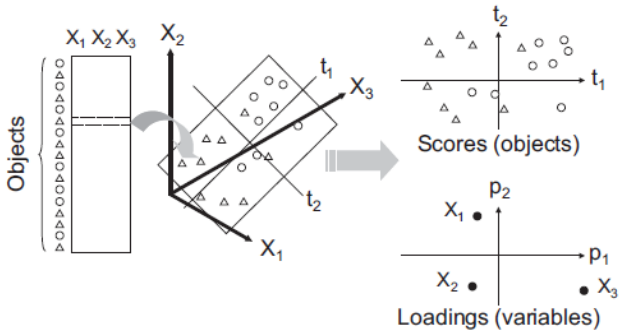

Figure II.6 principle of principal component analysis

b) Classification by supervised learning

In supervised learning methods each object is considered with respect to an observed response. Regression and classification problems are tackled depending on the output type under consideration, i.e. a numerical value in the first case and a class label in the second. Hence, classification aims to produce general hypotheses based on a training set of examples that are described by several variables and identified by known labels corresponding to the class information. The task is to learn the mapping from the former to the latter and numerous techniques, based either on statistics or on artificial intelligence, have been developed for that purpose, such as partial least squares (PLS), partial least squares discriminant analysis (PLS-DA), orthogonal projection to latent structures discriminant analysis (OPLS-DA), linear discriminant analysis (LDA), artificial neural networks (ANN), and support vector machines (SVM).

Partial least squares

Partial least squares (PLS) is a regression-based method, which builds a low-dimensional sub-space based on linear combinations of the original X variables. Additional Y information is used by adjusting the model to capture the (Y)-related variation into the original X variables. PLS-based classification

and PLS-discriminant analysis (PLS-DA) have widely been applied to sharpen the separation between groups or observations. This is realized by rotating principal components to maximize the separation between known classes, and to elucidate the variables that carry the class separating information. Similar to loading plots in PCA, S-plots visualize both the covariance and the correlation between metabolites and the modeled class designation. Consequently, the S-plot helps to identify statistically significant and potentially biochemically significant metabolites, based both on contributions to the model and their reliability. Despite its powerful ability to separate classes, care must be taken during fitting of PLS-DA to the training datasets, which exaggerate generalization ability. The ability of the trained PLS-DA model is usually assessed by cross-validation or permutation tests. Orthogonal projections to latent structures (OPLS)-DA is an extension of PLS-DA featuring an integrated orthogonal signal correction filter to remove variability not relevant to class separation, which results in a better robustness against noise.

An overview of the data analysis methods which are frequently employed in disease diagnosis and biomarker discovery studies is given in Table II.3 [138].

Table II.3 Data analysis methods frequently employed for disease diagnosis and biomarker discovery

Technique	Typical use	Description and comments
Univariate testing	Identification of potential biomarkers	Univariate analysis where the corresponding *p*-values are the standard scientific measure of significance. Multiple testing is prone to false positives unless corrected significance limit is used
Principal component analysis (PCA)	Data overview	Standard multivariate analysis method to provide an overview of a large dataset. Useful for identifying outliers, clusters and trends in the data. Is not a classification method
Hierarchical cluster analysis (HCA)	Clustering method	Cluster analysis that groups together observations that are similar to one another by producing a hierarchical structure dividing the instance space
Linear discriminant analysis (LDA)	Classification and biomarker identification	Discrimination method related to multiple linear regression. Number of variables must be smaller than the number of observations
Partial least squares discriminant analysis (PLS-DA)	Classification and biomarker identification	Classification variant of PLS. Works best with homogeneous classes. Biomarker identification possible, but not as straight forward as with OPLS-DA
Orthogonal projection to latent structures discriminant analysis (OPLS-DA)	Classification and biomarker identification	Classification variant of OPLS method. Ability to separate between-class from within-class variability. Straightforward interpretation and identification of potential biomarkers
Artificial neural networks (ANN)	Classification (non-linear)	High flexibility in modeling non-linear data, but prone for overfitting. Potential biomarker identification more complicated
Self organizing maps (SOM)	Clustering method	A neural network based method for identifying trends and clusters in the data. Can account for non-linearities in the data
Support vector machines (SVM)	Classification (non-linear)	High flexibility in modeling non-linearities. Careful model selection reduces possibility of overfitting. Potential biomarker identification more complex compared to linear methods

II.2.7. Metabolite identification

The process of metabolite identification in MS-based non-targeted metabolomic studies is a major bottleneck in deriving biological knowledge from data [144]. When mass spectrometry is applied to pure chemicals or relatively simple mixtures, it offers a range of powerful tools for characterization, structural elucidation, and identification of compounds. These tools comprise the accurate measurement of the mass-to-charge ratio (m/z) of molecular, fragment and associated ions; the determination of relative isotopic abundances (e.g., the relative abundance of ^{12}C and ^{13}C isotopomers) of molecular and fragment ions; fragmentation of molecular and fragment ions to define dissociation patterns related to chemical structure; and the comparison of experimental data to either databases containing physicochemical properties (e.g., molecular formulas and monoisotopic masses) or mass spectral libraries containing experimentally acquired chromatographic (e.g., retention times or retention indices) and mass spectrometry data (e.g., fragmentation mass spectra). However, the challenge of metabolite identification is considerable in non-targeted metabolomic studies as samples are complex and contain hundreds or thousands of chemical species, depending on the biological system and sample type under research. Human biofluids contain endogenous metabolites as well as exogenous metabolites derived from diet [145], gut microflora [146], pharmaceuticals [147], lifestyle and physical activity [148]. The knowledge on the total composition of many metabolomes is currently limited. Databases contain large lists of expected metabolites in different organisms, based on experimental, genomic and/or bibliographic data, but these lists are far from complete. Moreover, as already mentioned, the physicochemical diversity of the metabolome is significantly greater than that of the proteome, impeding generally applicable identification strategies. In addition, metabolites with the same nominal and monoisotopic mass, but different structures should be discriminated. Since single metabolites are often detected as multiple different derived species in MS, correct assignment to the 'parent' metabolite is essential. For instance, in GC-MS chemical derivatization with TMS reagents, can lead to the detection of amino acids with 1, 2 or 3 TMS groups [149]. This illustrates that the identification challenge is immense and that confident unambiguous identification is not always achievable. Four levels of metabolite identification confidence have been defined by the Chemical Analysis Working Group of the Metabolomics Standards Initiative (MSI) [150]:

1. Confidently identified compounds (i.e. comparison of two or more orthogonal properties with an authentic chemical standard analyzed under identical analytical conditions)
2. Putatively annotated compounds (i.e. without chemical reference standards, based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries).

3. Putatively characterized compound classes (i.e. based upon characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class).
4. Unknown compounds (although unidentified or unclassified these metabolites can still be differentiated and quantified based upon spectral data).

As mentioned in section II.2.4, there are some differences in metabolite identification in GC-MS and in LC-MS (/CE-MS).

II.2.7.1. Metabolite identification in GC-MS

Most of the GC-MS and the GCxGC-MS metabolic platforms operate with EI ionization, which is a highly reproducible process. The retention time/index and the EI-derived mass spectrum for each detected feature are typically used for identification purposes. Although widely applied, one limitation of EI mass spectra for metabolite identification, especially with TMS-derivatization, is the low abundance and sometimes absence of the molecular ion. Defining the mass of the molecular ion is of importance in de novo metabolite identification to assist in search for the molecular formula. The EI mass spectrum can be compared to mass spectra obtained from chemical standards accessible in commercially or freely available mass spectral libraries to facilitate identification. Several libraries, some of which are listed in Table II.4, are available containing either a generalized collection of chemicals (e.g. NIST) or only metabolites (e.g. Golm Metabolome Database [151]). Fragmentation patterns depend on the derivatization; most metabolite-specific libraries contain information on TMS-derivatized metabolites. As the EI ionization and fragmentation are highly reproducible processes across different GC-MS platforms, they can provide putative annotations (level 2). The mass spectra of two different metabolites are in some cases very similar. Although identification of the metabolite is then not possible at level 2, classification to metabolite class is possible (level 3). Monosaccharides and disaccharides are common examples. To obtain confident identification (level 1), the information obtained from EI mass spectra is combined with comparison of retention times or even better with retention indices. Retention indices are normalized retention times, taking into account differences in column length, film thickness, internal diameter, flow rate of the carrier gas, and oven temperature ramp. Many mass spectral libraries contain retention index data.

Table II.4 Databases for metabolite identification

Name	Content	Web address
Fiehn GC-MS Database	GC-MS spectra and retention indices	fiehnlab.ucdavis.edu/Metabolite-Library-2007
Golm Metabolome Database (GMD)	GC-MS spectra, retention time, experimental methods and protocols	gmd.mpimp-golm.mpg.de
Human Metabolome Database	Metabolites found in the human body, NMR, GC-MS and MS/MS data, physical, clinical and physicochemical properties, chemical structure, concentration data, pathway information, experimental methods	www.hmdb.ca
Madison Metabolomic Consortium Database	NMR and LC-MS spectra, physical properties, links to chemical and metabolomic databases, chemical structure	mmcd.nmrfam.wisc.edu
MassBank	GC, ESI, and fast atom bombardment (FAB)-MS, MS/MS spectra, chemical structure and name, separation method, experimental conditions	www.massbank.jp
MetaboLights Database	cross-species, cross-technique database with metabolite structures and reference spectra, biological roles, locations, concentrations and experimental data from metabolic experiments	www.ebi.ac.uk/metabolights/index
METLIN	LC-MS, LC-MS/MS, fourier transform (FT)-MS spectra, chemical structures, retention time, link to Kyoto Encyclopedia of Genes and Genomes (KEGG), chemical formula	metlin.scripps.edu
NIST Spectral Database	EI-MS and MS/MS spectra, retention time	webbook.nist.gov/chemistry

II.2.7.2. Metabolite identification in LC-MS and CE-MS

a) The use of accurate m/z measurements to define molecular formula and to search electronic resources

Accurate m/z measurement is often the first process applied in the chemical identification of metabolic features detected in LC-MS and CE-MS data sets. As mentioned above, ESI sources are typically utilized, although other ion sources, such as APCI are sometimes used. A metabolic feature can be matched to a single molecular formula or a small number of these based on measurements of m/z. The accuracy of this measurement determines the number of molecular formula matches; the greater the accuracy the lower the number of matches. Mass spectrometers applied to this task, such as TOF and Fourier Transform-based instruments have to operate at high mass resolution and mass accuracy. The molecular formulas are matched to metabolites via an on-line database search. A single molecular formula can correspond to multiple known metabolites. The application of accurate measurements of m/z is therefore an appropriate first step, but only provides putative annotation (level 2 or 3) requiring further verification. The reduction in the number of potential molecular formulas is achieved by the matching of experimentally derived m/z information to the equivalent m/z of specific molecular formulas. The efficiency of this reduction process depends on the resolution and accuracy of the mass spectrometer. The majority of mass spectrometers operate with mass resolutions of 5,000-50,000, which allows the resolution of metabolites with the same nominal

mass but different monoisotopic masses. As mass resolution increases, the ability to resolve ions of the same nominal mass but different monoisotopic mass raises. Even with high mass resolution, high mass accuracy is not necessarily achieved and appropriate mass calibration is required to provide high mass accuracy in these measurements [152]. As mass accuracy increases, the mass error range decreases and the number of proposed molecular formulas decreases. When m/z increases, the number of possible molecular formulas matching a given mass for a defined mass error will also increase [153]. In this process, unambiguous determination of a single molecular formula is not always achievable even with high mass resolution and the achievement of sub-ppm mass accuracies [153].

In LC-MS and CE-MS thousands of metabolic features are detected, defined as m/z–RT pairs. Significant complexity is entwined in these data. A single metabolite is typically (but not always) detected as multiple metabolic features, each having the same RT but a different m/z. The different m/z values relate to different derivative ions of the same metabolite. These can include protonated and deprotonated ions, adducts, fragments, isotopomers, dimers, and multiply charged ions. The derivative ions depend on the chemical properties of the metabolite as well as on the sample matrix, solvents, metabolite concentrations, and mass spectrometry platform and parameters. The complexity of ESI data can lead to a large number of false positive identifications, especially when derivative ions are falsely identified. The automated matching of metabolic features deriving from the same metabolite (including the automated determination of the ion type) has recently been applied in metabolite identification. Metabolic features derived from the same metabolite are identified, annotated, and grouped together using accurate m/z, m/z differences, RT similarity, pairwise correlation between measured responses, known adduct lists and chromatographic peak shape similarity. Metabolite identification for one of the metabolic features can then be linked to all other derived metabolic features for that metabolite. The freely available software platforms developed for this purpose include PUTMEDID-LCMS [154], CAMERA [155], PeakML/mzMatch [156] and IDEOM [157].

Following the identification of single or multiple molecular formula, the matching of these molecular formulas to known metabolites is performed, typically by searching an array of online or laboratory-specific resources, such as HMDB (Table II.3).

b) Application of chemical, biological and other experimentally derived MS data

As discussed above, the majority of m/z measurements in a single complex biological mass spectrum cannot be assigned to a single molecular formula based on accurate measurements of m/z only [153]. A range of bioinformatics approaches have been developed in the past few years to exploit relationships between signals in high-mass resolution mass spectra. These include peak-pair m/z

differences (for example, peak m/z patterns and prior biological knowledge) and peak-pair intensity ratios (for example, isotope abundance ratios and peak area correlations) as tools to reduce the number of putative molecular formulas or metabolite assignments for a single metabolic feature and thus aid in its identification. Other tools developed include ionization behavior rules, applying chemical knowledge of metabolites, which can be used to determine the probability of the formation of specific derivative ions for a metabolite and to eliminate metabolites with chemically infeasible ion types [158].

The majority of the bioinformatics approaches and methods focus primarily on the two principal variables measured in a typical MS experiment, i.e. m/z and signal intensity. MS analysis is usually preceded by chromatographic or electrophoretic separation of complex biological mixtures. The RT or migration time is predictive of metabolite structure, primarily hydrophobicity, hydrophilicity and/or charge for LC-MS, and charge and cross-sectional diameter for CE-MS. For example, in reversed-phase LC, hydrophilic metabolites elute at earlier RTs compared to hydrophobic metabolites. In GC-MS, RTs or indices are routinely applied across different platforms to aid identification by comparison of experimental data to mass spectral libraries, but in LC-MS retention behaviors are far less reproducible and their use for identification much more restricted; changes in LC column phase (for example, reversed-phase compared to HILIC), the manufacturer of columns of the same stationary phase, solvents and gradient elution conditions influence the RT significantly in many cases. Therefore, although LC-MS focused mass spectral libraries or databases are available (for example, METLIN and MassBank, Table II.3), comparison of RT data is only applicable for data acquired with the same analytical method on the same equipment and columns. Mass spectral libraries applying RT data are not as readily transferable as is observed for GC-MS applications.

c) Application of experimentally derived MS/MS and MS^n data

The processes that have been described to reduce large search space of molecular formulas down to a single or small set of metabolites (or molecular formulas) are successful in reducing the search space size but do not necessarily lead to unambiguous (level 1) identifications. Multiple possible metabolites are often reported for a single metabolic feature, in particular for stereoisomers. To provide further data for structural elucidation or to aid in de novo structure elucidation, fragmentation of molecular ions is applied.

CID leads to an activation of ions by increasing their internal energy and the subsequent loss of internal energy through the fission of covalent bonds. Weaker bonds are more likely to break. Hence, the type and strength of different covalent bonds in a metabolite will lead to a specific structure-defined fragmentation pattern. The resulting fragments can either retain the ion charge (and therefore be detected by mass spectrometers) or be neutral species (not directly detectable). In

tandem mass spectrometry the process is two-stage and provides a fragmentation mass spectrum for a chosen ion (defined as the precursor or parent ion). This is known as MS/MS or MS^2. In certain cases, when ion trap systems are used, additional levels of fragmentation can be applied, defined as multi-stage mass spectrometry or MS^n, where n is the number of successive fragmentation experiments. The processes of MS/MS and MS^n can be important when attempting to discriminate metabolites of the same molecular formula and similar chemical structure where, for instance, the type or position of a fatty acid is the only difference between two different metabolites. MS/MS and MS^n data can be compared to data available in mass spectral libraries. MS^n experiments can also be applied to deduce valid molecular formulas.

Table II.3 is by no means a complete overview of the existing databases for metabolite identification. For a more extensive overview the reader is referred to the database webpage of the metabolomics society [159], [160] and [161].

Additionally, *in silico* fragmentation software tools are available for LC-MS to enable the matching of *in silico* derived mass spectra (instead of mass spectra derived from authentic chemical standards) to the experimentally derived mass spectra [162-164].

II.2.8. Biological interpretation

In addition to the crucial step of metabolite identification, the set of metabolites has to be analyzed and put in a biological context. Therefore, it is essential to understand and interpret metabolite data in terms of the underlying biochemical mechanisms, their phenotypic and physiological consequences. Information on cell metabolism has traditionally been represented in pathways. These pathways are graphical representations of the relationships among enzymes, metabolites and catalyzed reactions. More recently, similar graph-based representations are being used to describe different types of biological networks, such as signaling pathways and protein-protein interactions. An obvious step in the interpretation of metabolic experiments is to map and visualize the identified metabolites and associated experimental measurements in the context of metabolic pathways and other general biological networks. Several software tools are available for pathway mapping and visualization [165], as listed in Table II.5.

Table II.5 Pathway mapping and visualization software

Name	Content	Web address
BioCyc	Pathway and genome database of organisms with completely or partially sequenced genome, database of chemical compounds, links to other databases	www.biocyc.org
HumanCyc	Human metabolic pathways, genes, metabolic enzymes, links to other databases	www.humancyc.org
KEGG	Metabolic and regulatory pathways, protein–protein interactions, genes of completely and partially sequenced genome, drugs, glycans, small molecules, reactions, functional hierarchies	www.genome.jp
MetaCyc	Metabolic pathways of organisms determined from experiments, enzymes, genes, chemical compounds, biochemical reactions, links to other databases	www.metacyc.org
Metscape	Plug-in for Cytoscape, visualization and management of biological networks, representation of the whole metabolism, in contrast to pathway-based representation	metsape.ncibi.org
Reactome	Human metabolic pathways, reactions, proteins, links to other databases	www.reactome.org

II.3. Metabolomics in CKD

The development of metabolomics technologies holds the promise to significantly impact on patient management by improving diagnosis, unravelling more appropriate therapeutic targets and enabling to predict disease development and to define more precisely prognosis. Although several reviews have been published on the contribution of metabolomics to CKD research [166-172], the recent explosion in metabolomic studies in this area renders a new overview useful. Additional potential biomarkers were found, which were not included in previous reviews. On the one hand, animal models in the search for new biomarkers of CKD will shortly be discussed here and, on the other hand, clinical studies will be treated in more detail.

III.3.1. Metabolomics in animal model research

In the search for new biomarkers of CKD, experimental studies have been performed in rodent CKD models on different biological samples by GC-MS [173-175], LC-MS [176-188], CE-MS [189,190] and NMR [191-193]. Table II.6 summarizes the sample type, species and population, analytical technique used and most importantly the candidate biomarkers that were revealed. We excluded metabolomic studies on specific rare or acute toxic kidney failure such as those induced by morning glory seed, cisplatin, melamine and cyanuric acid.

Recently, Zhao et al. conducted several studies on adenine-induced CKD rats [180-188]. The adenine-induced CKD model has the advantage of being closer to the development of chronic kidney disease in comparison to genetic models [194]. Perturbations were found in among others the phospholipid, amino acid and creatinine metabolism as displayed in Table II.5.

Apart from the search for new CKD biomarkers, some studies investigated the renoprotective effects of therapeutic agents such as fosinopril [180], poria cocos epidermis (fu-ling-pi, FLP) [181,186-187], ergosta-4,6,8(14),22-tetraen-3-one (ergone) [183,184,196], Zhibai Dihuang Pill (ZDP) [193], and

cordyceps sinensis [194] and significant metabolic changes were found when renoprotection was established. Furthermore, metabolomic studies were performed in the search for indicators of the therapeutic effect of an oral sorbent, AST-120, which is shown to delay CKD progression. Kikuchi et al. found indoxylsulfate to be the best indicator of this therapeutic effect, while hippuric acid, phenyl sulfate and 4-ethylphenyl were suggested as additional indicators [178]. Akiyama et al. revealed 6 anions and 17 cations that were significantly decreased and 2 cations that were significantly increased by AST-120 [189]. Among them, 4 anions, apart from indoxylsulfate and hippurate, and 19 cations were newly identified. A metabolomic study on plasma and urine from wild-type and organic anion transporter-1 (Oat1/Slc22a6) knockout mice identified a number of physiologically important metabolites, including several not previously linked to Oat1-mediated transport [197]. Oat1 is expressed on the brush border and basolateral membrane of proximal tubular cells of the kidney and plays as a major role as a drug transporter in the rate-limiting step of excretion of toxins and metabolites from the body into urine. In this study indoxylsulfate, pantothenic acid, 4-pyridoxic acid, uric acid, and metabolites involved in the tryptophan and nucleoside pathways were identified as discriminators between OAT knock-out and wild type mice. Toyohara et al. demonstrated that overexpression of human kidney-specific organic anion transporter SLCO4C1 in rat kidney decreased plasma levels of the uremic toxins guanidino succinate, asymmetric dimethylarginine, and the newly identified trans-aconitate and that it reduced hypertension, cardiomegaly, and inflammation in the setting of renal failure [198].

From Table II.6 we can conclude that the application of metabolomics in rodent CKD models has rapidly increased over the last five years. However, species differences between rats and humans have been detected, which suggests careful interpretation while extrapolating data from rodent experiments into humans. In humans for example, uric acid is the final oxidation product of purine metabolism, while in rat uric acid is further oxidized to allantoin through the action of the enzyme uricase. Therefore, this overview mainly focuses on clinical studies.

Table II.6 CKD metabolomics in animal model research

Analytical Technique	Sample type	Species, population	Candidate biomarkers	Remarks	Reference
GC-MS (methoximation + trimethylsilylation)	rat urine	6 LPK rats and 7 normal Lewis rats (male)	2-ketoglutaric acid, uric acid, allantoin, carbohydrate, lactose, threitol, glucose, phosphoric acid, hippuric acid, D-pinitol	PKD	Abbis et al. 2012 [173]
GC-TOF MS (methoximation + trimethylsilylation)	mice serum and urine	40 *C57BL/Ks db/db* mice and 40 *db/m* mice (male)	perturbations in the TCA cycle (TCA, citrate, malate, succinate, aconitate), lipid metabolism, glycolysis, and amino acid turnover	DN	Li et al. 2013 [174]
GC-TOF MS (methoximation + trimethylsilylation)	mice urine	urine from 10, 9, 10 female *jck* mice and 9, 10, 9 wild-type mice from each of the 3 time points: 26, 45, and 64 days of age, respectively	7 pathways, pentose phosphate pathway, glucosinolate biosynthesis, amino, sugar and nucleotide sugar metabolism, purine metabolism, pentose and glucuronate interconversions, galactose metabolism, tryptophan metabolism	PKD	Taylor et al. 2010 [175]
LC-QTRAP	mice plasma and kidney tissue	9 female Balb/c mice in the model group (injected with SEB) and 5 female Balb/c control mice	PS C18:0/C18:0, PS C18:0/C22:5, PI C18:0/C20:4 ↑	IgA nephropathy	Jia et al. 2006 [176]
LC-IT-TOF	rat serum	14 male SD rats with CRF induced by 5/6-nephrectomy and 13 age-matched normal male SD rats*	indoxylsulfate, phenylsulfate, hippuric acid, p-cresylsulfate ↑		Kikuchi et al. 2010 [177]
LC-IT-TOF	rat serum	7 rats with CRF induced by 5/6-nephrectomy with comparison before and after AST-120 administration and 7 healthy control rats (male SD)	indoxylsulfate, phenylsulfate, hippuric acid, 4-ethylphenylsulfate, p-cresylsulfate ↑	uremic toxins as indicator of the effect of an oral sorbent AST-120	Kikuchi et al. 2010 [178]
LC-IT-TOF MS	rat plasma	10 rats with adenine-induced CKD and 5 healthy control rats (male SD)	N6-succinyl adenosine, lysoPE 20:4, glycocholic acid, indoxylsulfate, phenylsulfate, p-cresylsulfate, phenylacetylglycine ↑ tryptophan ↓		Kobayashi et al. 2013 [179]
GC-TOF MS (methoximation + trimethylsilylation) and UPLC-TOF MS	renal cortex tissue	12 rats with streptozocin-induced DKD, 15 rats with streptozocin-induced DKD treated with fosinopril and 16 healthy control rats (male Wistar rats)	39 metabolites by GC-MS (20 up and 19 down) and 26 metabolites by LC-MS (19 up and 7 down) including hippurate, indoxylsulfate, 4-pyridoxic acid, 2-phenylethanol glucuronide, 2,8-dihydroxyquinoline-beta-D-glucuronide/3-indole carboxylic acid glucuronide, 6-hydroxy-5-methoxyindole glucuronide/5-hydroxy-6-methoxyindole glucuronide, D-fructose, D-sorbitol, maltose, allantoin, glycocholic acid, cholic acid/other isomers ↑	DKD, effects of fosinopril treatment	Zhao et al. 2012 [180]

UPLC-Q-TOF MS	rat serum	21 rats with adenine-induced CRF and 21 healthy control rats (male SD)	PC(16:0/18:2), creatinine, lysoPC(18:1), lysoPC(17:0), lysoPC(16:0), m/z 274.2745, and m/z 355.2634 ↑; dihydrosphingosine, tryptophan, ceramides(18:0/16:0), ceramides(18:0/14:0), L-acetylcarnitine, phytosphingosine ↓		Zhao et al. 2012 [181]
UPLC-Q-TOF MS	rat urine	12 rats with adenine-induced CRF and 12 healthy control rats (male SD)	phytosphingosine, adrenosterone, tryptophan, 2,8-dihydroxyadenine, creatinine, dihydrosphingosine, and m/z 144.1021 ↑; N-acetylleucine, 3-O-methyldopa, ethyl-N2-acetyl-L-argininate, dopamine, phenylalanine, kynurenic acid ↓		Zhao et al. 2012 [182]
UPLC-Q-TOF MS	rat serum	12 rats with adenine-induced CRF, 12 rats with adenine-induced CRF treated with ergone and 12 healthy control rats (male SD)	adenine, m/z 202.0079, PC(16:0/18:2), dopamine, creatinine, aspartic acid, N-acetyl-L-aspartic acid, phenylalanine, m/z 266.0518, lysoPC(18:1) ↑; lysoPC(16:1), lysoPC(18:4), lysoPC(16:0) ↓	therapeutic effects of ergone	Zhao et al. 2012 [183]
UPLC-Q-TOF MS	rat feces	12 rats with adenine-induced CRF and 12 healthy control rats (male SD)	chenodeoxycholic acid, palmitic acid, adenine, phytosphingosine, MG(24:1/0:0/0:0), 12-hydroxy-3-oxocholadienic acid, lysoPE(18:2/0:0), lysoPE(16:0/0:0)↑; chenodeoxycholic acid fragment, 7-ketolithocholic acid ↓	therapeutic effects of ergone	Zhao et al. 2013 [184]
UPLC-Q-TOF MS	rat kidney tissue	12 rats with adenine-induced CRF and 12 healthy control rats (male SD)	docosapentaenoic acid, adrenic acid, p-cresolsulfate, phenylacetylglycine, m/z 359.2941, allantoin, 4-hydroxybenzenesulfonic acid, indoxylsulfate ↑; docosahexaenoic acid, eicosapentaenoic acid, arachidonic acid, linoleic acid, xanthine ↓	TGF-β1 mechanism of tubulointerstitial fibrosis	Zhao et al. 2013 [185]
UPLC-Q-TOF MS	rat kidney tissue	8 rats with adenine-induced CKD, 8 rats with adenine-induced CKD treated with FLP and 8 control rats (male SD)	adrenic acid, indoxylsulfate, lysoPC(15:0), lysoPE(20:0/0:0), hippuric acid, uric acid, p-cresolsulfate, allantoin, tetracosahexaenoic acid ↑; eicosapentaenoic acid, xanthine, docosahexaenoic acid, lysoPC(20:4), lysoPC(18:2), arachidonic acid, docosapentaenoic acid, hypoxanthine, inosine, palmitic acid ↓	renoprotective effects of FLP	Zhao et al. 2013 [186]
UPLC-Q-TOF MS	rat urine	8 rats with adenine-induced CKD, 8 rats with adenine-induced CKD treated with FLP and 8 control rats	adenine, 2,8-dihydroxyadenine, L-acetylcarnitine, 8-hydroxyadenine, hypoxanthine, creatine, methionine, phytosphingosine ↑; indole-3-carboxylic acid, 3-methyldioxyindole, ethyl-N2-acetyl-L-argininate, 3-O-methyldopa, xanthurenic acid, uric acid, phenylalanine ↓	renoprotective effects of FLP	Zhao et al. 2013 [187]
UPLC-Q-TOF MS	rat serum	8 rats with adenine-induced CKD, 8 rats with adenine-induced CKD treated with FLP and 8 control rats	lysoPC(18:0), tetracosahexaenoic acid, prostaglandin PGE2 glyceryl ester, lysoPC(18:2), creatinine, lysoPE(22:0/0:0) ↑; palmitic acid, phytosphingosine, PC(16:0/18:2), tryptophan, lysoPC(20:4), lysoPC(16:1), lysoPC(16:0), valine ↓	renoprotective effects of FLP	Zhao et al. 2013 [188]

CE-TOF MS	rat plasma	13 rats with CRF induced by 5/6-nephrectomy, 12 rats with CRF induced by 5/6-nephrectomy treated with AST-120 and 3 sham-operated rats (Wistar rats)	16 anionic compounds (4-oxopentanoate, hippurate, N-acetylneuraminate, 4-pyridoxate) and 20 cationic compounds (glycine, gamma-guanidinobutyrate, N-gamma-ethylglutamine, allantoin, cytosine, 5-methylcytosine, imidazole-4-acetate) ↑; 2 cationic compounds (tryptophan) ↓	effect of AST-120 treatment	Akiyama et al. 2012 [189]
CE-TOF MS	rat plasma	5 heterozygous (Cy/+) and 5 normal littermate control (+/+) Han:SPRD rats	22 cations and 19 anions accumulated significantly, cationic compounds: γ-guanidino butyric acid, creatinine, anthranilate, 3-methylhistidine, allantoin, TMNO, pipecolate, 5-methyl-2'-deoxycytidine, homoarginine, α-aminoadipate, hydroxyproline, glucosamine, citrulline, ectoine, methionine sulfoxide, glycine, ADMA, N,N-dimethylglycine, guanidinosuccinic acid ↑; 2'-deoxycytidine, carnitine, o-acetylcarnitine ↓ ;anionic compounds: hippurate, allantoate, citrate, α-hydroxybenzoate, cis-aconitate, iso-citrate, phenaceturate, 3-phenylpropionate, 3-indoxylsulfate, trans-aconitate, isethionate, pantothenate, pimelate ↑; decanoate, 2-oxoglutarate, octanoate, 2-oxoisopentanoate, 4-methyl-2-oxopentanoate, 10-hydroxydecanoate ↓	ADPKD	Toyohara et al. 2011 [190]
1H-NMR	rat urine	15 rats with streptozotocin-induced DN and 5 healthy control rats (male Wistar)	differentiation was achieved, but except for glucoses no identification was performed		Nemoto et al. 2010 [191]
1H-NMR	rat urine and kidney tissue	6 rats with streptozotocin-induced DN (2 weeks), 6 rats with streptozotocin-induced DN (8 weeks) and 6 healthy control rats (male SD)	urine: lactate, succinate, citrate, allantoin, hippurate ↑; alanine, dimethylamine, creatine ↓; tissue: 3-hydroxybutyrate, lactate ↑; alanine, glutamate, glycerophosphocholine, succinate, myo-inositol, creatine ↓	DN	Zhao et al. 2011 [192]
1H-NMR	rat urine, serum and kidney tissue	9 rats with streptozotocin-induced DN, 9 rats with streptozotocin-induced DN treated with ZDP and healthy control rats (male SD)	VDL/LDL, 3-hydrobutyrate, leucine+isoleucine, valine ↑; creatine, methionine, lactate, citrate, pyruvate ↓	therapeutic effect of ZDP	Zhao et al.2012 [193]
1H-NMR	rat kidney tissue	16 rats with CRF induced by 5/6 nephrectomy, 16 rats with CRF induced by 5/6 nephrectomy treated with cordyceps sinensis and 16 sham-operated rats (male SD)	isoleucine, leucine, valine, lactate, proline + glutamic acid, glycine, allantoin ↑; aspartate, creatine, carnitine, sn-glycero-3-phosphocholine, betaine, taurine, myo-inositol, adenine partment signals in adenosine-5'-triphosphate, adenosine diphosphate, adenosine monophosphate, adenosine ↓	renal protective effects of cordyceps sinensis	Zhong et al. 2012 [194]

↑ increased levels versus reference group; ↓ decreased levels versus reference group

*14 CRF-induced rats: 3 rats used for identification and 11 for quantification; 13 normal rats; 3 rats used for identification and 10 for quantification

LPK: Lewis polycystic kidney; PKD: polycystic kidney disease; TCA: tricarboxylic acid, DN: diabetic nephropathy; SEB: staphylococcal enterotoxin B; PS: phosphatidylserine; PI: phosphatidylinositol; IgA: immunoglobulin A; CRF: chronic renal failure; SD: Sprague-Dawley; PE: phosphatidylethanolamine; DKD: diabetic kidney disease; PC: phosphatidylcholine; ergone:ergosta-4,6,8(14),22-tetraen-3-one; MG: monoacylglycerol; TGF-β1: transforming growth factor-beta 1; FLP: poria cocos epidermis, Fu-Ling-Pi; TMNO: trimethylamine N-oxide; ADMA: asymmetric dimethylarginine; ADPKD: autosomal dominant polycystic kidney disease; ZDP: zhibai dihuang pill; VLDL/LDL: very low density lipoprotein/low density lipoprotein

III.3.2. Metabolomics in clinical research

Different biological samples have been studied in patients with kidney failure by an arsenal of analytical techniques i.e. GC-MS [199-204], LC-MS [205-213], CE-MS [214-216] and NMR [217-223]. Table II.7 displays the sample type, population, analytical technique and the potential biomarkers.

II.3.2.1. Non-targeted approaches

a) GC-MS

Gao et al. performed a GC-MS-based study with oximation and trimethylsilylation derivatization that revealed metabolic distinctions between membranous nephropathy (MN) patients with urine protein lower than 3.5g/24h and those higher than 3.5g/24h [199]. In total, 26 urine metabolites and 9 serum metabolites were identified that account for the differences. The majority of the metabolites were significantly increased in patients with high urine protein for both urine and serum. Citric acid and 4 amino acids were markedly increased only in the serum samples of patients with high urine protein, implying more impaired filtration function of kidneys in patients with high urine protein than in patients with low urine protein. Dicarboxylic acids, phenolic acids, and cholesterol were significantly increased only in urine of patients with high urine protein, suggesting more severe oxidative status than in patients with low urine protein. Han et al. applied a GC-MS-based metabolic profiling method to assess the levels of non-esterified fatty acids (NEFAs) and esterified fatty acids (EFAs) in plasma [200]. Diabetic mellitus (DM) patients, DM patients with diabetic nephropathy (DN), which is the leading cause of chronic renal disease, and healthy controls were studied. The analysis showed that levels of some FAs in plasma samples were discriminating between the different stages in disease progress. Sharma et al. used GC-MS in a urinary metabolome screening and validation study of patients with DM and CKD (DM+CKD), in patients with DM without CKD (DM-CKD), and in healthy controls [201]. Compared with levels in healthy controls, 13 metabolites, i.e. 3-hydroxy isovalerate, aconitic acid, citric acid, 2-ethyl 3-OH propionate, glycolic acid, homovanillic acid, 3-hydroxyisobutyrate, 2-methylacetoacetate, 3-methyladipic acid, 3-methyl crotonylglycine, 3-hydroxypropionate, tiglylglycine, and uracil, were significantly reduced in the DM+CKD cohorts, and 12 of the 13 remained significant when compared with the DM-CKD cohort. Further analysis suggested that renal organic ion transport and mitochondrial function are dysregulated in diabetic kidney disease. Tao et al. developed a GC-MS method with ethyl chloroformate derivatization for the analysis of metabolites in serum and applied it to uremia. Significantly decreased levels of valine, leucine and isoleucine and increased levels of myristic acid and linoleic acid were observed in the patient group compared to the healthy control group [202].

Next to conventional sample types such as urine, serum, and plasma, breath analysis by GC-MS has recently been applied in the context of CKD. Grabowska-Polanowska et al. applied thermal desorption (TD) GC-MS to determine the breath composition in patients suffering from CKD [203]. Trimethylamine (TMA) was detected in all patients suffering from CKD, but not in the control group. TMA and isoprene, acetone, dimethylamine (DMA), some hydrocarbons and sulfur compounds were subsequently quantified. Apart from TMA, statistically significant differences were only found for pentane. Pagonas et al. investigated breath of patients in CKD stage 2 to 5HD and healthy controls by ion mobility spectrometry after GC preseparation [204]. Identification of the compounds of interest was performed by thermal desorption (TD) GC-MS. Significant differences between breath of patients with and without renal failure were revealed. Thirteen compounds were chosen for further evaluation. Some compounds including hydroxyacetone, 3-hydroxy-2-butanone and ammonia accumulated with deteriorating renal function and were eliminated by dialysis. The concentrations of these compounds allowed a significant differentiation between healthy, chronic renal failure with an eGFR of 10-59 ml/min, and ESRD. Other compounds including 4-heptanal, 4-heptanone, and 2-heptanone preferentially or exclusively occurred in patients undergoing hemodialysis.

b) LC-MS

Jia et al. identified 19 potential plasma phospholipid biomarkers for chronic glomerulonephritis by LC-QTRAP MS after a specific sample treatment [205]. The same group performed a serum metabolomic study on chronic renal failure by UPLC-QTOF MS [206]. Seven potential biomarkers, namely creatinine, tryptophan, phenylalanine, kynurenine, and 3 lysophosphatidylcholines were identified. Rhee et al. applied LC-QTRAP-based metabolite profiling to plasma of patients with ESRD and healthy controls [207]. In addition to confirming the elevation of numerous previously identified uremic toxins, several additional markers of ESRD were identified, including dicarboxylic acids (adipate, malonate, methylmalonate, and maleate), biogenic amines, nucleotide derivatives, phenols, and sphingomyelins. The pattern of lipids was notable for a universal decrease in lower-molecular-weight triacylglycerols, and an increase in several intermediate-molecular-weight triacylglycerols in ESRD compared with controls; with standard measurement of total triglycerides this heterogeneity could not be discovered. These observations suggest disturbed triglyceride catabolism and/or beta-oxidation in ESRD. Furthermore, Rhee et al. showed in another study that the addition of LC-MS-based metabolite profiling to clinical data may significantly improve the ability to predict whether an individual will develop CKD by identifying predictors of renal risk that are independent of eGFR [208]. An UPLC-orthogonal acceleration (oa) TOF MS-based platform was used by Zhang et al. to distinguish serum profiles of type 2 DM patients, DN patients and healthy controls [209]. Apart from some unidentified metabolites, significant changes in the serum level of leucine, dihydrosphingosine and

phytosphingosine were noted, indicating perturbations of amino acid metabolism and phospholipid metabolism in diabetic diseases. Zhu et al. identified potential phospholipid biomarkers in plasma associated with type 2 DM and DN [210]. Two novel biomarkers, i.e. PI C18:0/22:6 and SM dC18:0/20:2 were shown to discriminate healthy controls, type 2 DM patients and DN patients from each other.

c) Combination

In an attempt to obtain a comprehensive coverage of the metabolome, several studies combined the previous 2 analytical techniques, i.e. GC-MS and LC-MS [211-213]. A metabolomic discovery study combining RPLC-QTOF MS in both negative and positive ionization mode and GC-MS on blood serum samples of patients in different stages of CKD was described by Boelaert et al [211]. Eighty-five metabolites were shown to evolve with CKD progression of which 43 metabolites were a confirmation of earlier reported uremic retention solutes and/or uremic toxins. Thirty-one unique metabolites were revealed which were increasing significantly throughout CKD progression, by a factor surpassing the level observed for creatinine, the currently used biomarker for kidney function. High fold changes were found for aminohydroxyhippuric acid, 2-hydroxyhippuric acid glucuronide, 2-/3-hydroxyhippuric acid sulfate, hydroxypyridine, methoxy-hydroxyphenylglycol glucuronide, lactose, a tetrasaccharide, and trihydroxypentenoic acid. Hydroxyhippurate compounds, here detected as conjugates, are among the most discriminating metabolites, emphasizing their relevance in CKD. Additionally, 11 unique metabolites, mainly fatty acids, showed a decreasing trend. Shah et al. performed a GC-MS and LC-MS-based cross-sectional metabolomic study to ascertain whether plasma metabolite profiles are significantly different in CKD stages 2, 3, and 4 [212]. Comparison of stage 3/stage 2 identified 62 metabolites that differed, with 39 higher and 23 lower in stage 3 compared with stage 2; comparisons of stage 4/stage 2 identified 111 metabolites, with 66 higher and 45 lower; and comparisons of stage 4/stage 3 identified 11 metabolites, with 7 higher and 4 lower. Major differences in metabolite profiles with increasing stage of CKD were observed, including altered arginine metabolism, elevated coagulation/inflammation, impaired carboxylate anion transport, and decreased adrenal steroid hormone production. A discovery study for urinary biomarkers that differentiate the non-progressive form of albuminuria from the progressive form of albuminuria, i.e. from normoalbuminuria to microalbuminuria or to macroalbuminuria (diabetic kidney disease, DKD), in humans was conducted by van de Kloet et al [213]. Metabolite profiles of baseline 24 h urine samples were obtained by GC-MS and LC-MS to detect potential early indicators of pathological changes. A number of metabolites that had been associated with DKD already in literature were confirmed, but also new candidate biomarkers were found. The discriminating

metabolites included acyl-carnitines, acyl-glycines and metabolites related to tryptophan metabolism.

d) CE-MS

Three CE-MS-based metabolome studies have been reported in the context of CKD. Firstly, a preliminary survey of metabolic changes occurring in patients with CKD stage 1-2 was performed by Hayashi et al [214]. Serum and urine metabolomic profiles of 15 patients with stage 1-2 CKD were analyzed by CE-TOF MS, and compared to 7 healthy volunteers. In cation analysis mode, several increases in non-essential amino acids were identified in patients with CKD stage 1-2. Free-radical scavengers carnosine and hypotaurine were decreased in the urine, whereas serum hypotaurine and taurine were increased, consistent with changes in renal and/or systemic oxidative stress. In addition, hypoxanthine, which has the potential to be toxic to the endothelium by its pro-oxidant characteristics [224], was markedly increased in serum, whereas serum and urine adenosine and urine guanine were decreased, suggesting perturbations in purine nucleotide metabolism which could affect cardiovascular prognosis. Second, Hirayama et al. used CE-TOF MS to explore new serum biomarkers for DN diagnosis, through comprehensive analysis of serum metabolites [215]. Nineteen metabolites could be identified distinguishing between DN with macroalbuminuria and diabetic patients without albuminuria. These metabolites included creatinine, aspartic acid, γ-butyrobetaine, citrulline, symmetric dimethylarginine (SDMA), kynurenine, azelaic acid, and galactaric acid. Third, Toyohara et al. found 22 cations and 30 anions that accumulated significantly in plasma as the eGFR decreased by CE-MS [216]. These compounds included 9 cations and 27 anions that were newly identified. In contrast, 7 cations (2 new) and 5 anions (all new) that decrease significantly as eGFR declines were found. Every metabolite was evaluated for its suitability to detect early CKD.

e) NMR

Gronwald et al. used 2D NMR in a step towards identifying urinary markers of autosomal dominant polycystic kidney disease (ADPKD) [217]. Based on the average of 51 out of 701 NMR features, ADPKD patients with moderately advanced disease could reliably be discriminated from ADPKD patients with end-stage renal disease, patients with chronic kidney disease of other etiologies, and healthy controls with an accuracy of >80%. Of the 35 patients with ADPKD receiving medication for hypertension, most showed increased excretion of proteins and also methanol. In contrast, elevated urinary methanol was not found in any of the control and other patient groups. Hence, NMR fingerprinting of urine differentiates ADPKD from several other kidney diseases and individuals with normal kidney function. Hao et al. applied ^{1}H-NMR-based metabolomics for the urinary metabolic profile in the patients with primary focal segmental glomerulosclerosis (FSGS), membranous

nephropathy, minimal change disease, immunoglobulin A (IgA) nephropathy, and healthy controls [218]. FSGS patients were clearly distinguished from healthy controls and the other three types of glomerulopathies with good sensitivity and specificity based on their global urinary metabolic profiles. In FSGS patients, urinary levels of glucose, DMA and TMA increased compared with healthy controls, while pyruvate, valine, hippurate, isoleucine, phenylacetylglycine, citrate, tyrosine, 3-methylhistidine and β-hydroxyisovalerate decreased. Moreover, FSGS patients had lower urine N-methylnicotinamide levels compared with other glomerulopathies. Makinen et al. investigated serum lipids in 3 type 1 diabetes patient groups: patients with a normal albumin excretion rate (AER), microalbuminuria patients and overt kidney disease patients (macroalbuminuria) [219]. Lipoprotein subclass lipids and low-molecular-weight metabolites were quantified from native serum, and individual lipid species from the lipid extract of the native sample, using a ^{1}H-NMR metabonomics platform. Sphingomyelin, large VLDL cholesterol, total triglycerides, omega-9 and saturated fatty acids, glucose disposal rate, large HDL cholesterol and glomerular filtration rate were associated with kidney disease. Sphingomyelin was a significant regressor of urinary albumin in multivariate analysis with kidney function, glycemic control, body mass, blood pressure, triglycerides and HDL cholesterol. Mutsaers et al. applied ^{1}H-NMR spectroscopy, following three distinct deproteinization strategies, to determine differences in the plasma metabolic status of CKD stage 3-4 patients and healthy controls [220]. Ultrafiltration and acetonitrile precipitation were shown to be complementary protein removal techniques and both were required to obtain a clear metabolome profile. This approach revealed 14 upregulated metabolites in uremic plasma. In addition to confirming the retention of several previously identified uremic toxins, including p-cresylsulfate, two novel uremic retention solutes were detected, namely dimethyl sulphone and 2-hydroxyisobutyric acid. Furthermore, exposure of a human renal proximal tubule cell line (ciPTEC) to clinically relevant concentrations of both solutes resulted in an increased protein expression of the mesenchymal marker vimentin with more than 10%. In addition, the loss of epithelial characteristics significantly correlated with a loss of glucuronidation activity. Posada-Ayala et al. performed a proof of principle ^{1}H-NMR study to discover new markers of CKD [221]. In the discovery stage, the urine metabolome of 15 patients with CKD was analyzed and compared to that of 15 healthy individuals. A classification pattern clearly indicative of CKD was found. A validation cohort of urine samples from an additional 16 patients with CKD and 15 controls was then analyzed by LC-MS/MS and indicated that a group of seven urinary metabolites differed between CKD and non-CKD urine samples. This profile consisted of 5-oxoproline, glutamate, guanidoacetate, α-phenylacetylglutamine, taurine, citrate, and trimethylamine N-oxide (TMNO). A ^{1}H-NMR-based metabolomics approach was applied to serum of CKD stage 1-4 patients and healthy controls by Qi et al [222]. The significant endogenous metabolites that contributed to distinguish CKD in the different stages included the products of glycolysis (glucose, lactate), amino acids (valine,

alanine, glutamate, glycine), organic osmolytes (betaine, myo-inositol, taurine, glycerophosphcholine). Based on these metabolites, the model for diagnosing patients with CKD achieved the sensitivity and specificity of 100%. Sui et al. looked for biomarkers of IgA nephropathy, a common cause of chronic renal failure and the most frequent glomerulopathy [223]. Serum samples of healthy controls, low-risk patients in whom IgA nephropathy was confirmed as grades I-II by renal biopsy, and high-risk patients with nephropathies of grades IV-V were analyzed by ^{1}H-NMR. Compared with the healthy controls, both the low-risk and high-risk patients had higher levels of phenylalanine, myo-inositol, lactate, lipids as well as lower levels of β-glucose, α-glucose, valine, tyrosine, phosphocholine, lysine, isoleucine, glycerolphosphocholine, glycine, glutamine, glutamate, alanine, acetate, 3-hydroxybutyrate, and 1-methylhistidine.

Inconsistencies on metabolite changes are sometimes reported in literature such as for glutamic acid [211,214,216,222]. Sample collection, sample storage conditions and sample preparation methods can affect and modify metabolite structure, confounding already complex data sets and introducing substantial additional variability. Different analytical approaches could also be at the origin of these inconsistencies. Another explanation for inconsistencies may be the low number of samples analyzed in some studies such as the ones by Shah et al. [212] and Hayashi et al. [214], consequently decreasing the confidence of observation. As levels of biomarkers will likely be influenced by the complex and highly variable molecular changes associated with CKD, which are expected to be even more pronounced in patients with different etiologies of CKD and different comorbidities, a substantially large number of samples has to be analyzed to detect truly significant changes.

II.3.2.2. Targeted approaches

Since omics methods aim at the non-biased detection of unknowns and are by definition non-targeted, we do not discuss targeted methods here. However, since some studies succeed in quantifying an extensive array of metabolites, they are worth mentioning. Goek et al. performed a cross-sectional observational study of the general population quantifying 151 serum metabolites by flow injection MS/MS [225]. Metabolites and their 22,650 ratios were analyzed for their association with eGFR. Significant metabolites were meta-analyzed with independent data from the second study. Replicated associations with eGFR were observed for 22 metabolites and 516 metabolite ratios. Acylcarnitines such as glutarylcarnitine were associated inversely with eGFR. The replicated ratio with the strongest association was the ratio of serine to glutarylcarnitine. Across categories of a metabolic score consisting of 3 uncorrelated metabolites, the prevalence of decreased eGFR increased from 3% to 53%. The same group examined longitudinal associations of baseline concentrations of 140 metabolites and their 19,460 ratios with kidney function decline and CKD

incidence over 7 years in 1104 participants using flow injection- and LC-MS/MS methods [226]. A significant association with annual change in the eGFR was observed for spermidine and two metabolite ratios, the phosphatidylcholine diacyl C42:5-to-phosphatidylcholine acyl-alkyl C36:0 ratio and the kynurenine-to-tryptophan ratio. The kynurenine-to-tryptophan ratio was also associated with significantly higher incidence of CKD at the follow-up visit. In separate analyses, the predictive ability of the metabolites was assessed: both the three significantly associated metabolite (ratios) as well as a panel of 35 metabolites selected from all metabolites in an unbiased manner provided as much but not significantly more prognostic information than selected clinical predictors as judged by the area under the curve. The studies by Rhee et al. mentioned above in the LC-MS section also consist mainly of targeted methods.

Table II.7 CKD metabolomics in clinical studies

Analytical Technique	Sample Type	Species, population	Candidate biomarkers	Reference
GC-MS (oximation + trimethylsilylation)	human urine and serum	14 MN patients with urine protein lower than 3.5g/24h and 15 MN patients with urine protein higher than 3.5g/24h	urine: erythritol, xylitol, galactitol, inositol, glyceric acid, 2,4-dihydrobutyric acid, threonic acid, 2-deoxyribonic acid, 2-ketogluconic acid, glutaric acid, 3-methylglutaric acid, adipic acid, 2-hydroxyglutaric acid, suberic acid, 3-hydroxysebacic acid, mandelic acid, 4-hydroxyphenylacetic acid, vanillic acid, 3,4-dihydroxybenzoic acid, 4-hydroxyphenyllactic acid, vanillactic acid, cytosine, quinolinic acid, cholesterol ↑; cis-aconitic acid, lactose ↓; serum: L-asparagine, L-serine, L-threonine, pyroglutamic acid, citric acid, glucose, cholesterol ↑; m-cresol, 2-keto-3-methylvaleric acid ↓	Gao et al. 2012 [199]
GC-MS (ion trap) with H_2SO_4/CH_3OH (5%, v/v)	human plasma	30 type 2 diabetes patients without nephropathy, 30 type 2 diabetes patients with DN stage III, 30 DN stage IV patients and 30 DN stage V patients and 30 healthy controls.	EFAs: Control–DM: C10:0, C14:0, C16:1n-9, C16:0, C18:2, C18:1, C18:0, C20:4, C20:5, C20:3, C20:2, C22:6 ↓; DM–DNIII: C14:0, C16:1n-9, C16:0, C18:2, C18:1, C18:3, C18:0, C20:4, C20:5, C20:3, C20:2, C20:0, C22:6 ↑; DNIII–DNIV: C14:0, C16:0, C18:2, C18:0, C20:4, C20:0, C22:6 ↓; DNIV–DNV: C10:0, C18:2, C20:0 ↑ NEFAs: Control–DM: C16:0, C18:2, C18:1, C18:3, C18:0, C20:4, C20:3, C20:2, C20:0, C22:6 ↑; C10:0↓; DM–DNIII: C12:0, C20:0 ↑; DNIII–DNIV: C12:0, C14:0, C16:1n-9, C16:0, C18:1, C18:3, C18:0, C20:4, C20:5, C20:3, C20:0, C22:6 ↓; DNIV–DNV: C16:0, C18:3, C18:0, C20:4, C20:3, C20:0, C22:6 ↑	Han et al. 2011 [200]
GC-MS (oximation + trimethylsilylation)	human urine	32 type 1 DM patients without CKD, 41 type 2 DM patients without CKD, 24 DM+CKD patients (screening cohort), 61 DM+CKD patients (validation cohort) and 23 healthy controls	3-hydroxyisovalerate, aconitic acid, citric acid, 2-ethyl-3-OH propionate, glycolic acid, homovanillic acid, 3-hydroxy isobutyrate, 2-methylacetoacetate, 3-methyladipic acid, 3-methylcrotonylglycine, 3-hydroxypropionate, tiglylglycine, uracil ↓	Sharma et al. 2013 [201]
GC-MS (ethyl chloroformate derivatization)	human serum	24 uremic patients and 23 age and sex-matched healthy controls	tetradecanoic acid, 9,12-octadecadienoic acid ↑; valine, leucine, isoleucine ↓	Tao et al. 2008 [202]
TD-GC-MS	human breath	14 CKD patients and 9 healthy controls	TMA, pentane ↑	Grabowska-Polanowska et al. 2013 [203]
TD-GC-MS	human breath	26 CRF stage 2-4 patients, 28 CRF stage 5 patients on HD and 28 healthy controls	ammonia, 3-hydroxy-2-butanone, hydroxyacetone ↑	Pagonas et al. 2012 [204]

LC-QTRAP	human plasma	18 patients with chronic glomerulonephritis, 17 with CRF without RRT and 18 healthy controls	PI: m/z 833 C16:0–C18:2, m/z 857 C16:0–C20:4, C18:0–C18:4, m/z 861, C18:0–C18:2, C18:1-C18:1, m/z 863 C18:0–C18:1, m/z 883 C18:1–C20:4, m/z 885 C18:0–C20:4, m/z 887 C18:0–C20:3, m/z 909 C18:0–C22:6, m/z 911 C18:0–C22:5, m/z 913 C18:0–C22:4 PS: m/z 786 C18:0–C18:2,C18:1–C18:1, m/z 788 C18:0–C18:1, m/z 790 C18:0–C18:0, m/z 808 C18:1–C20:4, m/z 810 C18:0–C20:4, m/z 832 C18:1–C22:6, m/z 834 C18:0–C22:6, m/z 836 C18:0–C22:5, m/z 838 C18:0–C22:4	Jia et al. 2007 [205]
UPLC-QTOF MS	human serum	32 CRF patients without RRT and 30 healthy controls	creatinine, phenylalanine, kynurenine ↑; tryptophan, 3 LPC: C16:0, C18:1, C18:0 ↓	Jia et al. 2008 [206]
LC-QTRAP	human plasma	44 ESRD patients (before and after hemodialysis) and 10 age-matched at-risk controls	hippurate, 5-adenosylhomocysteine, sucrose, cytosine, adipate, MOPEG, 5-hydroxyindoleacetic acid, aminoisobutyric acid, homogentisate, 4-pyridoxate, 4-hydroxybenzoate, 3-hydroxykynurenine, phenylacetylglycine, sorbitol, creatinine, lactose, kynurenic acid, homovanillate, indoxylsulfate, homocystine, cGMP, allantoin, pantothenate, xanthosine, adenine, thymidine, TMNO, ureidopropionic acid, orotate, dimethylglycine, methylmalonate, isocitrate, maleate, ADMA/SDMA, malonate, 2-deoxyadenosine, 18:2 sphingomyelin, kynurenine, choline, 18:1 sphingomyelin ↑	Rhee et al. 2010 [207]
LC-QTRAP	human plasma	123 individuals who developed CKD during 8 years of follow-up (eGFR < 60 mL/min per 1.73 m^2), 1311 individuals who did not develop CKD	kynurenic acid, kynurenine, citrulline, choline, xanthosine, β-aminoisobutyric acid, aconitate, isocitrate ↑; 5-hydroxyindoleacetic acid ↓	Rhee et al. 2013 [208]
UPLC-oaTOF MS,	human serum	8 DN patients, 33 type 2 DM patients and 25 healthy controls	m/z 546.3627, 496.3484, 282.2815 ↑; phytosphingosine, dihydrosphingosine, leucine, m/z 415.2240, 297.2933 ↓	Zhang et al. 2009 [209]
LC-MS (LC-TOF MS + LC-Iontrap MS)	human plasma	30 type 2 DM patients, 52 type 2 DM patients with DN and 30 healthy controls	Control-DM: LPC m/z 564.3 C18:2, LPC m/z 568.3 C18:0, LPC m/z 588.3 C20:4, PC m/z 802.5 C16:0/18:2, PE m/z 738.5 C16:0/20:4, PG m/z 773.5 C18:0/18:2, SM m/z 801.5 dC18:0/20:2 ↑; PC m/z 806.5 C16:0/18:0, PC m/z 854.5C18:0/20:4, PI m/z 837.5 C16:0/18:0, PI m/z 885.5 C18:0/20:4, PI m/z 909.5 C18:0/22:6 ↓ Control-DN: LPC m/z 540.3 C16:0, LPC m/z 566.3 C18:1, LPC m/z 568.3 C18:0, LPC m/z 588.3 C20:4, PC m/z 802.5 C16:0/18:2, PE m/z 716.5 C16:0/18:1, PE m/z 738.5 C16:0/20:4, PG m/z 773.5 C18:0/18:2, SM m/z 747.5 dC18:1/16:0, SM m/z 801.5 dC18:0/20:2 ↑; C16:0/18:0, C18:0/20:4, C16:0/18:0, C18:0/22:6, C18:0/18:0 ↓ DM-DN: PE m/z 716.5 C16:0/18:1, SM m/z 801.5 dC18:0/20:2 ↑; LPC m/z 564.3 C18:2, PE m/z 750.5 pC18:0/20:4, PI m/z 909.5 C18:0/22:6, PS m/z 790.5 C18:0/18:0 ↓	Zhu et al. 2011 [210]
GC-MS (methoximation + trimethylsilylation) and LC-QTOF MS	human serum	20 CKD3 patients, 20 CKD5 patients and 20 healthy controls	43 already described uremic retention solutes, 42 newly identified: 31 metabolites (among others aminohydroxyhippuric acid, 2-hydroxyhippuric acid glucuronide, 2-/3-hydroxyhippuric acid sulfate, hydroxypyridine, methoxy-hydroxyphenylglycol glucuronide, lactose, a tetrasaccharide, and trihydroxypentenoic acid) ↑; 11 metabolites (mainly fatty acids) ↓	Boelaert et al. 2013 [211]

GC-MS (trimethylsilylation) and UPLC-LTQMS	human plasma	10 CKD2, 10 CKD3 and 10 CKD4 patients	117 metabolites differ significantly, thematic changes in arginine metabolism: ADMA, SDMA, citrulline, arginine ↑ ornithine ↓; elevated coagulation/inflammation: fibrinopeptide A, phosphorylated fibrinopeptide A, proline-hydroxyproline ↑; impaired carboxylate anion transport: γ-glutamylleucine, γ-glutamylisoleucine, γ-glutamylglutamine, γ-glutamylphenylalanine, CMPF ↑; decreased adrenal steroid hormone production: dehydroisoandrosterone sulfate, 4-androsten-3-β,17-β-diol disulfate ↓	Shah et al. 2013 [212]
GC-MS (oximation and trimethylsilylation) and LC-MS (LC-LTQ-FT and LTQ-orbitrap)	human urine	26 non-progressive DKD patients and 26 progressive DKD patients (after an average follow-up of 5.5 years)	pseudouridine, deoxyfructose, 3-hydroxy-3-(3-hydroxyphenyl) propanoic acid, L-valine, 2-hydroxyvaleric acid, 2-hydroxyglutaric acid, N-(3-hydroxybenzoyl) glycine, glucuronide compound, gluconic acid, L-cystine, tryptophan, salicyluric acid, substituted carnitine, S-(3-oxododecanoyl)cysteamine, substituted carnitine, N-methylguanosine, substituted carnitine, kynurenic acid, 2-(2-phenylacetoxy) propionylglycine, 3-methylcrotonylglycine, heptanoylcarnitine ↑; 4-oxoproline, 3,4,5-trihydroxypentanoic acid, 2,3-dihydroxy-3-methylbutanoate, 5-hydroxymethyl-2-furancarboxylic acid, galactonic acid, N-formylproline or N-ethylproline, L-arabinose, benzoic acid, 3-hydroxyphenylacetic acid, D-glutamic acid, glycolic acid, hippuric acid, substituted carnitine, indoleacetic acid ↓	van der Kloet et al. 2012 [213]
CE-TOF MS	human serum and urine	15 CKD1 + CKD2 patients and 7 healthy controls	serum: glutamate, aspartate, ornithine, hypotaurine, taurine, hypoxanthine, lactate, m/z 132.0307, m/z 185.0141, m/z 293.1231, m/z 295.0670, m/z 341.1358, m/z 437.1800, m/z 561.2548, m/z 710.0462 ↑; glutamine, hydroxyproline, adenosine ↓; urine: glutamate, aspartate ↑; histidine, glutamine, carnosine, hypotaurine, adenosine, cAMP, guanine, citrate, fumarate, 3-phosphoglycerate, m/z 380.1087, m/z 385.0955 ↓	Hayashi et al. 2011 [214]
CE-TOF MS	human serum	78 type 2 DN patients: 20 DM patients without nephropathy and albuminuria, 32 early DM patients with microalbuminuria, 26 overt DN patients with macroalbuminuria	creatinine, aspartic acid, γ-butyrobetaine, citrulline, SDMA, kynurenine, m/z 129.067, 244.106, 96.960, 103.014, 149.049 ↑ azelaic acid, galactaric acid, m/z 156.139, 158.154, 243.184, 276.128, 302.197, 316.213 ↓	Hirayama et al. 2012 [215]
CE-TOF MS	human plasma	41 CKD patients with eGFR 5.9-57.0 (CKD stage 3 to 5)	22 cations (9 new): N-acetylglucosamine, γ-butyrobetaine, opthalmate, N-ε-acetyllysine, cytosine, hypotaurine, 7-methylguanine, methionine sulfoxide, aspargine↑; 30 anions (25 new): isethionate, gluconate, pimelate, isocitrate, N-acetyl-β-alanine, sebacate, 4-oxopentanoate, cis-aconitate, homovanillate, adipate, citramalate, 2-isopropylmalate, threonate, N-acetylaspartate, 4-hydroxy-3-methoxymandelate, oxamate, glutarate, azelate, phthalate, malonate, citraconate, quinate, succinate, cysteine S-sulfate, 4-hydroxy-3-methoxybenzoate ↑; 7 cations (2 new): 2-aminobutyrate, glutamic acid↓; 5 anions (all new): 4-methyl-2-oxopentanoate, 2-oxoisopentanoate, lactate, octanoate, 2-oxoglutarate ↓	Toyohara et al. 2010 [216]

2D NMR	human urine	54 ADPKD patients (35 received blood pressure-lowering medication), 10 stable ADPKD patients with ESRD on HD with residual urine excretion, 16 patients 3 months after renal transplantation, 30 patients with DM type 2 with an eGFR ranging from 60 to 89 ml/min per 1.73 m^2 and an albumin/creatinine ratio of 30–300 mg/g, 22 patients with DM type 2 with an eGFR <50 ml/min per 1.73 m^2, although no microalbuminuria and 46 healthy controls	methanol, tartaric acid, threonine, sucrose ↑; alanine, tyrosine, citrate, D-saccharate, 3-hydroxyisovalerate ↓	Growald et al. 2011 [217]
1H-NMR	human urine	25 FSGS patients, 24 MN patients, 14 minimal change disease patients, 26 IgA nephropathy patients and 35 healthy controls	glucose, DMA, TMA ↑; pyruvate, valine, hippurate, isoleucine, phenylacetylglycine, citrate, tyrosine, 3-methylhistidine, β-hydroxyisovalerate ↓	Hao et al. 2013 [218]
^{1}H-NMR	human serum	326 type 1 diabetes patients: 240 without kidney disease and 86 with DKD	sphingomyelin, (large VLDL cholesterol, total triglycerides, omega-9 and saturated fatty acids) ↑	Makinen et al. 2012 [219]
^{1}H-NMR	human plasma	10 CKD3 + CKD4 and 4 healthy controls	1-methylhistidine, 3-methylhistidine, hippuric acid, p-cresylsulfate, creatinine, dimethyl sulphone, 2-hydroxyisobutyric acid, N,N-dimethylglycine, trigonelline, pseudouridine, betaine, myo-inositol, DMA , TMNO↑	Mutsaers et al. 2013 [220]
2D NMR	human urine	15 CKD patients (3 CKD3, 8 CKD4, 4 CKD5) and 15 healthy controls in discovery phase	TMNO, glutamate, guanidinoacetate, alpha-phenylacetylglutamine ↑; taurine, 5-oxoproline, citrate ↓	Posada-Ayala et al. 2014 [221]
1H-NMR	human serum	20 CKD1, 20 CKD2, 20 CKD3, 20 CKD4 patients and 28 healthy controls	myo-inositol, lactate ↑; betaine, glucose, taurine, glycerophosphocholine ↓; valine, alanine, glutamate, glycine	Qi et al. 2012 [222]
1H-NMR	human serum	35 IgA patients and 23 healthy controls	phenylalanine, myo-inositol, lactate, L6 lipids, L5 lipids, L3 lipids ↑;β-glucose, α-glucose, valine, tyrosine, PC, lysine, isoleucine, glycerolphosphocholine, glycine, glutamine, glutamate, alanine, acetate, 3-hydroxybutyrate, 1-methylhistidine ↓	Sui et al. 2012 [223]

↑ increased levels versus reference group; ↓ decreased levels versus reference group

MN: membranous nephropathy; DN: diabetic nephropathy; DM: diabetes mellitus; EFA: esterified fatty acid; NEFA: non-esterified fatty acid; TMA: trimethylamine; CRF: chronic renal failure; PI: phosphatidylinositol; PS: phosphatidylserine; LPC: lysophosphatidylcholines; MOPEG: 3-methoxy-4-hydroxyphenylethyleneglycol sulfate; cGMP:

cyclic 3’,5’-guanosine monophosphate; TMNO: trimethylamine N-oxide; ADMA/SDMA: asymmetric/symmetric dimethylarginine; oaTOF: orthogonal acceleration time-of-flight; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; SM: sphingomyelin; DKD: diabetic kidney disease; AER: albumin excretion rate; cAMP: cyclic 3'-5'- adenosine monophosphate; ADPKD: autosomal dominant polycystic kidney disease; FSGS: focal segmental glomerulosclerosis; IgA: immunoglobulin A; DMA: dimethylamine; VLDL: very low density lipoprotein

Apart from the search for biomarkers of CKD, metabolomic studies have also been used for other applications in CKD. Metabolite profiling of hemodialysate with GC-TOF-MS was for example applied by Qi et al. to differentiate patients treated with high-flux hemodialysis from those with low-flux hemodialysis, revealing higher concentrations of uremic retention solutes, and thus better removal, in patients under high-flux dialysis compared to those under low-flux dialysis [227]. Furthermore, ^{1}H-NMR was used by Choi et al. to investigate dialysis modality-dependent changes in serum metabolites. Inosine and hypoxanthine were only present in serum of patients receiving hemodialysis and not in peritoneal dialysis patients [228]. Solutes generated by colon microbes may contribute to uremic illness. Aronov et al. used a metabolic approach to compare plasma from hemodialysis patients with and without colon to identify colon-derived uremic solutes [229]. This study allowed to clarify the origin of some known uremic solutes such as p-cresolsulfate and indoxylsulfate and to demonstrate the presence of many additional uremic solutes of microbial origin generated by the intestinal flora. Li et al. developed an improved online two-dimensional (2D) LC-QTOF MS system for the lipid profiling of human plasma, in which different lipid classes were separated by the first dimensional normal-phase (NP) LC and different lipid molecular species were separated by the second dimensional RPLC [230]. This method was applied to identify the differences in lipid profile between plasma from peritoneal dialysis patients with bad volume status (overhydration as measured with bioimpedance below 2 kg) and peritoneal dialysis patients with good volume status (overhydration >2 kg for 3 consecutive months). Thirty potential biomarkers could be identified in that specific study distinguishing patients with different volume status. Dunn et al. performed a GC-MS- and direct injection (DI) MS-based proof-of-principle holistic profiling study of endogenous metabolites in a prospective collection of PD effluent collected over 6 years in order to investigate metabolic differences in PD effluent between PD therapy patients who later developed clinically defined encapsulating peritoneal sclerosis (EPS), characterized by extensive sclerosis of the peritoneum with bowel adhesions often causing obstruction, and controls [231]. Changes in the metabolomic profiles including amino acids, amines and derivatives, short-chain fatty acids and derivatives and sugars were observed prior to EPS development could be detected. Psihogios et al. used a ^{1}H-NMR-based approach to investigate the correlation of histopathologically assessed tubulointerstitial lesions with the urinary metabolite profile in patients with glomerulonephritis submitted to renal biopsy [232]. The presence of renal damage was predicted with a sensitivity of 96% and a specificity of 99%. Patients with mild, moderate, and severe tubulointerstitial lesions became progressively differentiated from the healthy individuals. The onset of the tubulointerstitial lesions was characterized by decreased excretion of citrate, hippurate, glycine, and creatinine, whereas further deterioration was followed by glycosuria, selective aminoaciduria, total depletion of citrate

and hippurate, and gradual increase in the excretion of lactate, acetate, and TMNO. Sato et al. aimed at identifying markers to estimate adequate dialysis dose [233]. Therefore, low molecular weight molecules were analyzed by LC-IT-TOF in plasma samples of patients with ESRD before and after hemodialysis, and in healthy subjects. In samples obtained after haemodialysis, the relative quantities of 54 peaks were significantly decreased when compared with those in the plasma before haemodialysis. The candidate biomarkers were allocated to three groups. Molecules in group A decreased completely with a large variance, molecules in group B decreased partially but with a large variance, and molecules in group C decreased partially with low variance after haemodialysis. A small cohort validation study consisting of the patients with ESRD undergoing haemodialysis demonstrates that 3 candidate biomarkers in group C would be a very useful marker to estimate haemodialysis dose, i.e. 1-methylinosine and two unidentified molecules.

In 2003, the European Uremic Toxin (EUTox) Work Group proposed the classification of uremic retention solutes, including the 90 retention solutes known and quantified in uremia at that time [234]. This list has been extended with an additional 56 solutes in 2012 [235]. We compared the recent findings from metabolome studies with these reviews. Similar to these overviews, we only considered metabolites that were increased in serum and plasma. Nevertheless, metabolites that decrease throughout CKD might also be clinically relevant and provide new insights in the perturbation of metabolic pathways. A substantial amount of new retention solutes were found as displayed in Table II.8. However, a considerable disadvantage of this overview compared to the previous reviews is that no concentrations are provided since non-targeted metabolomic studies are at best semi-quantitative, providing relative differences instead of absolute concentrations. If concentrations applied in further toxicity tests exceed those encountered in uremia, conclusions might have relatively little clinical relevance. Therefore quantification of these metabolites of interest should be performed. It is also interesting to remark that several metabolites have been identified by more than one study.

In conclusion, this overview, which to the best of our knowledge is the most up to date and thus very likely also the most comprehensive one, shows that an extensive list of potential biomarkers of CKD has recently been generated by metabolome discovery studies. The data currently available indicate a clear potential for metabolomics in the search for biomarkers of CKD, but at the same time they unfortunately also highlight that the studies published to date run short of delivering the expected objective, namely identification of molecules outperforming conventional biomarkers such as serum creatinine and albuminuria, and are ultimately qualitatively so good that they are ready to be translated into clinical practice, although in our own study some substances were found that rose

earlier than creatinine and thus have a potential for faster tracing of CKD [211]. The lack of comparability between studies due to different technologies, different sample types, and a low number of samples further complicates this quest. In addition, the route to valid biomarkers necessitates subsequent study of the initially detected potential markers applying independent analytical methods in independent populations [236], and this validation step is unfortunately rarely taken. Hence, discovery studies flooding the literature with biomarkers that show potential but never graduate to validation studies is a potential pitfall. Therefore, future research should focus more on the validation of identified potential biomarkers. Another shortcoming is the observational character of most described studies, not considering correlations with hard endpoints such as progression of CKD, mortality, or inflammation. However, also an optimistic view on metabolomics in CKD is appropriate. To begin with, technological improvements on instrument sensitivity and accuracy allow identification of metabolites by far higher confidence and at quantitative levels that were undetectable only a few years ago. Furthermore, the identified retention solutes offer a valuable input for uremic toxicity research. Assessment of these newly detected metabolites' role in pathophysiology will enable identification of key culprits in the uremic syndrome and starting from there advancement in their removal by focus on strategies eliminating essentially the most toxic elements. Moreover, the application of metabolomics is not only limited to the identification of potential biomarkers and/or uremic retention solutes, but the data displayed in this section also provides various other new insights related to drug mechanism, progression of CKD, evaluation of removal by high- and low-flux dialysis, contribution of colon to uremia, differentiation between different degrees of volume status, development of encapsulating peritoneal sclerosis in PD patients, adequate dialysis dose, and role of Oat1-mediated transport and thus of the tubular system in renal elimination of uremic toxins. Hence, metabolomics holds great promise to significantly impact CKD research in future.

Table II.8 New uremic retention solutes found by metabolome studies

Solute	Sample type	Ref	Solute	Sample type	Ref	Solute	Sample type	Ref	Solute	Sample type	Ref
1-Methylhistidine	P	[220]	Arginine	P	[212]	Homovanillate	P,P	[207,216]	p-Cresol glucuronide	S	[211]
2-/3-Hydroxyhippuric acid sulfate	S	[211]	Aspartic acid leucine / Aspartic acid isoleucine	S	[212]	Hydroxyindole	S	[211]	Phenylalanine phenylalanine	S	[211]
2-Deoxyadenosine	P	[207]	Asparagine	S	[199]	Hydroxypyridine	S	[211]	Phenylacetylglycine	P	[207]
2-Furoylglycine	S	[211]	Aspargine	P	[216]	Hypotaurine	S,P	[214,216]	Phenylalanine	S	[223]
2-Hydroxyhippuric acid	S	[211]	Aspartate	S	[214]	Indole-3-lactate	S	[211]	Phosphorylated fibrinopeptide A	P	[212]
2-Hydroxyhippuric acid glucuronide	S	[211]	Azelate	P	[216]	Isethionate	P	[216]	Phthalate	P	[216]
2-Hydroxyisobutyric acid	P	[220]	Betaine	P	[220]	Isocitrate	P,P,P	[207,208, 216]	Pimelate	P	[216]
2-Isopropylmalate	P	[216]	C5:0-Glycine	S	[211]	Lactate	S,S,S	[214,222, 223]	Proline	S	[211]
3-Hydroxhippuric acid	S	[211]	cGMP	P	[207]	Lactose	P,S	[207,211]	Proline-hydroxyproline	P	[212]
3-Hydroxykynurenine	P	[207]	Cholesterol	S	[199]	Lysine	S	[211]	Pyroglutamic acid	S,S	[199,211]
3-Methylhistidine	P	[220]	Choline	P,P	[207,208]	Maleate	P	[207]	Quinate	S,P	[211,216]
3-Methyluridine / ribothymidine	S	[211]	Cinnamoylglycine	S	[211]	Malonate	P,P	[207,216]	Sebacate	P	[216]
4-Acetamidobutanoate	S	[211]	Cis-aconitate	P	[216]	Maltose	S	[211]	Serine	S	[199]
4-Hydroxy-3-methoxybenzoate	P	[216]	Citraconate	P	[216]	Mercaptolactic acid	S	[211]	Sialyllactose	S	[211]
4-Hydroxy-3-methoxymandelate	P	[216]	Citramalate	P	[216]	Methionine	S	[211]	Succinate	P	[216]
4-Hydroxybenzoate	P	[207]	Citric acid	S	[199]	Methionine sulfoxide	P	[216]	Succinoadenosine	S	[211]

4-Oxopentanoate	P	[216]	Citrulline	P,P	[208,212]	Methoxy-hydroxyphenylglycol glucuronide	S	[211]	Sucrose	P,S	[207,211]
4-Pyridoxate	P,S	[207,211]	Cysteine S-sulfate	P	[216]	Methylglutarylcarnitine	S	[211]	Taurine	S	[214]
5-Adenosylhomocysteine	P	[207]	Cytosine	P,P	[207,216]	Methylmalonate	P	[207]	Tetradecanoic acid	S	[202]
5-Hydroxyindoleacetic acid	P	[207]	D-Glucuronic acid-N-acetyl-D-glucosamine	S	[211]	Methyluric acid	S	[211]	Threonate	P	[216]
5-Methoxysalicylic acid	S	[211]	Dimethyl sulphone	P	[220]	MOPEG	P	[207]	Threonine	S	[199]
7-Methylguanine	P	[216]	Dimethyluric acid	S	[211]	N-acetylaspartate	P	[216]	Thymidine	P	[207]
9,12-Octadecadienoic acid	S	[202]	Erythronic acid	S	[211]	N-Acetylglucosamine	P	[216]	Trigonelline	P	[220]
Acetylhomoserine	S	[211]	Fibrinopeptide A	P	[212]	N-Acetylneuraminic acid	S	[211]	Trihydroxypentenoic acid	S	[211]
Aconitate	P	[208]	Galacturonic acid / Glucuronic acid	S	[211]	N-acetyl-β-alanine	P	[216]	Ureidopropionic acid	P	[207]
Adenine	P	[207]	Gluconate	S,P	[211,216]	Nicotinuric acid / isonicotinylglycine	S	[211]	β-Aminoisobutyric acid	P	[208]
Adipate	P,P	[207,216]	Glucose	S	[199]	N-ε-acetyllysine	P	[216]	γ-Butyrobetaine	P	[216]
Alanine	S	[211]	Glutamate	S,S	[211,214]	Opthalmate	P	[216]	γ-Glutamylglutamine	P	[212]
allantoin	P	[207]	Glutamine	S	[211]	Ornithine	S	[214]	γ-Glutamylisoleucine	P	[212]
Aminohydroxyhippuric acid	S	[211]	Glutamylphenylalanine	S	[211]	Oxamate	P	[216]	γ-Glutamylleucine	P	[212]
Aminoisobutyric acid	P	[207]	Glutarate	P	[216]	Oxoprolylproline	S	[211]	γ-Glutamylphenylalanine	P	[212]
a-N-Acetylneuraminyl-2,6-b-D-galactosyl-1,4-N-acetyl-b-D-glucosamine	S	[211]	Homogentisate	P	[207]	Pantothenate	P,S	[207,211]			

Ref: reference; P: plasma; S: serum; cGMP: cyclic 3',5'-guanosine monophosphate; MOPEG: 3-methoxy-4-hydroxyphenylethyleneglycol sulfate

II.4. References

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Chapter 3

State-of-the-art non-targeted metabolomics in the study of chronic kidney disease*

III.1. Abstract

Here we report a metabolomics discovery study conducted on blood serum samples of patients in different stages of chronic kidney disease (CKD). Metabolites were monitored on a quality controlled holistic platform combining reversed-phase liquid chromatography coupled to high-resolution quadrupole time-of-flight mass spectrometry in both negative and positive ionization mode and gas chromatography coupled to quadrupole mass spectrometry. A substantial portion of the serum metabolome was thereby covered. Eighty-five metabolites were shown to evolve with CKD progression of which 43 metabolites were a confirmation of earlier reported uremic retention solutes and/or uremic toxins. Thirty-one unique metabolites were revealed which were increasing significantly throughout CKD progression, by a factor surpassing the level observed for creatinine, the currently used biomarker for kidney function. Additionally, 11 unique metabolites showed a decreasing trend.

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III.2. Introduction

Chronic kidney disease (CKD) is characterized by a progressive loss in renal function over a period of months or years. The kidneys lose the capacity to remove potentially toxic compounds from the blood into the urine, resulting in their accumulation in the body. The compounds that interact negatively with biological functions are called uremic toxins [1]. In 2003, the European uremic toxin (EUTox) work group proposed a classification of 90 identified uremic retention solutes [2]. In 2012, an update of the list was provided, proposing 56 additional solutes [3]. Recent professional guidelines classify the severity of CKD into five stages based on the estimated glomerular filtration rate (eGFR), with stage 1 being the mildest and usually causing few symptoms and stage 5 (end stage) being a severe illness with poor life expectancy if untreated [4]. Current biomarkers available for the detection of an impaired kidney function, such as serum creatinine or urinary albumin, lack the sensitivity for early detection of CKD. By way of example, blood serum creatinine stays within the reference range until a third or even half of the kidney function has been lost [5]. Early detection is primordial towards disease management; hence, there is a clear need for novel, more sensitive and specific biomarkers.

In recent years, the so-called omics approach emerged as a powerful tool for biomarker discovery [6]. Genomics has indispensably initiated the omics cascade and paved the way for transcriptomics, proteomics and metabolomics. This holistic approach is revolutionary since it allows interrogating thousands of potential biomarkers simultaneously without a priori knowledge of the underlying biology or pathophysiology of the system being studied. There have been several kidney disease studies employing genomics, transcriptomics and proteomics contributing to the quest for better disease markers [7-10]. In addition, metabolomics has been proposed to hold great promise to identify novel uremic toxins and biomarkers of CKD [11]. A gas chromatography–mass spectrometry (GC-MS) method with ethyl chloroformate derivatization has been optimized for the comprehensive analysis of metabolites in serum and has been applied to human uremia [12]. Depletion of three amino acids, i.e. valine, leucine, and isoleucine, and an abnormal accumulation of fatty acids were observed in uremic serum. Several liquid chromatography–mass spectrometry (LC-MS) metabolomics studies have recently been performed on CKD in humans [13-17]. Plasma phospholipid profiles of chronic glomerulonephritis were investigated by LC-Ion Trap MS and it was suggested that these phospholipids can be used as a potential biomarker for the progression of primary chronic glomerulonephritis [13]. A serum based ultra-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF) study on end stage renal disease (ESRD) revealed seven potential biomarkers, i.e. creatinine, tryptophan, phenylalanine, kynurenine and three lysophosphatidylcholines [18]. Next to confirming the elevation of numerous previously

identified uremic toxins, several additional markers of ESRD were identified in plasma by a LC–MS/MS based metabolite profiling platform [15]. These markers include dicarboxylic acids, biogenic amines, nucleotide derivatives, phenols, and sphingomyelins. Another LC-Ion Trap-TOF study, focusing on estimation of adequate haemodialysis dose, found 1-methylinosine and 2 unidentified molecules as effective candidate biomarkers [16]. Recently, a combination of GC- and LC-MS was used to compare the plasma metabolomic profiles of CKD stages 2, 3 and 4. An altered arginine metabolism, elevated coagulation/inflammation, an impaired carboxylate anion transport and decreased adrenal steroid hormone production were revealed to be related to CKD severity [17]. In addition, some LC-MS based metabolomics studies have been performed on serum and urine of rats [19-21]. Apart from GC and LC hyphenated to MS, also CE-MS has recently been explored in the search for CKD biomarkers [22]. Twenty-two cations and 30 anions were found to accumulate significantly with decreasing eGFR. In addition, also five new anions and seven cations decreasing significantly with declining eGFR were identified. Each compound was evaluated for its suitability to detect early renal damage more sensitively than creatinine and cystatin C. 1-Methyladenosine, N-acetylglucosamine, γ-butyrobetaine, sebacate, cis-aconitate and homovanillate were reported to be candidate markers for detecting the early stages of renal disease in CKD patients.

Further contributing to the quest for better disease biomarkers and novel uremic retention solutes, the present essay describes a non-targeted metabolomics analysis based on reversed-phase (RP) LC-Q-TOF MS and GC-MS comparing a healthy control group and two patient groups suffering from different stages of CKD, i.e. CKD stage 3 (CKD3) and CKD stage 5 on haemodialysis (CKD5HD). Following the initial rapid increase in publications in the field of metabolomics, it is now becoming more and more important to provide evidence of the validity of the methodology [23]. As this aspect was often lacking in previous metabolomics studies on CKD, the current paper places great emphasis on the validity of the metabolomics dataset by using quality control samples and compares the findings with the reviews on uremic retention solutes and the previously mentioned metabolic profiling studies.

III.3. Experimental

III.3.1. Materials

III.3.1.1. Chemicals

Water (ULC–MS grade), acetonitrile (HPLC-S grade), methanol (ULC-MS grade), isopropanol (HPLC grade) and formic acid (LC-MS grade) were purchased from Biosolve BV (Valkenswaard, The Netherlands). Pyridine dried (max 0,0075 % H_2O), methoxyamine hydrochloride, N-methyl-N-

(trimethylsilyl)trifluoroacetamide with 1 % trimethylchlorosilane, myristic-d $_{27}$ acid were purchased from Sigma-Aldrich-Fluka (Sigma-Aldrich, Schnelldorf, Germany).

III.3.1.2. Study samples

Serum was collected at the University Hospital Ghent (Ghent, Belgium) by sampling blood by venipuncture from 40 patients diagnosed with chronic kidney disease (CKD) and 20 healthy controls using Venosafe™ Plastic tubes containing both a gel and a clot activator (VF-1065SAS, Terumo Europe NV, Leuven, Belgium). The blood was allowed to clot for 30 min on the bench after which it was centrifuged at 3,000 rpm for 10 min at room temperature. Aliquots of 500 µL serum were stored at −80 °C and shipped on dry ice to Metablys (Kortrijk, Belgium) for analysis. Table III.1 represents the clinical characteristics of the retained subjects. From the 40 patients included, 20 patients are classified into CKD stage 3 (CKD3) and 20 into CKD stage 5 on haemodialysis (CKD5HD), based on their glomerular filtration rate estimated from the serum creatinine values as prescribed by the KDOQI guidelines. The underlying etiology of the CKD patients was of vascular (n = 13), glomerular (n = 3) or interstitial (n = 7) origin, polycystic kidney disease (n = 4), nephrectomy (n = 4), transplant failure (n = 4) other origin (n = 4) or unknown (n = 1). Patients on HD received therapy three times a week and were sampled prior to the midweek session. All patients included were non-diabetic with the exception of 2 CKD5HD patients. Enrolment occurred after informed consent and serum samples were collected between January 5th and March 7th of 2011. The protocol was approved by the local Ethics Committee.

Table III.1 Clinical characteristics of the included patients

	Healthy	CKD3	CKD5HD	*p*-value
Number	20	20	19	-
Age	33.8 ± 13.6	60.3 ± 14.0*	67.9 ± 12.6*	1.00E-04
Male/Female	9/11	12/8	13/6	NS
BMI (kg/m²)	22.7 ± 3.9	26.0 ± 4.0	27.8 ± 6.3*	5.00E-03
Syst BP (mm Hg)	125 ± 16	134 ± 24	146 ± 26*	1.50E-02
Diast BP (mm Hg)	77 ± 10	82 ± 11	67 ± 15*°	1.00E-03
Pulse	69 ± 10	68 ± 8	70 ± 14	NS
CTN (mg/dL)	0.92 ± 0.19	1.49 ± 0.30	7.55 ± 2.52*°	1.00E-04
CRP (mg/L)	0.16 ± 0.17	0.21 ± 0.15	1.08 ± 1.47*°	1.00E-03

$p < 0.05$ in Tukey's test vs. Healthy*, vs. CKD3°; NS: not significant; BMI: body mass index; BP blood pressure; CTN: creatinine; CRP: C-reactive protein

III.3.2. Sample preparation

III.3.2.1. Preparation of QC samples

Hundred µL aliquots of the 60 study samples were collected in a quality control (QC) pool. This QC pool was then divided into 50 µL aliquots to obtain representative QC samples. QC samples were prepared simultaneously along with study samples and were analyzed throughout both the LC-MS and GC-MS analysis sequences every defined number of study samples (every four study samples in case of GC-MS, every five study samples in case of LC-MS). These samples did not contain any biological variability and can thus be considered as technical replicates.

III.3.2.2. GC-MS specific sample preparation

Sample preparation for GC-MS analysis is based on an earlier published method [24]. Briefly, 10 µL of a 3 µg/µL internal standard solution (myristic-d_{27} acid) was added to 50 µL of serum, thawed at room temperature. Proteins were precipitated using 500 µL of ice-cold methanol (−20 °C). After homogenization and conservation at −20 °C during 20 min, samples were centrifuged (10 min at 15,000 rpm). The upper phase was transferred to a 1.5 mL clear extreme recovery glass vial (Agilent Technologies, Waldbronn, Germany) and dried under nitrogen. Next, a two-step derivatization was performed. Methoxyamination was performed by adding 100 µL of a 20 mg/mL solution of methoxyamine hydrochloride in pyridine, vortexing for 1 min and heating at 30 °C for 90 min. An aliquot of 100 µL N-methyl-N-(trimethylsilyl) trifluoroacetamide with 1 % trimethylchlorosilane (MSTFA + 1 % TMCS) was subsequently added, vortexed for 1 min and heated at 37 °C for 60 min. Derivatized samples show limited stability [24], so injection was performed within 24 h after the derivatization process.

III.3.2.3. LC-MS specific sample preparation

Serum samples were thawed at room temperature. 50 µL serum was transferred to 0.5 mL Eppendorf tubes and 200 µL of ice-cold methanol (−20 °C) was added. After vortex mixing during 10 s, samples were placed at −20 °C for 20 min. Next, the samples were centrifuged (10 min at 15,000 rpm) and 200 µL of the supernatant was dried in a centrifugal vacuum evaporator (miVac duoconcentrator, Genevac Lim., Ipswich, UK). The dried extracts were redissolved in 100 µL water/acetonitrile 95/5 (v/v) and transferred in a new 0.5 mL Eppendorf tube. Next, the samples were centrifuged for 10 min at 15,000 rpm, after which 80 µL of supernatant was transferred to a vial with glass insert for LC-MS analysis.

III.3.3. Gas chromatography-mass spectrometry

GC-MS analyses with electron ionization (EI) were performed on an Agilent 7890A GC combined with an MSD detector (5975C inert XL MSD with Triple-Axis Detector, Agilent) controlled by MSD

Chemstation for data acquisition (Agilent Technologies). Separation was performed on a DuraGuard DB-5MS capillary column (30 m × 0.25 mm × 0.25 µm with a 10 m guard capillary; Agilent Technologies) [25]. Injections (1 µL) were performed at 250 °C in splitless mode using a MultiPurpose Sampler MPS (Gerstel, Mülheim, Germany). The oven temperature program was 60 °C for 1 min, 10 °C/min to 325 °C (10 min). Helium was used as carrier gas in constant flow mode at a linear velocity of about 35 cm/s (0.92 mL/min, about 46 kPa at 60 °C). Retention time was locked to myristic-d $_{27}$ acid at 16.727 min. These conditions are based on the library developed by Kind et al. [26]. Electron ionization (EI) was used and MS was performed in scan mode (m/z 50-600) with the MS quadrupole at 150°C and MS ion source at 250°C. The system was tuned using perfluorotributylamine (PFTBA).

The GC-MS analytical block has been divided into five analytical batches to fulfil the 24 h sample stability criterion with 12 study samples per batch, four conditioning samples (QC samples), four QC samples and eight blank samples, equally divided before and after the analytical batch. Derivatized samples were re-randomized for GC-MS analysis. During analysis, samples were stored on the autosampler tray at room temperature. Figure III.1 displays representative GC-MS chromatograms of serum from a healthy control and a CKD5 patient.

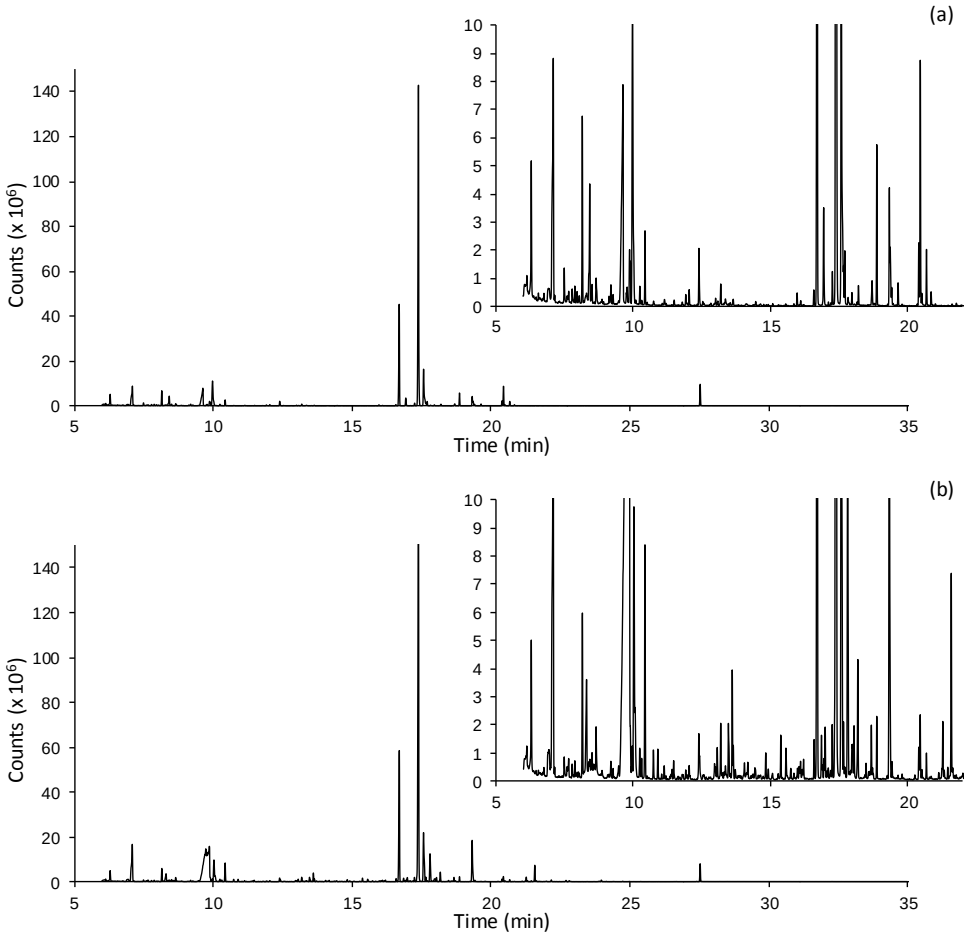


Figure III.1 Representative TIC chromatograms of serum from (a) healthy control and (b) CKD5 patient

III.3.4. Liquid chromatography-mass spectrometry

A 1200 RRLC system (Agilent Technologies) hyphened to a high-resolution accurate mass 6530 Q-TOF mass spectrometer (Agilent Technologies), equipped with a Jetstream ElectroSpray Ionization (ESI) source, was used for LC-MS measurements. All instruments were controlled by MassHunter Workstation Acquisition 4.0 (Agilent Technologies). Metabolites were separated on an Acquity HSS T3 C18 column (2.1 × 100 mm; 1.8 µm; Waters, Milford, MA, USA) at 40 °C. Elution was carried out with a gradient of (A) 0.1 % formic acid and (B) acetonitrile with 0.1 % formic acid, starting from 0 to 100 % B in 20 min. The flow rate was 0.35 mL/min and the injection volume 10 µL. The whole system was allowed to re-equilibrate under starting conditions for 5 min. The MS instrument was operated in both positive and negative ESI modes. MS/MS experiments were performed in both targeted MS/MS mode and auto MS/MS mode (i.e. data dependent acquisition mode or DDA). Needle voltage was optimized to +/- 3.5 kV, the drying and sheath gas temperatures were set to 300°C and the drying and sheath gas flow rates were set to 6 and 8 L/min, respectively. Data were collected in centroid mode from *m/z* 50–1,700 at an acquisition rate of 1 spectrum/sec in the extended dynamic range mode (2 GHz), offering an in-spectrum dynamic range of 10^5 and a resolution of ± 10,000 full width at half maximum (FWHM) in the metabolite *m/z* range. To maintain mass accuracy during the analysis sequence, a reference mass solution was used containing reference ions (121.0508 and 922.0097 for positive ESI mode, and 112.9856 and 1033.9881 for negative ESI mode). The Q-TOF instrument was tuned using the ESI-L low concentration tuning mix (Agilent Technologies) prior to the analysis sequence. For auto MS/MS mode, a survey MS scan was alternated with three DDA MS/MS scans resulting in a cycle time of 2 s (acquisition rate of 2 spectra/sec). Singly charged precursor ions were selected based on abundance. After being fragmented twice, a particular m/z value was excluded for 30 s, allowing the MS/MS fragmentation of chromatographically resolved isomers. The quadrupole was operated at narrow resolution and the collision energy was fixed at 10, 20 or 40 eV, respectively. Targeted MS/MS mode fragmented listed precursor ions in a defined retention time window (± 0.15 min) at fixed collision energies of 10 and 20 eV.

The LC-MS analysis was performed in one batch, separately for positive and negative ESI measurement. Each analysis sequence started with two blank runs and six conditioning samples (QC samples). Sixty study samples were analyzed in randomized order in both ionization modes, with QC samples (n = 12) analyzed every five study samples. Samples were kept at 4 °C in the autosampler tray. Representative TIC chromatograms (positive ESI) of serum from a healthy control and a CKD5 patient are shown in Figure III.2.

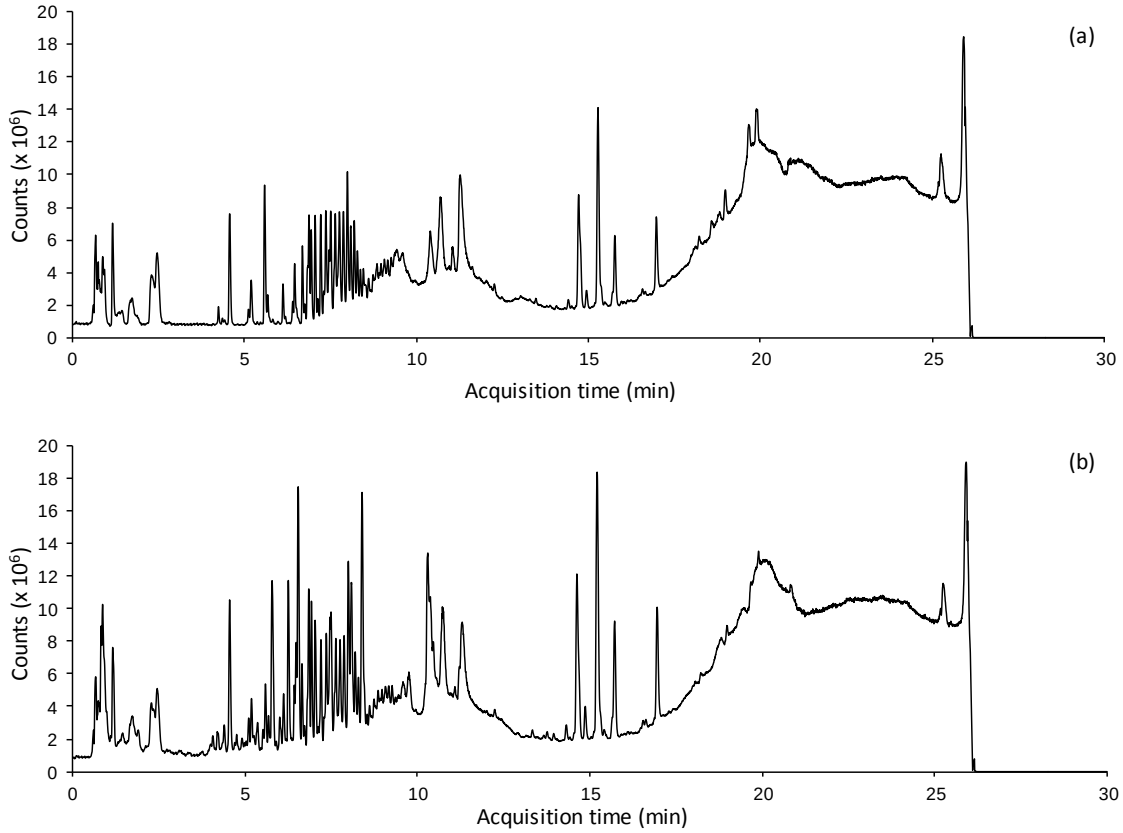


Figure III.2 Representative TIC chromatograms of serum from (a) healthy control and (b) CKD5 patient

III.3.5. Data analysis

Figure III.3 presents a schematic overview of the data analysis workflow applied for both LC-MS and GC-MS acquired data. LC-MS raw data files were first subjected to "naïve" untargeted data processing by the Molecular Feature Extraction (MFE) algorithm incorporated in the MassHunter Qualitative Analysis 5.0 software package (Agilent Technologies). This algorithm localizes and combines all related ions (molecular ion, isotopes and adducts) with the generation of a single mass (median), retention time (peak apex) and an abundance (sum of all ions). After MFE, MassProfiler Professional 12.0 (Agilent Technologies) was used to align, visualize and filter the extracted features. Subsequently, a targeted feature extraction, based on the Find by Ion algorithm in MassHunter Qualitative Analysis 5.0, searched the raw data files de novo for these listed features based on matching their composite spectrum (mass, isotopes, adducts) and their specific retention time in a user-defined mass and retention time window. This targeted approach greatly reduces the number of false negatives and positives generated by the MFE algorithm by re-evaluating the missing values in the data matrix hereby increasing the quality of the dataset. Targeted feature lists of each sample were again aligned and filtered in MassProfiler Professional, thereby obtaining a final dataset ready for statistical analysis. Statistically significant metabolites were identified using the capabilities of the Q-TOF mass spectrometer and its accompanying software tools. GC-MS data processing started with

XCMS based untargeted feature extraction and alignment. The XCMS algorithm does not classify spectral ions originating from the same compound. Hence, the resulting feature list encloses multiple ions for each individual metabolite detected. An in-house built feature reduction tool selected and retained the most abundant feature per defined retention time. Only the most abundant spectral ion for each detected metabolite was withdrawn in the reduced feature list. The aligned XCMS data matrix was subsequently imported in MassProfiler Professional for statistical analysis. Differential metabolites were investigated de novo in a targeted data processing step, which incorporates a deconvolution in AMDIS and manual integration in MSD ChemStation. After statistical confirmation of these metabolites, the identification process, based on EI (RTL) library searching, took place.

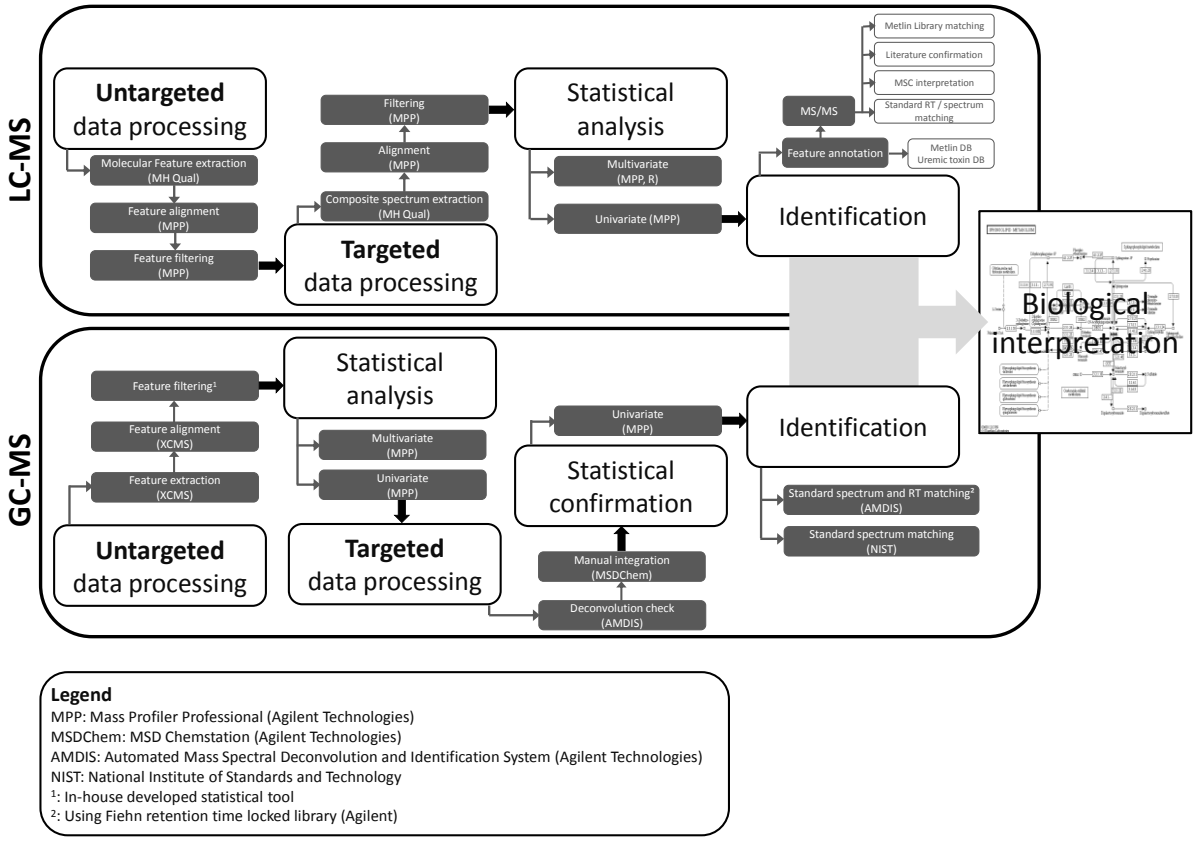


Figure III.3 Schematic representation of the applied data analysis approach for LC-MS and GC-MS. (MH Qual - MassHunter Qualitative Analysis; MPP - MassProfiler Professional; MSC – Molecular Structure Correlator; DB – database; AMDIS - Automated Mass Spectral Deconvolution and Identification System; MSDChem – MSD Chemstation; NIST - National Institute of Standards and Technology)

LC-MS data processing is documented in detail in Figure III.4.

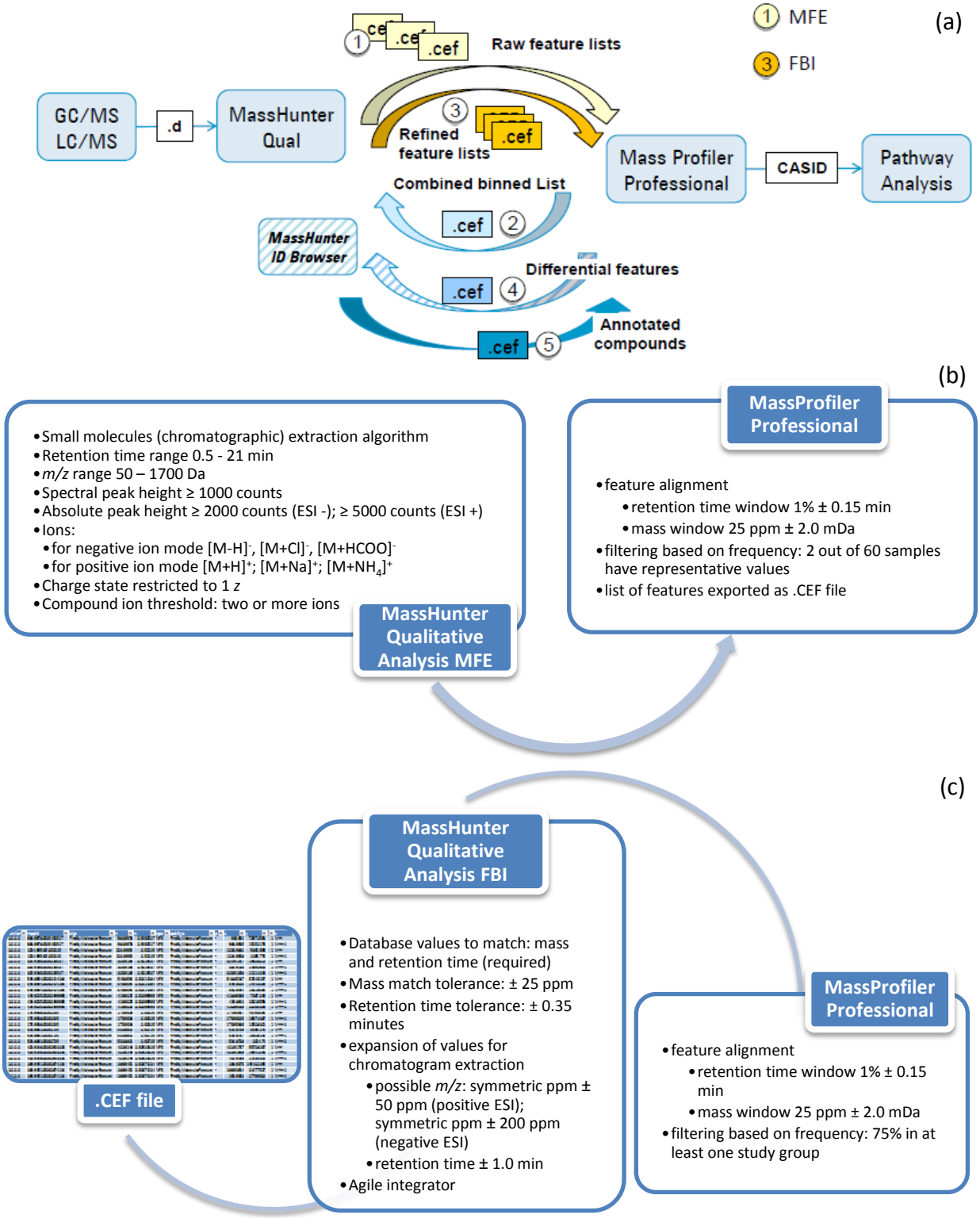


Figure III.4 Details of the data processing pipeline applied for LC-MS data: (a) visual representation of the workflow suggested by the instrument vendor, (b) untargeted feature extraction: features are detected using the Molecular Feature Extraction (MFE) algorithm in MassHunter Qualitative Analysis 5.0 (Agilent Technologies). The resulting feature list is imported into MassProfiler Professional 12.0 (Agilent Technologies), aligned and filtered. (c) Targeted feature extraction: compounds listed in a .CEF are extracted from the raw data files based on accurate mass and retention time by Find By Ion (FBI) in MassHunter Qualitative Analysis 5.0. The Find by Ion algorithm enhances the quality of the dataset by reducing the number of false negatives and positives

GC-MS total ion chromatogram (TIC) data were exported to AIA format data files by MSD ChemStation for Data Analysis (Agilent Technologies) and were then processed by XCMS software [27]. XCMS software, version 1.30.3 running under the R package version 2.14.1., is freely available under an open-source license at http://metlin.scripps.edu/download/, and incorporates nonlinear retention time alignment, matched filtration, automatic peak detection and peak matching. The matched filter algorithm was used with default values for all parameters, except for "fwhm" and "snthresh" which were respectively set at 6 and 3. In the grouping, "bw" was set to 1.5. The resulting data matrix was then exported as acsv file and reduced by an in-house built feature reduction tool. After grouping all features according to retention time, the tool only keeps the most intense ion per retention time group, based on the quality control samples. The reduced feature list was verified and features representing low reproducibility and/or originating from column bleeding were removed. In order to correct a loss of sensitivity of the detection along the experiment, all intensities were normalized by the average intensity of the next QC sample analyzed in the sequence.

Final LC- and GC-MS datasets were independently imported in MassProfiler Professional 12.0 for statistical data analysis. All sample groups were analyzed in parallel (Healthy vs CKD3, Healthy vs CKD5HD, CKD3 vs CKD5HD), using univariate statistics based on unpaired Mann-Whitney testing with Benjamini-Hochberg false discovery rate correction (FDR) [28] to reduce the probability of Type I errors (i.e. false positives). Statistically significant features from pairwise comparisons were combined in the Venn Diagram function, where the intersection, containing features that were significantly different between all studied sample groups, was maintained. Multivariate statistical analysis, i.e. principal component analysis (PCA), was performed in R [29].

Differential metabolites detected by LC-MS were first annotated by accurate mass (<5 ppm) using the Metlin database (Agilent Technologies) [30,31] and an in-house built uremic toxin database generated based on literature information [2,3,19,32,33]. MS/MS fragmentation data were used to confirm metabolite identification. MS/MS spectra were matched on spectra of standard compounds, on the Metlin spectral library (Agilent Technologies) [34-36] compared with literature or interpreted using MassHunter Molecular Structure Correlator (MSC; Agilent Technologies). Metlin spectral library matching was performed using the reverse scoring option, where library spectra are matched against the acquired spectra, after which false positives were removed by manual verification. MSC tries to explain each observed fragment ion into the proposed structure using a "systematic bond-breaking" approach as described by Hill and Mortishire-Smith [37]. Molecular structures from Metlin, Human Metabolome Database (HMDB) and ChemSpider were imported into MSC. Differential metabolites detected by GC-MS were identified in AMDIS by using the Fiehn retention time locked (RTL) library (Agilent Technologies) [26] and NIST 11 Mass Spectral Search Program.

III.4. Results and discussion

III.4.1. Quality of the analysis

III.4.1.1. LC-MS

The validity of the performed LC-MS based analyses has been monitored in both a targeted and a non-targeted manner using the QC samples. The former applies a list of randomly selected metabolites to determine the error of the measurement on signal intensity (peak area), retention time and mass accuracy (Table III.2). Peak area fluctuations are typically below 10 % relative standard deviation (RSD, n = 12). Metabolites that elute in the polar bulk of the chromatogram, e.g. creatinine, also show repeatable signal intensities. Chromatographic retention time reproducibility and mass accuracy are satisfactory, two characteristics primordial towards feature alignment.

Table III.2 Relative standard deviation (RSD) of peak area and retention time, and average mass accuracy of randomly selected metabolites measured in QC samples (n=12), detected in LC-MS. Creatinine, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), sucrose, tryptophan and warfarin were detected in positive ESI mode, while guanosine, pantothenic acid, phenyl sulphate, tyrosine and uric acid were detected in negative ionization mode

Metabolite	Mass	Average mass accuracy (ppm)	tR	RSD tR	RSD AUC
Creatinine	113.0589	1.91	0.84	0.28%	3.19%
CMPF	240.0998	0.99	10.48	0.26%	6.16%
Sucrose $[M+Na]^+$	364.0982	0.61	1.01	0.21%	7.22%
Tryptophan	204.0899	3.27	5.60	0.20%	4.18%
Warfarin	308.1049	2.67	12.24	0.84%	7.34%
Guanosine	283.0917	1.87	4.25	0.33%	4.24%
Pantothenic acid	219.1107	2.67	5.11	0.14%	5.62%
Phenyl sulphate	173.9987	4.96	5.60	0.21%	5.26%
Tyrosine	181.0739	2.64	2.35	0.41%	4.19%
Uric acid	168.0283	3.73	1.75	0.57%	4.62%

Next to this targeted approach, the reproducibility of the applied metabolomics analysis can also be visualized in a more comprehensive way by calculating the error on all detected features in the QC samples and representing the acquired RSD distribution (Figure III.5).

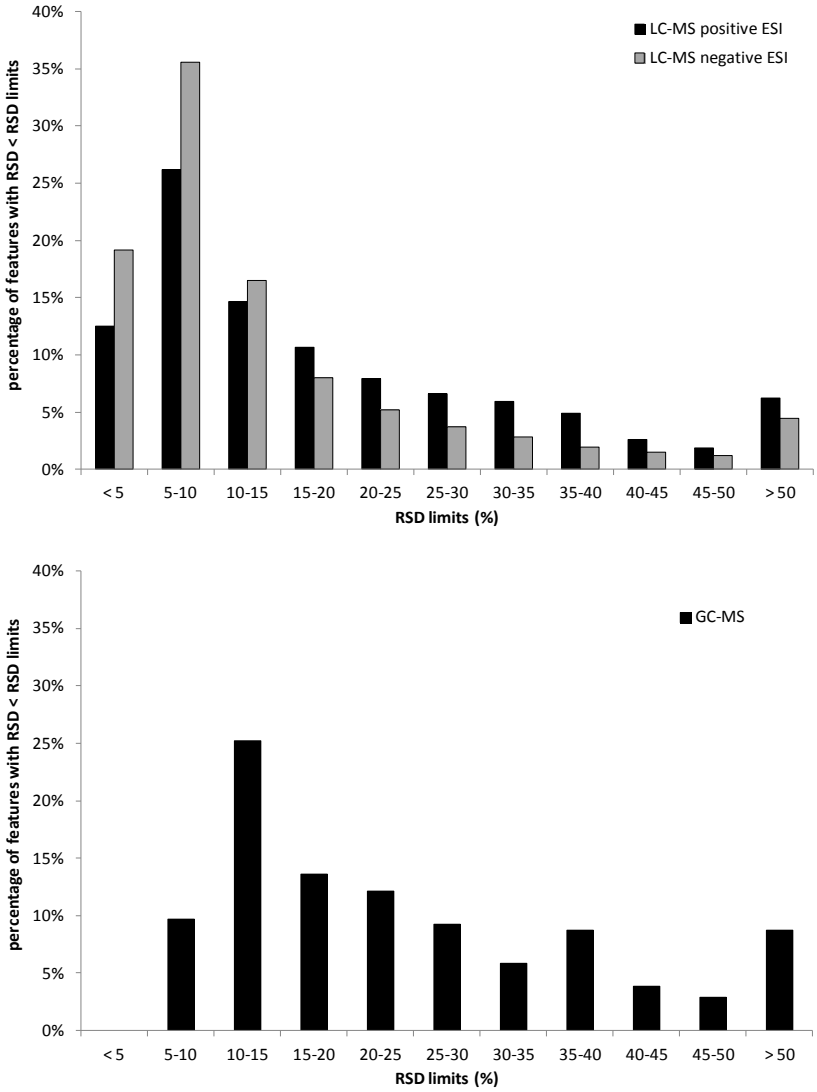


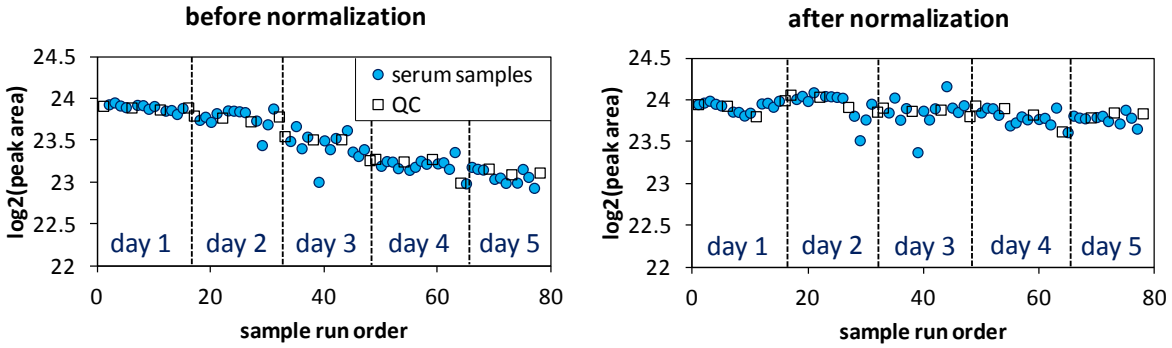
Figure III.5 RSD distribution plot displaying the technical repeatability of (a) the LC-MS analysis in both positive and negative ESI mode and (b) the GC-MS analysis. The stability of the feature signals is expressed as relative standard deviation (RSD) values, calculated for each feature as the standard deviation of the peak area in all QC samples divided by the average of the peak area in all QC samples. RSD is calculated for features with 100% frequency after all data processing steps

For LC-MS analysis in positive ESI; 4,043 features or 53.36 % of all features (7,577 features were detected with 100 % frequency in all QC samples) show RSD values below 15 %; the majority of features (78.49 % or 5,947 features) had an RSD below 30 %, which can be defined as the upper limit for untargeted or discovery metabolomics analysis [38]. LC-MS analysis in negative ESI displayed 2,665 features or 71.24 % of all features (3,741 features were detected with 100 % frequency in all QC samples) with an RSD value below 15 %, while 3,298 features or 88.16 % of features had an RSD value below 30 %.

III.4.1.2. GC-MS

The limited stability of derivatized compounds in GC-MS analysis demanded a "batched" type of analysis, opposite to LC-MS analysis [39]. QC samples demonstrated a low reproducibility of the GC–MS analysis due to a drop of the signal intensity along the sequence, more specific between the different batches. This largely impairs the true biological variation between the analyzed samples and thus the validity of the dataset. A classical normalization approach using the internal standard peak area (myristic-d_{27} acid) did not correct for the signal intensity drop [40]. After the evaluation of different approaches, normalization was performed by dividing the measured peak area of each detected metabolite by the total abundance of the next QC sample in the sequence. The latter corresponded best to the acquired decrease in signal intensity within and between the different batches. Figure III.6 displays the signal intensity of the retention time locked internal standard myristic-d_{27} acid plotted versus the analytical run order; next to a PCA based representation where the first principal component is plotted against the analytical run order, both before (left plots) and after normalization (right plots). Reproducibility for the retention time locked internal standard over the entire sequence (n = 78) improved from 22.36 to 8.79 %, respectively without and with normalization.

(a)



(b)

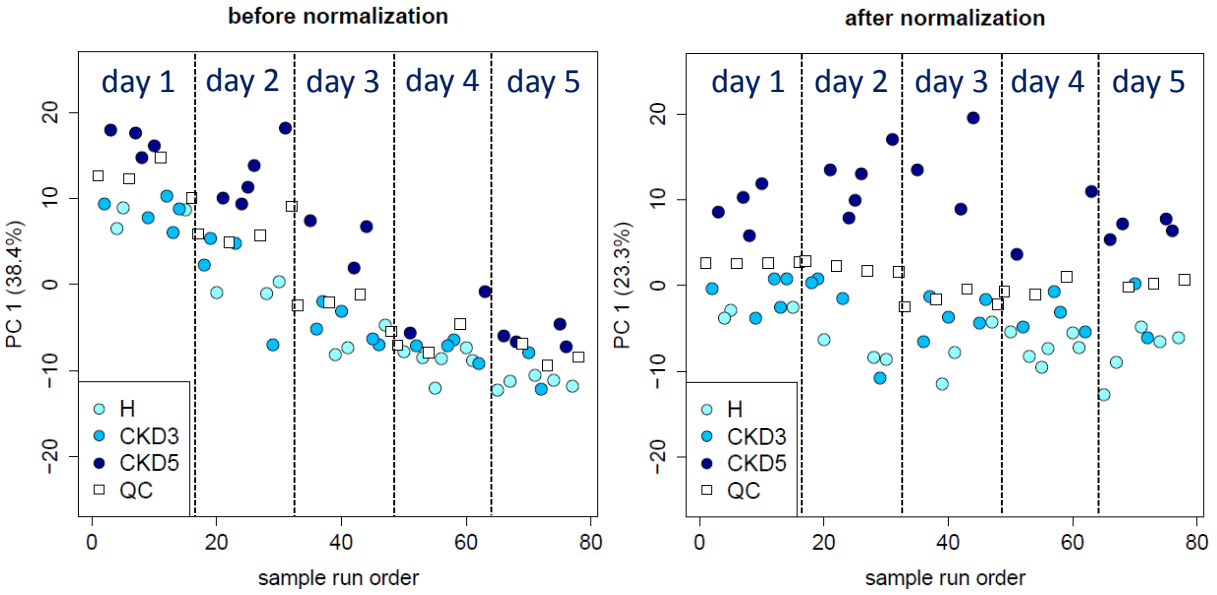


Figure III.6 Effect of normalization by total abundance of next QC on (a) the internal standard (myristic-d_{27} acid) peak area, shown as log_2(peak area) versus sample run order, and (b) on the first principal component from PCA. In both representations, the decreasing trend in signal intensity is neutralized by the normalization step, which makes samples comparable between different analysis batches

Targeted quality control for GC-MS revealed a peak area RSD generally lower than 15 %, both within-batch and between-batch (Table III.3), confirming the reproducibility of the GC-MS data after normalization. A more global picture by defining the RSD distribution on all detected features in the QC samples shows a lower reproducibility of the GC-MS analysis compared to the LC-MS analyses (Figure III.5). Only 34.95 % or 72 of the detected features show an RSD value below 15 %, while 144 features or 69.90 % of features had an RSD value below 30 %.

Table III.3 Relative standard deviation (RSD) of retention time (locked) and peak area of the target ion of randomly selected metabolites measured in QC samples (n=4 within-day, n=20 between-day), detected in GC-MS. The between-day RSD values are calculated as the averages of the within-day RSD of five days. The between-day RSD of the peak area has been subjected to normalization to correct for the between-day variability of the GC-MS analysis

Metabolite	Target Ion Mass	tR	RSD tR within-batch	RSD tR between-batch	RSD AUC within-batch	RSD AUC between-batch
L-(+) lactic acid	147	7.12	0.06%	0.09%	10.02%	10.33%
L-valine	144	9.20	0.01%	0.05%	8.10%	14.32%
Citric acid	73	16.59	0.01%	0.04%	8.43%	14.38%
Hippuric acid	105	16.86	0.01%	0.04%	9.29%	13.56%
D-sorbitol	205	17.83	0.01%	0.02%	4.07%	9.20%
Palmitic acid	117	18.88	0.00%	0.04%	3.39%	8.76%
L-tryptophan	202	20.44	0.01%	0.03%	9.47%	11.68%
Oleic acid	117	20.46	0.01%	0.02%	3.17%	8.68%
Pseudouridine	357	21.47	0.01%	0.02%	7.77%	12.43%
Cholesterol	129	27.54	0.00%	0.02%	3.52%	6.58%

III.4.2. Revealing and identifying differential metabolites

III.4.2.1. LC-MS

Generally, data analysis in metabolomics experiments comes to narrowing down complex data matrices to a list of biologically relevant metabolites. Table III.4 demonstrates the feature reduction in numbers throughout the successive LC-MS data processing steps.

Table III.4. Feature reduction throughout the LC-MS data processing

Data processing step	Positive ESI	Negative ESI
Molecular feature extraction (MFE)	24,735	11,312
Find by Ion; Frequency filter (75%; used for univariate statistics)	10,355	4,240
$p < 0.05^{\Phi}$	592	685

$^{\Phi}$ Only features that showed an increase or decrease throughout the different stages of CKD were withdrawn: Healthy > CKD3 > CKD5HD or Healthy < CKD3 < CKD5HD.

PCA models were built with exclusion of the QC samples and one CKD5HD outlier (Figure III.7). PCA of the CKD sample sets gives similar results for both positive and negative ionization acquired data sets, clearly classifying the three study groups from healthy up to CKD5HD. Statistical univariate analysis, based on Mann-Whitney unpaired analysis (Benjamini-Hochberg FDR corrected) was subsequently performed on the 75 % frequency datasets. This metabolomics inquiry mainly focused on identifying small molecules that showed an evolution related to the disease progression (Healthy > CKD3 > CKD5HD or Healthy < CKD3 < CKD5HD). Hence, only features were retained that were upregulated or downregulated from healthy to CKD5HD. In positive ESI, 495 features were mounting during CKD progression, while 97 features were decreasing with CKD progression (Table III.4). Negative ESI unravelled 435 features that were upregulated, next to 250 features that were downregulated with CKD progression.

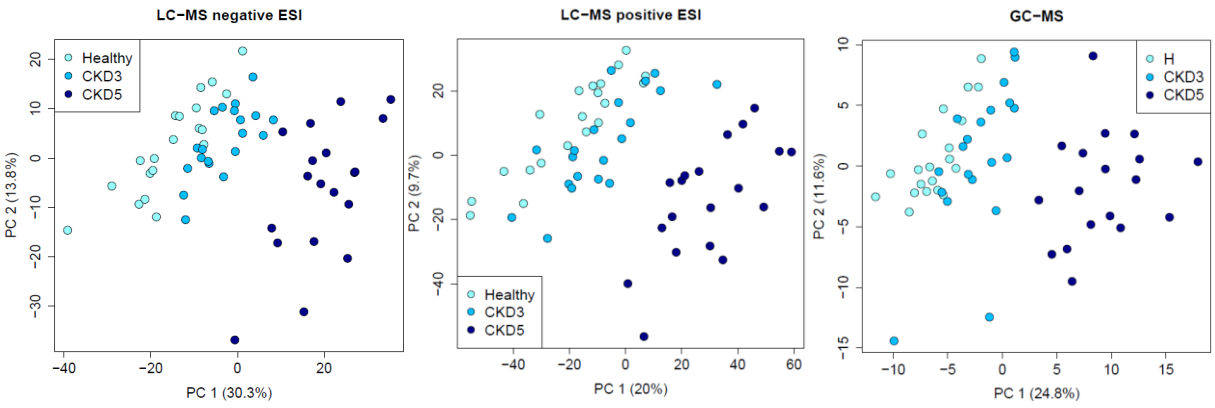


Figure III.7 Principal component analysis of all samples analyzed on the different analytical platforms: LC-MS in negative ESI mode, LC-MS in positive ESI mode and GC-MS. Samples are colour-coded according to their class: healthy (light blue); CKD3 (medium blue); CKD5HD (dark blue). PCA models were built with 100% frequency datasets representing 3,634 features for LC-MS positive ESI mode, 906 features for LC-MS negative ESI mode and 206 features for GC-MS. PCA distinguishes the sample groups according to the degree of CKD severity; from healthy individuals up to CKD5HD patients

A major bottleneck in LC-MS based discovery metabolomics is the accurate identification of low molecular weight compounds of interest. Generally, only a small fraction of significantly up- or downregulated metabolites can be identified. Our identification strategy fully exploited the features of the high-resolution Q-TOF-MS system. The generation of molecular formulas, based on accurate mass, isotopic abundance and spacing, was complemented with accurate mass (<5 ppm) database searching in the Metlin database and an in-house built uremic retention solute database. This so-called rough feature "annotation" resulted in 159 metabolite hits (26.9 %) for positive ESI and 213 annotations (31.1 %) for negative ESI. To validate the initial annotations of features, MS/MS fragmentation was performed. Acquired MS/MS spectra were verified using different approaches, ranked according to the degree of confidence: (a) retention time and MS/MS spectra matching with standard metabolites; (b) spectral matching with the Metlin library [34,35] (c) confirmation with fragmentation reported in literature; (d) interpretation of MS/MS spectra with Molecular Structure Correlator (MSC). Figure III.8 displays an example of spectral library matching and MSC spectral interpretation. MassHunter spectral library matching automatically searches tandem mass spectrometry (MS/MS) data against high quality experimental MS/MS data from known metabolites contained in the Metlin library, thereby generating a score based on shared peak count and accurate mass of fragment ions. Figure III.8a displays the acquired fragmentation spectrum for a compound with molecular formula $C_{11}H_{11}NO_3$, where Figure III.8b represents the reference Metlin library spectrum. Database searching based on accurate mass identified three possible compounds with different molecular structures, i.e. 3-indoleacetic acid, 5-methoxyindoleacetate and cinnamoylglycine. Matching the acquired fragmentation spectrum with the Metlin spectral library designated cinnamoylglycine as the correct identification with a matching score of 97.24. MSC correlates accurate mass MS/MS fragment ions for a compound of interest with one or more proposed molecular structures for that compound by using a systematic bond-breaking approach [37]. Figure III.8c displays the fragmentation spectrum acquired for 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), which is a well-known uremic toxin [2,3,32,33]. All fragment ions are labelled with the most plausible fragment structure calculated by MSC and their individual MSC score. In-source fragmentation of metabolites was investigated to avoid false positive identifications. By way of example, p-cresol and glutamine were detected as increasing metabolites in CKD progression ($p < 0.05$). A closer look in the data unravelled these metabolites as in-source fragments of p-cresyl sulphate and phenylacetylglutamine, respectively. Worth noting that p-cresol has long been reported as uremic toxin while it has typically been measured as a sample preparation artefact of p-cresylsulfate [41]. Several metabolites were identified as in-source fragments and excluded from further interpretation.

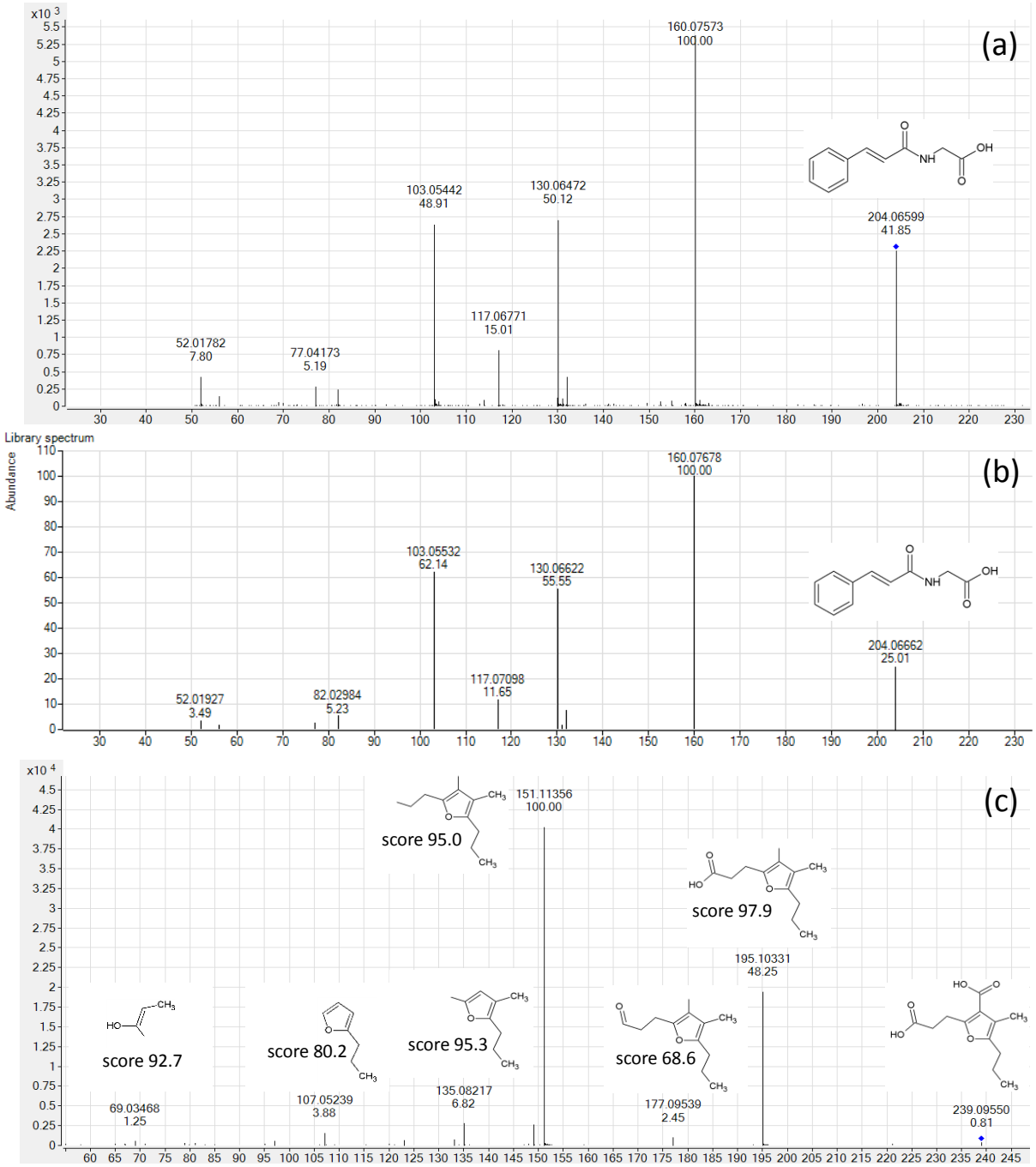


Figure III.8 MS/MS identification strategy using (a-b) Metlin library matching, where the acquired fragmentation spectrum a of $C_{11}H_{11}NO_3$ is matched against the Metlin library. The acquired MS/MS spectrum b matched with the Metlin library fragmentation spectrum of cinnamoylglycine with a reverse matching score of 97.24. c MS/MS identification strategy using molecular structure correlator, where the fragment ions of $C_{12}H_{16}O_5$ are associated to fragment ions of 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid with a total MSC score of 96.32. The most plausible fragment structure and the score of each individual fragment given by MSC are displayed. The accurate mass and relative abundance (compared to most intense ion, in percent) are given for each spectral ion

III.4.2.2. GC-MS

Feature reduction during GC-MS data processing is presented in Table III.5.

Table III.5 Feature reduction throughout the GC-MS data processing

Data processing step	Feature number
XCMS feature detection	2,657
Feature reduction tool	229
Removal of siloxane contaminant peaks	206
$p < 0.05^{\phi}$	23

$^{\phi}$ Only features that showed an increase or decrease throughout the different stages of CKD were withdrawn: Healthy > CKD3 > CKD5HD or Healthy < CKD3 < CKD5HD.

PCA on the resulting feature list clearly separates the studied sample groups (Figure III.7). Statistical univariate analysis maintained 23 metabolites that show either an increasing or decreasing evolution from healthy to CKD5HD. Differential metabolites were identified in AMDIS by using the Fiehn RTL library (e) and NIST standard spectrum library (f). In analogy to LC-MS, p-cresol was identified; again representing a sample preparation artefact of p-cresyl sulphate generated under the acidic derivatisation conditions used [41]. Glutamine, on the other hand, shown to be an in-source fragment in LC-MS, could be revealed by GC-MS.

III.4.3. Uremic retention solutes in CKD

Metabolites showing a significant change in concentration in serum from healthy controls to CKD patients at stage 3 (CKD3) to stage 5 on haemodialysis (CKD5HD) ($p < 0.05$), are listed in Tables III.6 and III.7. The identification of all these metabolites was confirmed as marked (a-f), in accordance to the above described identification strategy. A distinction has been made between (1) uremic retention solutes/uremic toxins that have already been described in literature (Table III.6) and (2) unknown compounds (Table III.7) which show an increasing trend, surpassing the expansion of creatinine, from healthy up to CKD5HD, or a decreasing trend.

Table III.6 List of earlier reported uremic retention solutes confirmed in the present study

Analysis	Metabolite	Formula	ID	Ref.	Mass[a]	RT	*p* (CKD3 vs. H)	FC (CKD3 vs. H)	*p* (CKD5HD vs. CKD3)	FC (CKD5HD vs. CKD3)	*p* (CKD5HD vs. H)	FC (CKD5HD vs. H)
1a	Alanine	$C_3H_7NO_2$	a	[16]	89.0477	2.41	4.63E-06	1.51	2.56E-07	2.88	1.75E-07	4.34
1a	CMPF	$C_{12}H_{16}O_5$	b	[2]	240.0998	10.47	1.42-02	2.35	2.54E-02	1.95	7.94E-05	4.57
1a	Cinnamoylglycine	$C_{11}H_{11}NO_3$	b	[44]	205.0738	7.75	2.46E-04	1.57	2.13E-04	1.71	4.23E-07	2.68
1b			b	[44]	205.0743	7.81	7.98E-04	1.54	7.79E-04	1.64	3.41E-06	2.52
1a	Creatinine	$C_4H_7N_3O$	a	[2]	113.0579	0.84	1.34E-02	1.21	2.61E-06	2.19	1.36E-06	2.65
2a			e	[2]	113.0579	13.63	1.92E-05	2.02	1.70E-06	6.25	3.70E-07	12.64
1b	*Dehydroisoandrosterone sulfate*	*$C_{19}H_{28}O_5S$*	*b*	*[17]*	*368.1682*	*10.07*	*4.75E-04*	*-3.16*	*2.80E-02*	*-2.58*	*4.33E-06*	*-8.13*
1a	Dimethylguanosine	$C_{12}H_{17}N_5O_5$	c, [52]	[2]	311.1225	5.19	4.06E-06	1.75	2.27E-07	10.12	1.69E-07	17.71
1a	Disaccharide (sodium adduct)[c]	$C_{12}H_{22}O_{11}$	-	[15]	364.0982	0.97	2.80E-05	3.61	1.28E-07	11.40	1.09E-07	41.12
1b	Disaccharide[c]		-	[15]	342.1162	0.98	1.77E-05	2.21	1.28E-07	9.07	1.09E-07	20.07
2a	Disaccharide: Sucrose		e	[15]	342.1162	24.00	3.97E-04	2.53	1.70E-06	49.50	3.70E-07	125.36
2a	Erythronic acid	$C_4H_8O_5$	e	[45]	136.0372	13.48	1.11E-05	2.13	1.70E-06	9.53	3.70E-07	20.33
1a	2-Furoylglycine	$C_7H_7NO_4$	d	[42]	169.0375	4.92	6.32E-05	36.42	3.31E-06	45.29	1.75E-07	1649.42
1b			d	[42]	169.0376	4.92	5.21E-05	26.12	3.74E-06	18.31	1.75E-07	478.34
1a	Glutamic acid[b]	$C_5H_9NO_4$	a	[22,46]	147.0538	1.13	1.58E-05	1.64	2.27E-07	6.70	1.69E-07	10.98
1b			a	[22,46]	147.0540	1.14	9.44E-04	1.45	3.45E-07	4.66	1.92E-07	6.75
2a			e	[22,46]	147.0532	14.38	3.78E-03	1.89	1.18E-04	2.49	5.12E-07	4.71
2a	Glutamine	$C_5H_{10}N_2O_3$	e	[22]	146.0691	14.05	1.93E-05	4.82	1.54E-04	2.31	3.70E-07	11.12
1a	Glutamylphenylalanine	$C_{14}H_{18}N_2O_5$	b	[17]	294.1217	6.18	3.65E-05	1.48	2.68E-07	2.18	1.69E-07	3.23
1b			b	[17]	294.1227	6.19	3.43E-04	1.48	1.12E-04	1.67	5.90E-07	2.48
1a	Hippuric acid	$C_9H_9NO_3$	a	[2]	179.0586	6.51	2.51E-02	1.63	5.53E-07	13.02	1.72E-07	21.26
2a			e	[2]	179.0582	16.86	7.49E-03	1.96	4.06E-06	20.43	3.70E-07	40.01
1b	4-Hydroxyhippuric acid	$C_9H_9NO_4$	c, [18]	[2]	195.0535	5.26	5.36E-04	1.41	3.45E-07	19.81	1.92E-07	27.96
2a	3-Hydroxhippuric acid	$C_9H_9NO_4$	f	[44]	195.0532	19.80	3.33E-03	2.99	2.69E-06	16.72	3.70E-07	49.94
1b			c, [18]	[44]	195.0532	5.61	2.88E-02	2.3	4.85E-06	11.32	2.08E-07	25.83
1b	2-Hydroxyhippuric acid	$C_9H_9NO_4$	b	[44]	195.0542	7.42	1.45E-02	4.9	1.07E-04	29.32	2.87E-07	143.60
2a	Hydroxyindole	C_8H_7NO	f	[44]	133.0528	15.39	3.79E-04	2.99	1.70E-06	18.57	3.70E-07	55.62

2a	Indole-3-lactate	$C_{11}H_{11}NO_3$	a	[44]	205.0739	20.06	2.05E-04	1.66	4.29E-05	2.02	3.70E-07	3.34
1b	Indoxyl sulfate	$C_8H_7NO_4S$	b	[2]	213.0102	6.77	4.48E-04	2.69	3.45E-07	10.09	1.92E-07	27.09
1a	Kynurenic acid	$C_{10}H_7NO_3$	a	[2]	189.0425	6.01	1.10E-02	1.73	2.27E-07	14.11	1.69E-07	24.44
1a	Kynurenine	$C_{10}H_{12}N_2O_3$	c, [53]	[2]	208.0850	4.42	1.03E-03	1.37	1.94E-04	1.55	5.35E-07	2.13
2a			e	[2]	208.0848	20.21	5.36E-04	1.78	4.29E-05	1.94	4.37E-06	3.46
2a	Lysine[b]	$C_6H_{14}N_2O_2$	e	[22]	146.1055	21.14	2.05E-04	2.76	8.92E-05	2.84	3.70E-07	7.84
1a	Methionine[b]	$C_5H_{11}NO_2S$	a	[22]	149.0504	1.80	1.06E-05	2.20	2.93E-07	2.27	1.69E-07	5.00
1a	Methyladenosine	$C_{11}H_{15}N_5O_4$	b	[2]	281.1114	4.81	4.06E-06	1.44	2.27E-07	2.66	1.69E-07	3.82
1a	Methyladenosine	$C_{11}H_{15}N_5O_4$	b	[2]	281.1118	2.24	4.17E-05	1.35	3.28E-05	1.58	5.33E-06	2.13
1b	Methylinosine	$C_{11}H_{14}N_4O_5$	c, [52]	[2]	282.0955	4.75	7.26E-05	1.74	3.45E-07	6.36	1.92E-07	11.05
1a			c, [52]	[2]	282.0962	4.75	4.06E-06	2.00	2.27E-07	9.99	1.69E-07	19.99
1a	N-Methyl-pyridone-carboxamide	$C_7H_8N_2O_2$	d	[3]	152.0586	4.08	1.15E-03	2.20	4.78E-07	3.48	2.60E-07	7.64
1a	N-threonylcarbamoyladenosine	$C_{15}H_{20}N_6O_8$	d	[2,47]	412.1343	5.98	4.63E-06	1.84	2.56E-07	9.97	1.75E-07	18.36
1b			d	[47]	412.1343	5.98	4.63E-06	1.70	2.56E-07	8.11	1.75E-07	13.75
1b	*Oleic acid*[b]	*$C_{18}H_{34}O_2$*	*a*	[17,48]	*282.2559*	*19.89*	*2.54E-03*	*-2.85*	*2.80E-02*	*-1.84*	*8.20E-05*	*-5.24*
1a	Pantothenic Acid	$C_9H_{17}NO_5$	a	[15]	219.1100	5.08	3.69E-04	1.37	1.01E-06	2.60	5.35E-07	3.57
1b			a	[15]	219.1123	5.09	4.18E-04	1.42	2.63E-06	2.13	7.78E-07	3.02
1b	4-Pyridoxic acid	$C_8H_9NO_4$	b	[43]	183.0536	4.15	2.00E-04	2.49	1.71E-06	25.46	1.75E-07	63.44
1b	p-Cresol glucuronide (pCG)	$C_{13}H_{16}O_7$	d	[49]	284.0904	7.31	1.21E-02	2.49	1.30E-06	24.50	2.38E-07	60.91
1b	p-Cresol sulfate (pCS)	$C_7H_8O_4S$	a	[3]	188.0171	7.23	1.18E-03	2.46	1.27E-05	2.99	3.49E-07	7.38
1b	Phenylacetylglutamine	$C_{13}H_{16}N_2O_4$	d	[3,16]	264.1128	6.62	1.34E-03	2.44	3.45E-07	10.96	1.92E-07	26.70
2a	Proline	$C_5H_9NO_2$	e	[50]	142.0979	10.31	5.98E-03	1.53	1.23E-03	1.55	5.99E-06	2.38
1a	Pseudouridine	$C_9H_{12}N_2O_6$	c, [54]	[2]	244.0700	1.44	4.87E-07	1.62	9.37E-08	3.94	9.37E-08	6.37
1b			c, [54]	[2]	244.0700	1.44	4.87E-07	1.47	9.37E-08	3.17	9.37E-08	4.65
2a			e	[2]	244.0595	21.47	2.55E-05	1.60	1.70E-06	6.40	3.70E-07	10.21
2a	Pyroglutamic acid	$C_5H_7NO_3$	e	[22]	129.0426	13.20	3.33E-03	1.27	1.18E-04	1.47	2.08E-06	1.87
1a	Quinic acid	$C_7H_{12}O_6$	a	[22]	192.0627	0.90	6.32E-05	4.43	1.14E-05	3.22	2.34E-07	14.24
2a			e	[22]	192.0634	17.00	5.97E-05	15.39	2.33E-05	7.48	3.94E-07	115.17
1b	Quinolinic acid	$C_7H_5NO_4$	b	[2]	167.0248	1.04	3.10E-03	1.26	3.45E-07	4.76	1.92E-07	6.00
1a	*Tryptophan*	*$C_{11}H_{12}N_2O_2$*	*a*	[22,51]	*204.0902*	*5.60*	*2.93E-03*	*-1.11*	*2.72E-07*	*-3.18*	*9.37E-08*	*-3.54*

2a	Urea	CH_4N_2O	e	[2]	60.0324	9.77	7.99E-05	1.94	3.17E-06	2.42	3.70E-07	4.70
2a	Uric acid	$C_5H_4N_4O_3$	e	[2]	168.0283	19.34	3.33E-03	2.04	1.70E-06	11.36	3.70E-07	23.22
1a	Xanthosine	$C_{10}H_{12}N_4O_6$	a	[2]	284.0757	4.56	6.24E-04	1.82	2.55E-06	2.87	1.75E-07	5.21
1b			a	[2]	284.0757	4.56	1.88E-03	1.65	4.26E-06	2.52	1.75E-07	4.14

(1a) LC-MS positive mode; (1b) LC-MS negative mode; (2a) GC-MS; (a) confirmation with retention time and MS/MS spectra from standard compounds; (b) confirmation of MS/MS spectra using Metlin library; (c) confirmation with MS/MS spectra in literature; (d) confirmation with MSC; (e) Fiehn library; (f) NIST library. Italics represent downregulated metabolites.

ID identification procedure, RT retention time, FC fold change, CMPF 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid, q FDR corrected p value

[a]Measured mass for LC-MS, theoretical monoisotopic mass for GC-MS

[b]Different change in concentration in CKD compared to literature

[c]Hexose based saccharide

Table III.7 List of novel uremic retention solutes revealed in the present study

Analysis	Metabolite	Formula	ID	Mass[a]	RT	*p* (CKD3 vs. H)	FC (CKD3 vs. H)	*p* (CKD5HD vs. CKD3)	FC (CKD5HD vs. CKD3)	*p* (CKD5HD vs. H)	FC (CKD5HD vs. H)
1a	4-Acetamidobutanoate	$C_6H_{11}NO_3$	b	145.0739	3.75	5.86E-06	2.76	2.56E-07	16.23	1.75E-07	44.79
1b			b	145.0740	3.76	4.86E-06	2.52	2.56E-07	15.49	1.75E-07	39.02
1a	Acetylhomoserine	$C_6H_{11}NO_4$	d	161.0686	1.93	2.80E-04	2.13	2.56E-07	11.07	1.75E-07	23.57
1b			d	161.0688	1.93	5.21E-05	1.52	2.56E-07	8.61	1.75E-07	13.10
1b	Aminohydroxyhippuric acid	$C_9H_{10}N_2O_4$	d	210.0641	4.77	1.43E-05	3.76	4.78E-07	41.57	1.75E-07	156.10
1a	a-N-Acetylneuraminyl-2,6-b-D-galactosyl-1,4-N-acetyl-b-D-glucosamine	$C_{25}H_{42}N_2O_{19}$	d	674.2371	1.08	2.78E-06	2.70	1.28E-07	11.81	1.09E-07	31.86
1b			d	674.2390	1.10	2.78E-06	1.85	1.28E-07	8.74	1.09E-07	16.18
1b	Asp Leu / Asp Ile	$C_{10}H_{18}N_2O_5$	b	246.1216	5.28	3.03E-02	2.43	3.73E-07	13.92	2.60E-07	33.88
1b	*C10:0-OH*	*$C_{10}H_{20}O_3$*	*d*	*188.1414*	*11.07*	*2.54E-03*	*-1.72*	*1.93E-03*	*-1.65*	*1.92E-06*	*-2.85*
1b	*C12:0-OH*	*$C_{12}H_{24}O_3$*	*d*	*216.1726*	*13.34*	*6.63E-04*	*-2.25*	*3.37E-03*	*-1.59*	*1.12E-06*	*-3.58*
1b	*C14:0-OH*	*$C_{14}H_{28}O_3$*	*d*	*244.2042*	*15.57*	*6.63E-04*	*-1.68*	*1.77E-03*	*-1.36*	*1.92E-06*	*-2.29*
1b	*C18:0-2OH*	*$C_{18}H_{36}O_4$*	*d*	*316.2611*	*14.51*	*1.31E-03*	*-1.63*	*2.25E-02*	*-1.32*	*9.36E-06*	*-2.15*
1b	*C22:4*	*$C_{22}H_{36}O_2$*	*d*	*332.2726*	*19.76*	*3.47E-03*	*-1.66*	*3.96E-02*	*-1.26*	*3.87E-04*	*-2.09*
1b	*C22:5*	*$C_{22}H_{34}O_2$*	*d*	*330.2559*	*19.07*	*2.96E-02*	*-1.73*	*1.19E-02*	*-1.68*	*2.57E-05*	*-2.90*
1a	C5:0-glycine	$C_7H_{13}NO_3$	d	159.0891	4.92	7.90E-04	2.02	6.26E-06	4.77	1.75E-07	9.66
1b	C5:0-glycine	$C_7H_{13}NO_3$	d	159.0891	4.93	1.72E-03	2.69	1.16E-06	10.51	1.75E-07	28.26
1b	Dimethyluric acid	$C_7H_8N_4O_3$	c, [55]	196.0603	5.46	4.36E-03	2.74	1.71E-06	7.46	2.08E-07	20.43
2a	Gluconic acid	$C_6H_{12}O_7$	e	196.0583	18.53	7.82E-04	1.55	1.11E-06	6.76	2.94E-07	10.46
2a	Galacturonic acid / Glucuronic acid	$C_6H_{10}O_7$	e	194.0427	17.85	1.93E-05	2.24	1.70E-06	14.90	3.70E-07	33.36
1a	D-glucuronic acid-N-acetyl-D-glucosamine	$C_{14}H_{23}NO_{12}$	d	397.1195	1.09	4.63E-06	3.81	2.56E-07	9.96	1.75E-07	37.95
1b			d	397.1195	1.09	5.91E-06	2.48	2.56E-07	7.82	1.75E-07	19.36
1b	*Hexacosanedioic acid*	*$C_{26}H_{50}O_4$*	*d*	*426.3711*	*20.32*	*1.65E-02*	*-1.86*	*6.26E-06*	*-4.60*	*2.34E-07*	*-8.57*
1a	Hydroxypyridine	C_5H_5NO	b	95.0371	1.37	4.96E-05	7.58	8.67E-05	5.71	2.87E-07	43.24
1b	2-hydroxyhippuric acid glucuronide	$C_{15}H_{17}NO_{10}$	b	371.0852	5.70	9.66E-04	8.66	4.62E-05	52.78	1.55E-06	457.21

1b	2-/3-hydroxyhippuric acid sulfate[b]	$C_9H_9NO_7S$	b	275.0100	4.98	9.66E-04	5.91	2.55E-07	62.95	1.55E-06	371.88
1b	*keto-C5:0*	*$C_5H_8O_3$*	*d*	*116.0473*	*3.91*	*8.40E-04*	*-1.33*	*1.29E-05*	*-1.56*	*1.75E-07*	*-2.07*
1b	*keto-C6:0*	*$C_6H_{10}O_3$*	*d*	*130.0636*	*6.40*	*8.40E-04*	*-1.38*	*3.08E-05*	*-1.71*	*2.34E-07*	*-2.36*
1b	*keto-C6:0*	*$C_6H_{10}O_3$*	*d*	*130.0645*	*5.99*	*2.23E-03*	*-1.35*	*1.16E-06*	*-2.26*	*1.75E-07*	*-3.05*
2a	Lactose	$C_{12}H_{22}O_{11}$	e	342.1162	24.46	1.14E-02	3.88	1.11E-06	19.66	2.94E-06	76.36
2a	Maltose	$C_{12}H_{22}O_{11}$	e	342.1162	24.78	1.97E-04	2.64	1.11E-06	10.07	2.94E-06	26.59
1a	Mercaptolactic acid	$C_3H_6O_3S$	d	122.0034	0.97	2.54E-03	3.91	2.95E-07	5.07	5.60E-07	19.85
1b	Methoxy-hydroxyphenylglycol glucuronide	$C_{15}H_{20}O_{10}$	d	360.1056	3.14	1.46E-04	6.25	2.69E-07	12.67	1.75E-07	79.10
1b	5-Methoxysalicylic acid	$C_8H_8O_4$	a	168.0427	7.98	2.00E-04	3.17	7.00E-06	5.41	1.75E-07	17.15
1a	Methylglutarylcarnitine	$C_{13}H_{23}NO_6$	d	289.1519	5.05	2.29E-05	3.74	1.04E-06	10.05	1.91E-07	37.61
1a	Methylglutarylcarnitine	$C_{13}H_{23}NO_6$	d	289.1519	4.90	1.08E-03	1.84	2.56E-07	15.40	1.75E-07	28.30
1a	3-Methyluridine / ribothymidine	$C_{10}H_{14}N_2O_6$	d	258.0856	1.16	5.21E-05	2.24	2.56E-07	8.04	1.75E-07	17.98
1a	Methyluric acid	$C_6H_6N_4O_3$	c, [55]	182.0440	4.45	3.74E-03	2.95	6.97E-07	12.04	2.08E-07	35.50
1b			c, [55]	182.0449	4.41	2.39E-03	2.14	5.39E-07	11.41	1.75E-07	24.39
1b	Monosaccharide[c]	$C_6H_{12}O_6$	b	180.0634	0.85	1.87E-05	2.01	2.95E-07	6.44	1.75E-07	12.92
1a	N-Acetylneuraminic acid	$C_{11}H_{19}NO_9$	b	309.1047	0.85	5.91E-06	2.32	2.56E-07	5.89	1.75E-07	13.70
1a	Nicotinuric acid/isonicotinylglycine	$C_8H_8N_2O_3$	d	180.0535	5.91	1.08E-03	1.63	2.56E-07	9.26	1.75E-07	15.10
1b			d	180.0535	5.91	9.87E-05	1.94	2.56E-07	7.03	1.75E-07	13.61
1a	Oxoprolylproline	$C_{10}H_{14}N_2O_4$	d	226.0954	5.04	1.22E-03	1.61	2.56E-07	9.94	2.60E-07	15.97
1b	*palmitic acid (C16:0)*	*$C_{16}H_{32}O_2$*	*a*	*256.2402*	*18.88*	*3.74E-03*	*-2.12*	*2.33E-02*	*-1.46*	*6.58E-05*	*-3.11*
1a	Phe Phe	$C_{18}H_{20}N_2O_3$	b	312.2065	8.60	3.47E-03	2.39	1.93E-03	2.0	3.47E-03	4.8
1a	Sialyllactose	$C_{23}H_{39}NO_{19}$	d	633.2103	0.93	6.26E-06	2.39	2.56E-07	9.30	1.75E-07	22.26
1a	Succinoadenosine	$C_{14}H_{17}N_5O_8$	d	383.1079	5.18	1.06E-05	1.86	2.56E-07	8.12	1.75E-07	15.08
1b			d	383.1076	5.19	4.68E-05	1.52	2.56E-07	6.17	1.75E-07	9.35
1a	Tetrasaccharide[c]	$C_{24}H_{42}O_{21}$	-	666.2219	1.18	4.75E-04	5.43	2.56E-07	16.23	1.75E-07	88.07
2a	Trihydroxypentenoic acid	$C_5H_6O_5$	f	146.0427	14.84	1.36E-05	4.46	1.70E-06	15.51	3.70E-07	69.12

(1a) LC-MS positive mode; (1b) LC-MS negative mode; (2a) GC-MS; (a) confirmation with retention time and MS/MS spectra from standard compounds; (b) confirmation of MS/MS spectra using Metlin library; (c) confirmation with MS/MS spectra in literature; (d) confirmation with MSC; (e) Fiehn library ; (f) NIST library; ID: identification procedure; italic: downregulated metabolites

[a]measured mass for LC-MS, theoretical monoisotopic mass for GC-MS; [b]identified as 2- or 3-hydroxyhippuric acid, MS/MS is not sufficiently differential and no commercial standard is available to discriminate between these isomers, 2-hydroxyhippuric acid = salicyluric acid; [c]hexose based saccharide

III.4.3.1. Confirmation of earlier reported uremic retention solutes

Table III.6 lists all 43 metabolites identified in the present analysis which were already reported in the literature, as referred in the table, and of which the concentration was significantly increasing or decreasing throughout CKD progression, irrespective of their fold change. Only three compounds were downregulated, i.e. dehydroisoandrosterone sulphate, oleic acid and tryptophan (reported in italic in Table III.6). For dehydroisoandrosterone sulphate and tryptophan, similar observations were reported in the literature [17,22]. Oleic acid on the other hand showed upregulation in two reported studies, comparing HD patients to normal or CKD stage 4 to CKD stage 2, respectively [17,48]. Next to oleic acid, our dataset reveals several other fatty acid related metabolites which were significantly decreased in function of disease (see Table III.7). The fact that haemodialysis patients have a relative depletion of essential fatty acids has already been described [56], and our findings extend this observation to even earlier stages of the disease (CKD3). Interesting to note, several additional fatty acids detected with GC (linoleic acid, stearic acid, palmitoleic acid, methyl palmitate and methyl stearate) were decreasing between healthy and CKD3 ($p < 0.05$), but were not significantly different in all parallel comparisons.

Forty of the earlier reported metabolites were increasing with CKD progression. Creatinine, which is typically used to diagnose kidney deterioration, has been confirmed as an uremic metabolite on both analytical platforms. It has been detected in the flow-through of the LC-MS analysis; thereby its relative differences measured by LC-MS are less reliable than the differences revealed by GC-MS. In that perspective, the fold changes of creatinine as quantified by GC-MS have been used as cut-off for the detection of novel uremic retention solutes. Two of the earlier reported metabolites, i.e. 2-furoylglycine and quinic acid, show a strong increase with decreased kidney function. They were already significantly upregulated in CKD3 compared to healthy individuals, showing potential for earlier detection of impaired kidney function weighed against creatinine. In addition, levels of both metabolites further increased in patients on haemodialysis and these metabolites were among the most increased ones in CKD5HD versus healthy controls. 2-Furoylglycine is an acylglycine compound. Dietary studies showed that 2-furoylglycine precursors are of exogenous origin, most probably from furan derivatives found in food prepared by strong heating (e.g. chocolate and heated fruit juice). The excess in furan derivatives, which might occur due to decreased renal clearance, will be conjugated to glycine, an important detoxification process mainly occurring in the liver [57]. Quinic acid is a natural sugar compound found in many plants, such as tobacco leaves, multi-coloured fruits and vegetables. It is a key metabolite generated in the shikimate pathway; this pathway only exists in plants and is responsible for the biosynthesis of aromatic amino acid such as tryptophan, phenylalanine and tyrosine, and these are essential amino acids for human nutrition and health. The

human gastrointestinal microflora is involved in the quinic acid metabolism, converting the dietary quinic acid to benzoic acid after which it is conjugated with glycine in the liver to form hippuric acid, another well-known protein bound uremic toxin also identified in the present study. Changes in diet and intestinal microbiota, as might occur in CKD, could increase both levels of quinic and hippuric acid [58-60]. The present study also confirmed the increase of four other colon-derived uremic solutes: phenylacetylglutamine, hydroxyindole, p-cresol sulphate and indoxylsulphate, previously identified in the study by Aronov et al. [44]. Reports in the literature on the metabolites marked with footnote b in Table III.6 are inconsistent, both confirming [61] and contradicting [22,62] our findings. Differences in sample type (plasma, serum) or analytical approach (CE-TOF-MS, ^{1}H-NMR) could be at the origin of these inconsistencies and thus these metabolites will need further confirmation.

Several isomers of hydroxyhippuric acid (OHHA) were identified as highly upregulated solutes related to CKD severity. Extraction of m/z 194.04589 from a CKD5HD sample in negative ion mode yielded seven peaks (Figure III.9). Two of these peaks could be immediately addressed as in-source fragmentation, i.e. of 2-/3-hydroxyhippuric acid sulphate and 2-hydroxyhippuric acid glucuronide. 2-, 3- and 4-hydroxyhippuric acid could be unravelled by their retention time, i.e. 4-OHHA < 3-OHHA < 2-OHHA, and specific fragmentation spectrum, based on the characteristics described by Chen et al. [18]. Two other isomers were also identified in the present study, but those could not be identified with the current setup, nevertheless they point to the excess of hydroxyhippuric acids in serum from CKD patients. The isomer p-hydroxyhippuric acid (4-hydroxyhippuric acid) is a known inhibitor of the erythrocyte plasma membrane Ca^{2+}-ATPase [63], it also attenuates apoptosis of healthy polymorphonuclear leukocytes without affecting [Ca^{2+}] [64]. A disturbed balance between anti-apoptotic and pro-apoptotic factors can affect immune response in case of increased apoptotic cell death or the inflammatory status in case of reduced polymorphonuclear leukocyte apoptosis. No biological effect of the other isomers listed in Table III.6 (2-hydroxyhippuric acid, 3-hydroxyhippuric acid) nor of their conjugates (sulphate and glucuronide) are reported in the literature. It is of note that 3-hydroxyhippuric acid seems to be a marker for the colonic metabolism and absorption of consumed polyphenols, ubiquitous in plants (fruits and vegetables). 3-Hydroxyhippuric acid apparently derives only from phenolic compounds that have a hydroxyl-group at the 3-position of their aromatic ring, such as caffeic acid derivatives, flavan-3-ol derivatives, cyanidin derivatives, quercetin derivatives, etc. [65]. Fruits and vegetables are also important sources of dietary salicylates (i.e. 2-hydroxyhippuric acid) [66].

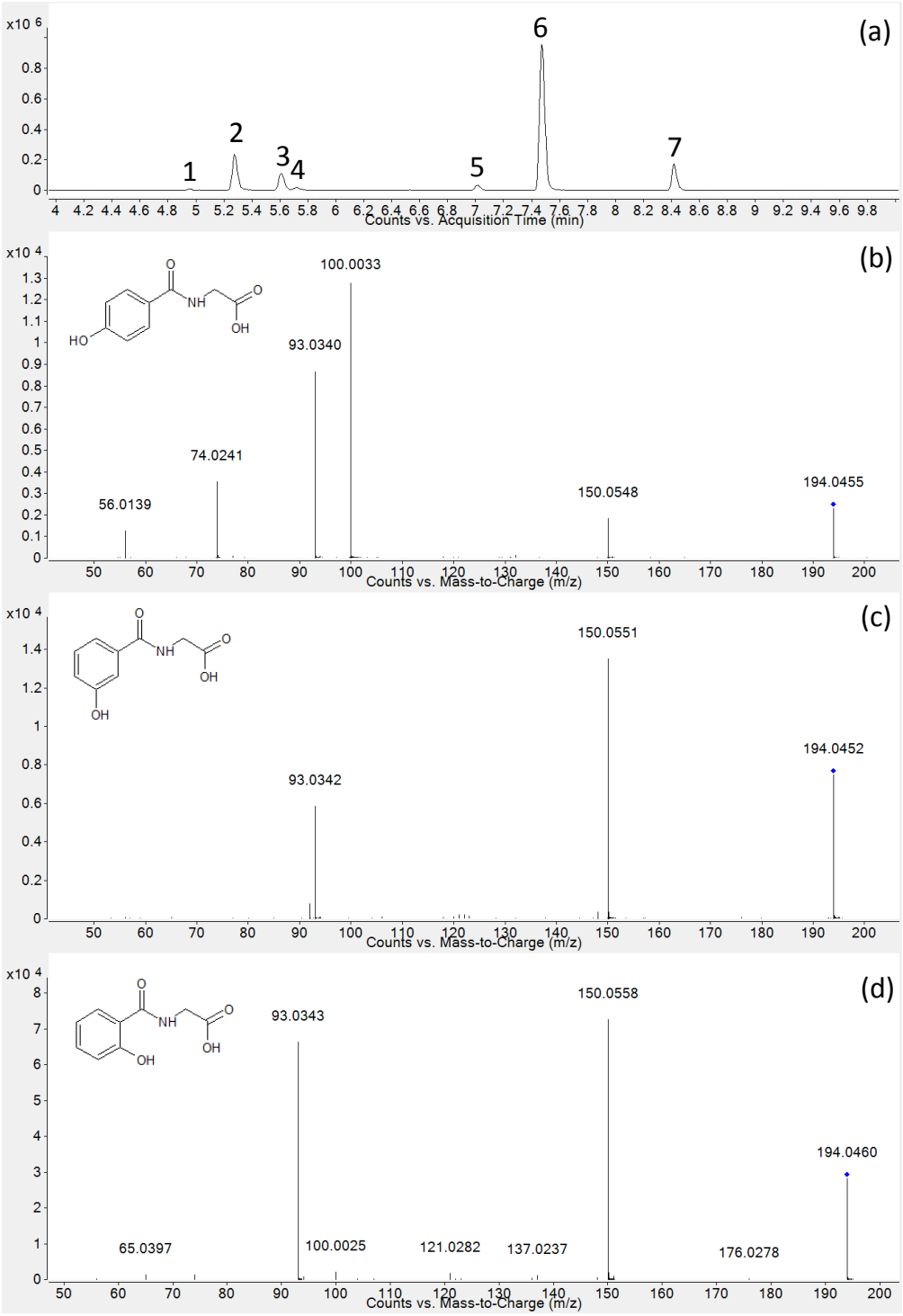


Figure III.9 Identification of $C_9H_9NO_4$ isomers in serum of a CKD5HD patient: (a) extracted ion chromatogram of *m/z* 194.04589 (25 ppm extraction window) revealing following compounds: 1) In-source fragment of hydroxyhippuric acid sulfate, 2) 4-hydroxyhippuric acid, 3) 3-hydroxyhippuric acid, 4) in-source fragment of 2-hydroxyhippuric acid glucuronide, 5) unknown, 6) 2-hydroxyhippuric acid and 7) unknown; (b) fragmentation spectrum of 4-hydroxyhippuric acid ($[M-H]^-$; CE 10); (c) fragmentation spectrum of 3-hydroxyhippuric acid ($[M-H]^-$; CE 10); (d) fragmentation spectrum of 2-hydroxyhippuric acid ($[M-H]^-$; CE 10)

III.4.3.2. Detection of novel uremic retention solutes

The as yet unknown uremic retention solutes are listed in Table III.7. Restrictions were set on the fold change between different sample groups in accordance with the relative differences uncovered for creatinine. Significantly upregulated unknown metabolites are listed only when they met at least one of the following criteria: (1) metabolites revealing at least a significant twofold change (FC ≥ 2) in CKD3 versus H; OR (2) metabolites revealing at least a significant sixfold change (FC ≥ 6) in CKD5HD versus CKD3; OR (3) metabolites revealing at least a significant 12-fold change (FC ≥ 12) in CKD5HD versus H. No restrictions were placed on metabolites that show a decreasing trend.

Thirty-one unknown metabolites were significantly upregulated in CKD and met at least one of the above criteria. Again, hydroxyhippurate compounds, here detected as conjugates, are among the most discriminating metabolites, emphasizing their relevance in CKD. After their absorption and passage through the intestinal mucosa or even further on in the liver, hydroxyhippurates can be metabolized by a cytosolic sulphotransferase or by a glucuronosyltransferase, as is known for other intestinally generated metabolites such as p-cresol [67]. Similar to sucrose, several carbohydrates were identified as increasing species with CKD progression. Using the LC platform, carbohydrates are detected in the flow-through which does not allow to make a distinction between the type of hexose, i.e. glucose, galactose, mannose, etc. Therefore, several carbohydrates are annotated as mono-, di- and tetrasaccharide. The disaccharide annotation reported in Table III.6, hence, represents the read-out of all disaccharides, i.e. sucrose, lactose and maltose. The identification of the latter species results from the GC-MS measurement. Typical glycoprotein or glycolipid derived carbohydrates such as N-acetylneuraminic acid and N-acetylneuraminyl-galactosyl-N-acetylglucosamine, next to the repeating unit of hyaluronic acid, i.e. glucuronic acid-N-acetylglucosamine are detected. Sialic acid has already been shown to be elevated in plasma of patients suffering from renal disease [68] while serum hyaluronic acid levels have been shown to increase in patients with end-stage renal failure treated by haemodialysis [69,70]. It should be mentioned that several carbohydrate species are used as excipient in pharmaceutical formulations, e.g. lactose, thereby concealing their actual contribution in the kidney deterioration. Two isomers of methylglutarylcarnitine are shown to increase significantly in function of disease progression. Free carnitine, on the other hand, shows a lower concentration in CKD5HD compared to H and CKD3 (FC -3.22 and -2.85 respectively). It is not reported in Table III.7 since it is not discriminating between H and CKD3. Carnitine depletion has been shown to occur in haemodialysis patients, a phenomenon confirmed in the present study [71].

III.5. Concluding remarks

A metabolomics platform, based on a combination of reversed-phase LC coupled to high-resolution Q-TOF-MS and GC coupled to quadrupole MS, has been evaluated as discovery tool to reveal potential uremic toxins and biomarkers for CKD. Next to 43 already described uremic retention solutes and/or toxins, 42 yet unknown metabolites could be related to CKD progression. Thirty-one unique metabolites were revealed which were increasing significantly throughout CKD progression. Next to this, 11 metabolites showed a decreasing trend in function of disease. This discovery study shows the potential of metabolomics for studying CKD. The next step is to develop targeted analytical approaches to enable quantification of the identified metabolites at the different and preferably earlier stages of CKD (CKD1 and 2). Quantitative data will allow (a) the evaluation of their disease related predictive value, as well as (b) the evaluation of the metabolite's biological activity at, for CKD, relevant concentrations, revealing their possible role in inflammation and cardiovascular pathophysiology of CKD and finally (c) the evaluation of their kinetics in CKD and during renal replacement therapy (haemodialysis and peritoneal dialysis).

III.6. References

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Chapter 4

Metabolic profiling of human plasma and urine in chronic kidney disease by hydrophilic interaction liquid chromatography coupled with time-of-flight mass spectrometry: a pilot study

IV.1. Abstract

Chronic kidney disease (CKD) is often characterized by a progressive loss in renal function over a period of months or years and by the accumulation of uremic retention solutes in the body. Current biomarkers, such as serum creatinine or urinary albumin, lack the sensitivity for early detection of CKD, which is primordial towards disease management. In the search for new uremic retention solutes and/or biomarkers of CKD, a hydrophilic interaction liquid chromatography time-of-flight mass spectrometric (HILIC-TOF MS) platform was developed. Urine and plasma samples from CKD patients at stage 3 (n=20), at stage 5 not yet receiving dialysis (n=20) and from healthy controls (n=20) were monitored in both positive and negative electrospray ionization mode. The validity of the metabolomics dataset was ensured by quality control (QC) samples. Data were treated with XCMS followed by multivariate statistical analysis. Differentiation was achieved between the metabolic profile of the CKD patients and healthy controls. Moreover, 4 metabolites that showed a significant increase or decrease throughout the different stages of CKD, i.e. cinnamoylglycine, glycoursodeoxycholic acid, 2-hydroxyethane sulfonate, and pregnenolone sulfate, could be identified by the use of authentic standards, the latter three of which are newly detected uremic retention solutes.

IV.2. Introduction

Chronic kidney disease (CKD) is a worldwide public health problem often characterized by a gradual loss of kidney function over time [1]. A multitude of compounds which are normally secreted by the healthy kidneys into the urine, are no more or insufficiently removed and accumulate in the body. These substances are called uremic retention solutes. If these compounds exert biological or biochemical activities, they are referred to as uremic toxins [2-4]. In 2003, the European Uremic Toxin (EUTox) Work Group composed an encyclopedic list of 90 uremic retention solutes, divided into 3 classes based on their physico-chemical properties [5]. In 2012, an update of the list provided 56 newly identified solutes. Nevertheless, many uremic retention solutes remain unknown. CKD is classified into 5 stages, with stage 1 being the mildest and stage 5 (end stage) being a severe illness with poor life expectancy if untreated [6]. Monitoring CKD activity requires non-invasive, specific and sensitive biomarkers that provide clinicians with information correlating with pathophysiologic processes occurring within the kidney. Current biomarkers of CKD and its progression that are in widespread clinical use, such as serum creatinine and albuminuria, have limitations in serving these goals and reliance on these biomarkers may result in an extensive time lapse between the start of the disease and the moment when alarm signs are prominent enough to incite therapeutic interventions [7-12]. Hence, the search for new relevant biomarkers should be continued to better stratify patients with CKD.

Metabolomics, a recent systems biology approach, complements the genomic, transcriptomic, and proteomic efforts to characterize a biological system and can be regarded as the end point of the "-omics"-cascade. Since metabolites represent end products, metabolomics holds the promise of providing an integrated physiologic phenotype of a system [13,14]. The main analytical techniques in metabolomics studies involve mass spectrometry (MS), usually preceded by a chromatographic separation step, and nuclear magnetic resonance spectroscopy (NMR). In the search for novel biomarkers of CKD, NMR [15-17] and hyphenated techniques such as gas chromatography-mass spectrometry (GC-MS) [18-20], liquid chromatography-mass spectrometry (LC-MS) [21-23] and capillary electrophoresis-mass spectrometry (CE-MS) [24-27] have been applied. As no single analytical technique is entirely competent in covering the broad metabolic picture, combining multiparallel technologies, has become indispensable, aiming at a comprehensive metabolome coverage. Recently, several studies on CKD have combined LC-MS and GC-MS to enlarge this coverage [28,29]. Within LC-MS-based metabolomics studies the metabolome coverage can be extended by combining multiple LC separation modes. The majority of the LC-MS-based studies employ reversed-phase liquid chromatography (RPLC). The use of reversed-phase columns provides efficient separation and retention of relatively nonpolar metabolites across a large molecular weight

range. Polar metabolites, being mainly primary metabolites, elute in the column void or early in the chromatographic run which is problematic as a good chromatographic separation prior to MS is essential to reduce the impact of matrix-induced effects. The latter species represent metabolite classes of high significance, such as amino acids and organic acids, which are directly involved in the normal growth, development, or reproduction of an organism and are thus important for the diagnosis of diseases. Hydrophilic interaction chromatography (HILIC) has become increasingly popular for the analysis of polar metabolites and several metabolomics studies have confirmed that the addition of HILIC is a useful tool to increase the metabolome coverage [30-34]. However, no HILIC-based metabolomics studies have been reported in the search for novel uremic retention solutes or potential biomarkers of CKD. Therefore, the goal of this study is to develop and apply a HILIC-time-of-flight (TOF) MS metabolic platform in the search for novel uremic retention solutes/potential biomarkers by comparing urine and plasma from a healthy control group and two patient groups suffering from different stages of CKD, i.e. CKD stage 3 (CKD3) and CKD stage 5 (CKD5).

IV.3. Materials and methods

IV.3.1. Chemicals

Water (LC-MS grade), methanol (LC-MS grade), ammonium acetate (ULC-MS grade) and formic acid (ULC-MS grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Acetonitrile (HPLC grade) was purchased from Sigma-Aldrich (Schnelldorf, Germany). Metabolite standards were obtained from The Metabolomics Innovation Centre (TMIC, Edmonton, Canada).

IV.3.2. Study samples

Urine samples were obtained by centrifugation of fresh urine from 40 patients diagnozed with chronic kidney disease (CKD) and 20 healthy controls at 1800 rpm for 10 min at room temperature. Aliquots of 1000 µl were stored at -80°C until analysis. Blood was sampled by venipuncture from the same study group using K_2EDTA Vacutainer™ tubes (Becton Dickinson, San Jose, CA, USA) after informed consent. Plasma was obtained by centrifugation, immediately after sampling, at 3,000 rpm for 10 min at room temperature. Aliquots of 500 µl plasma were stored at -80°C until analysis. The clinical characteristics of the included patients are tabulated in Table IV.1. From the 40 patients included, 20 were classified in CKD stage 3 (CKD3) (estimated glomerular filtration rate - eGFR - 30-60 mL/min/1.73 m^2 body surface) and 20 in CKD stage 5 not receiving dialysis (CKD5) (eGFR < 15 mL/min/1.73 m^2), based on their glomerular filtration rate estimated from the serum creatinine values as prescribed by the KDOQI guidelines. The underlying etiology of CKD was of vascular (n=8),

glomerular (n=5) or interstitial (n=5) origin, polycystic kidney disease (n=5), nephrectomy (n=8), transplant failure (n=4), other (n=4) or unknown (n=1).

Table IV.1 Clinical characteristics of the included patients

	Healthy	CKD3	CKD5
Number	20	20	20
Age	33.8 ± 13.6	61.0 ± 14.4	64.3 ± 20.0
Male/Female	9/11	12/8	10/10
BMI (kg/m²)	22.7 ± 3.9	26.9 ± 4.0	26.2 ± 4.4
Syst BP (mm Hg)	125 ± 16	134 ± 24	141 ± 24
Diast BP (mm Hg)	77 ± 10	82 ± 11	83 ± 11
Pulse	69 ± 10	68 ± 8	75 ± 15
CTN (mg/dL)	0.92 ± 0.19	1.49 ± 0.30	4.28 ± 0.93
CRP (mg/L)	0.16 ± 0.17	0.20 ± 0.16	1.44 ± 2.68

BMI: body mass index; BP blood pressure; CTN: creatinine; CRP: C-reactive protein

IV.3.3. Sample preparation

IV.3.3.1. Sample preparation: urine

The frozen urine samples were thawed, and were prepared by adding 180 µL of water to 60 µL of urine in Eppendorf tubes, briefly vortexing, and centrifuging at 13,000 rpm for 10 min. Subsequently, 180 µL was dried under nitrogen at room temperature and re-dissolved in 120 µL of the initial mobile phase (see below).

IV.3.3.2. Sample preparation: plasma

The frozen plasma samples were thawed. Subsequently, 150 µL of plasma was transferred to Eppendorf tubes and 300 µL of ice-cold methanol (-20°C) was added. After vortex mixing during 10 seconds, samples were placed at -20°C for 20 minutes. Afterwards, the samples were centrifuged for 10 minutes at 13,000 rpm and 300 µL of the supernatant was dried under nitrogen at room temperature. The dried extracts were re-dissolved in 150 µL of the initial mobile phase and transferred to a new Eppendorf tube. The samples were centrifuged for 10 min at 13,000 rpm, after which 120 µL of supernatant was transferred to a vial with glass insert for LC-MS analysis.

IV.3.3.3. Preparation of QC samples

A quality control (QC) pool was constructed by collecting 100 µL of all the study samples. Subsequently, this QC pool was divided into aliquots to acquire representative QC samples. QC samples were prepared simultaneously along with study samples and were analyzed throughout the LC-MS analysis every 5 study samples. Since these samples did not contain any biological variability, they could be considered as technical replicates. For both plasma and urine, study and QC samples were prepared in random order.

IV.3.4. Liquid chromatography-mass spectrometry conditions

All samples were analyzed on a 1290 Infinity LC coupled to a 6230 TOF MS (Agilent Technologies), equipped with a Jetstream ESI source as interface. Separation was performed on an Acquity UPLC ethylene bridged hybrid (BEH) HILIC column (1.7 µm, 2.1 mm x 150 mm) with an Acquity UPLC BEH HILIC VanGuard precolumn (1.7 µm, 2.1 mm x 5 mm). The mobile phase consisted of (A) 0.1% formic acid in 50 mM ammonium acetate and (B) 0.1% formic acid in acetonitrile. Elution was carried out with a gradient starting from 2% B to 20% A in 30 min, followed by an increase to 100% A and re-equilibration. The flow rate was 0.3 mL/min and the injection volume 10 µL. The column temperature and the autosampler temperature were kept at 40°C and 4° C, respectively. The MS instrument was operated in both positive and negative electrospray ionization (ESI) modes. Needle voltage was optimized to 4 kV in positive ESI mode and -3.5 kV in negative ESI mode. The drying and sheath gas temperatures were set to 325°C and the drying and sheath gas flow rates were set to 8 and 7 L/min, respectively. Data were collected in centroid mode from *m/z* 50–1,200 at an acquisition rate of 1 spectrum/sec in the extended dynamic range mode (2 GHz). To maintain mass accuracy during the analysis sequence, a reference mass solution was used containing reference ions (121.0508 and 922.0097 for positive ESI mode, and 112.9856 and 1033.9881 for negative ESI mode). The TOF instrument was tuned using the ESI-L low concentration tuning mix (Agilent Technologies) prior to the analysis sequence. The LC-MS analysis was performed in one batch, separately for positive ESI and negative ESI measurement. Each analysis sequence started with 2 blank runs and 6 conditioning samples (QC samples). All study samples were analyzed in randomized order in both ionization modes, with QC samples (n=12) analyzed every 5 study samples. All instruments were controlled by the MassHunter Workstation Acquisition 4.0 (Agilent Technologies) software. Figure IV.1 displays representative chromatograms (positive ESI mode) of plasma obtained from (a) a healthy control and (b) a CKD5 patient.

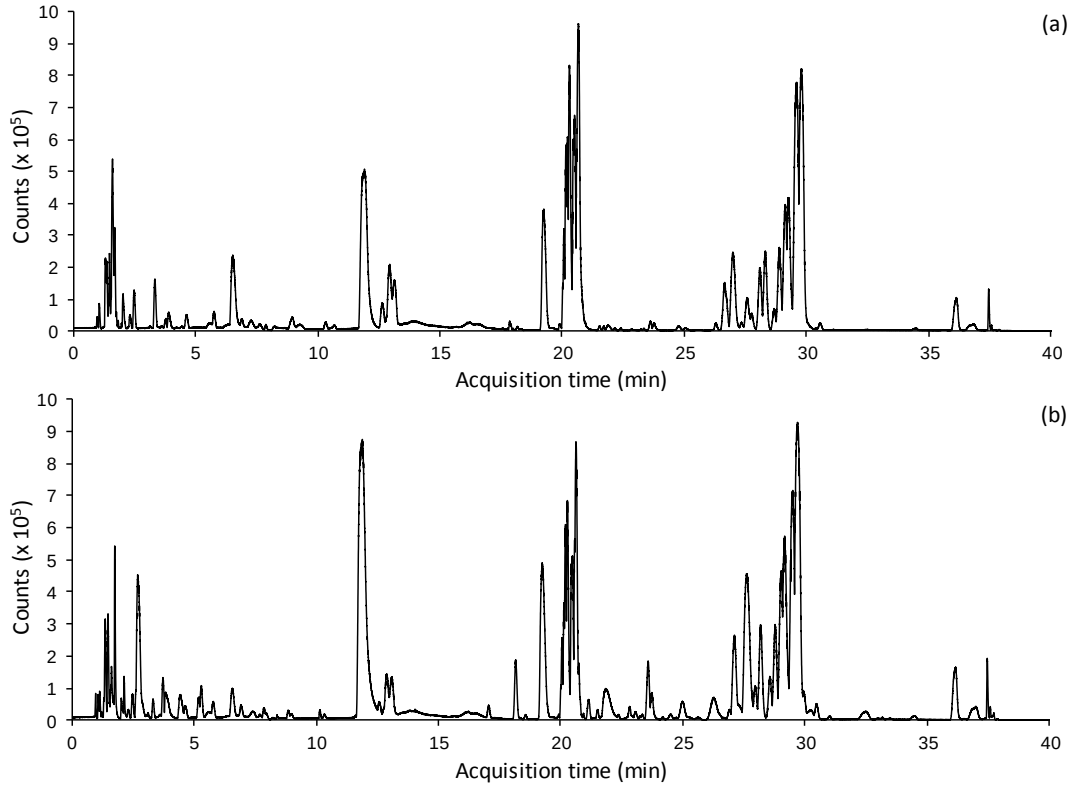


Figure IV.1 Representative chromatograms of plasma from (a) healthy control and (b) CKD5 patient

IV.3.5. Data analysis

The LC-MS total ion chromatogram (TIC) data were exported to mzData format data files by MassHunter Qualitative Analysis B.04.00 (Agilent Technologies) and pairs of sample groups were subsequently processed by XCMS software using default parameters [35]. XCMS software (version 1.34.0) running under the R package (version 2.15.3), incorporates nonlinear retention time alignment, matched filtration, automatic peak detection and peak matching and is freely available under an open-source license. Subsequently, all sample groups were analyzed pairwise (healthy versus CKD3, healthy versus CKD5, CKD3 versus CKD5) in R, using univariate statistics based on unpaired Mann-Whitney testing with Benjamini-Hochberg corrected p-values to control the false discovery rate (FDR) [36]. Next, urine data were normalized to the total intensity of components that are common to all samples, which is also called MS "total useful signal" (MSTUS) [37]. A second order analysis was performed to identify metabolites that are up- or downregulated across the different CKD stages. Therefore, the processed XCMS output files were used as input for metaXCMS (version 0.1.20), where they were realigned, statistically evaluated and compared for shared differences. MetaXCMS is freely available as an open-source R-package that includes a graphical user interface [38]. Feature lists were filtered by a fold change (FC) ≥ 1.5 and corrected p-value (q) ≤ 0.05 (see 3.3). The second-order comparison was applied using a tolerance of 0.01 *m/z* and 60 s retention time. Results were visualized as a Venn diagram with the number of common features to all sample groups

contained within the intersection. Since this study focused on identifying small molecules that showed an evolution related to the disease progression (Healthy > CKD3 > CKD5 or Healthy < CKD3 < CKD5), only features were retained that were upregulated or downregulated from healthy to CKD5. The number of features was further reduced by removing features that were not present in at least 75 % of one of the sample groups. The XCMS algorithm does not classify spectral ions originating from the same compound. Hence, the resulting feature list encloses multiple ions for each individual metabolite detected. Therefore, the CAMERA package, which is freely available from the Bioconductor repository, was used for grouping related features and for the annotation of ion species [39]. Data were processed with CAMERA functions in the following order xsAnnotate, groupFWHM, groupCorr, findIsotopes, and findAdducts using default parameters. Data were uploaded into freely available MetaboAnalyst software to construct PCA plots. Molecular formulas were generated by Find by Molecular Feature and Generate Formulas in the MassHunter Qualitative Analysis B.04.00 software. The accurate mass and molecular formula were then matched to metabolites via searching of the on-line Metlin database. Finally, identification was confirmed with commercially available authentic standards.

IV.4. Results and discussion

IV.4.1. Quality of analysis

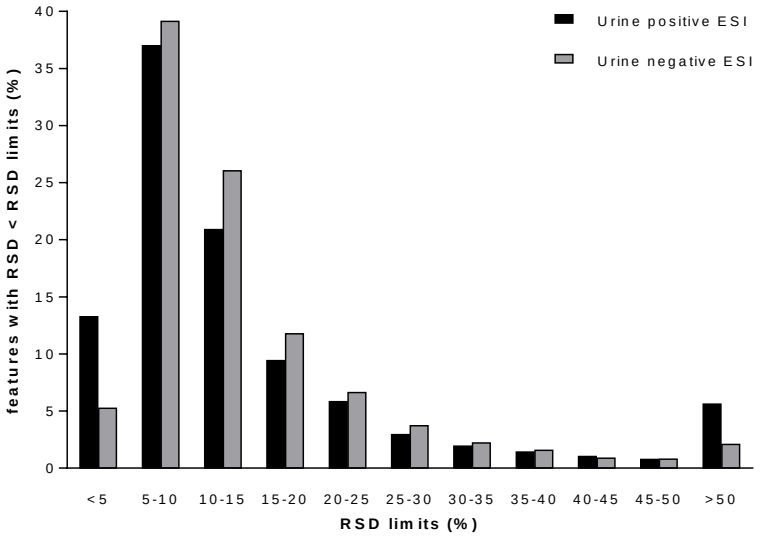
QC data were examined post-run in both a targeted and a non-targeted way for evidence of changes during sequences. Targeted monitoring was performed by determining the error of the measurement on signal intensity (peak area), retention time and mass accuracy for a list of randomly selected metabolites. Table IV.2 summarizes the results of this targeted validity verification. Peak area fluctuations, originating from both the sample preparation step and the LC-MS analysis, are typically below 15% relative standard deviation (RSD, n=12) and are generally better for urine than for plasma. Chromatographic retention time reproducibility is in general satisfactory. High mass accuracy is expected for the TOF instrument (< 5 ppm), which is advantageous for identification purposes since the greater the accuracy the lower the number of molecular formula matches. We only found one out of twelve mass accuracies exceeding 5 ppm for the list of randomly selected metabolites, which indicates overall acceptable accuracies.

Table IV.2 Relative standard deviation (RSD) of area under the curve (AUC) and retention time (t_R), and average mass accuracy of randomly selected metabolites measured in QC samples (n=12)

Analysis	Metabolite	Theoretical Mass	Average mass accuracy (ppm)	t_R (min)	RSD tR (%)	RSD AUC (%)
1a	Adenosine	180.0634	0.89	7.65	0.17	5.43
1a	Hydroxyindole	267.0968	4.70	7.57	0.56	6.34
1a	Pseudo-uridine	133.0528	1.30	1.03	0.32	2.51
2a	Urea	60.0324	5.47	3.62	0.80	6.18
2a	Creatinine	113.0589	4.77	12.22	0.96	6.88
2a	Panthothenic acid	219.1107	4.50	5.15	1.06	11.74
1b	2-Furoylglycine	169.0375	0.79	3.73	0.28	5.71
1b	4-Hydroxyhippuric acid	195.0532	4.94	3.94	0.46	9.7
1b	Phenylacetylglutamine	264.1110	4.68	15.03	0.36	5.66
2b	Pseudo-uridine	244.0695	3.22	2.49	0.31	11.33
2b	Xanthosine	284.0757	3.17	2.82	0.30	13.81
2b	Hypoxanthine	136.0385	2.68	6.03	0.58	10.13

(1a) urine positive ESI; (1b) urine negative ESI; (2a) plasma positive ESI; (2b) plasma negative ESI

Apart from this targeted approach, the reproducibility of the applied metabolomics analysis was examined in a more comprehensive way by calculating the error on the peak area of all detected features in the QC samples and representing the acquired RSD distribution as depicted in Figure IV.2. For urine in positive mode 71.12% of all features show RSD values below 15% and 89.29% of all features had an RSD below 30%, which can be defined as the upper limit for untargeted or discovery metabolomics analysis [32]. Urine analysis in negative ESI mode displayed 70.43% of all features with a RSD value below 15%, while 92.52% of the features had an RSD value below 30%. For plasma in positive ESI mode 36.35% of all features showed RSD values below 15% and 73.91% of all features had a RSD below 30%. Plasma analysis in negative mode displayed 46.62% of all features with RSD values below 15%, while 87.91% of the features had a RSD below 30%. Hence, the reproducibility of the urine analyses is better than the reproducibility of the plasma analyses.



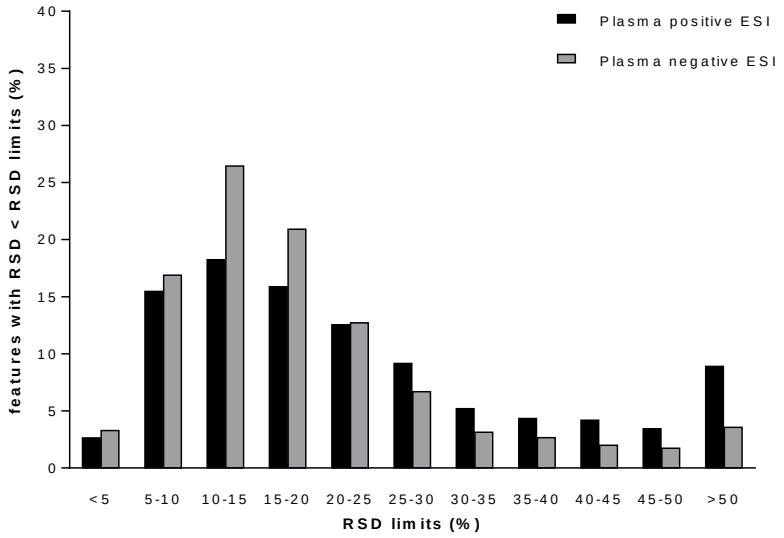


Figure IV.2 relative standard deviation (RSD) distribution plot displaying the technical repeatability of the LC-MS analysis of urine and plasma in both positive and negative ESI mode. The stability of the feature signals is expressed as RSD values, calculated for each feature as the standard deviation of the peak area in all QC samples divided by the average of the peak area in all QC samples

IV.4.2. Revealing differential metabolites

PCA was used as a first exploratory step in the data-processing pipeline. Grouping of the samples originating from the different CKD stages and controls can be seen from the score plots in Figure IV.3. Healthy controls are clearly separated from CKD5 samples. Nevertheless, there is overlap between CKD3 samples and the other sample groups, for both urine and plasma.

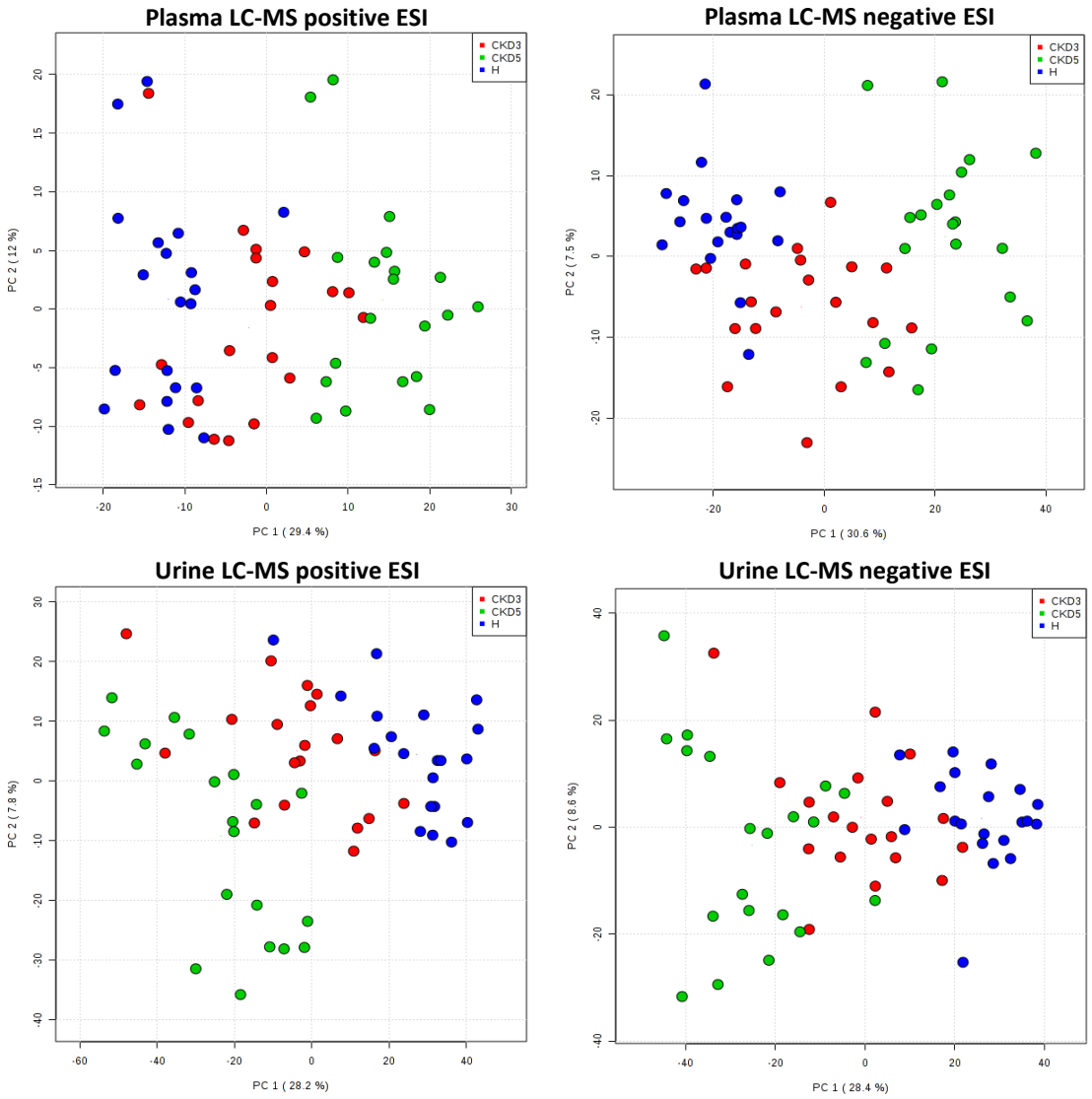


Figure IV.3 Principal component analysis score plots obtained from the urine and serum data. Samples are colour-coded according to their group: healthy (blue); CKD3 (red); CKD5 (green)

Data analysis in metabolomics experiments boils down to reducing complex data matrices to a list of biologically relevant metabolites. Table IV.3 summarizes the feature reduction throughout the successive data-processing steps.

Table IV.3 Feature reduction throughout data-processing

	Feature number			
Data processing step	**urine positive ESI**	**urine negative ESI**	**plasma positive ESI**	**plasma negative ESI**
XCMS feature detection (CKD3vsH, CKD5vsH and CKD5vsCKD3)	4,442	3,628	3,481	3,508
Differential features MetaXCMS Filter by corrected p-value ($q \leq 0.05$) and fold change ($FC \geq 1.5$)	1,382	1,126	714	992
MetaXCMS common features (m/z tolerance 0.01 and retention time tolerance 60 s)	94	49	20	76
(H < CKD3 < CKD5 or H > CKD3 > CKD5) and 75% frequency criterion	92	47	20	69
CAMERA	74	36	18	51

Creatinine, a clinically widespread biomarker of CKD, was well retained in the HILIC mode and was confirmed as an uremic metabolite. The fold changes of creatinine and several well-known uremic toxins are listed in the upper part of Table IV.4. In general, fold changes were more substantial for protein bound solutes, such as indoxylsulfate or p-cresylsulfate, compared to the current marker serum creatinine, especially during the early stages of CKD (CKD3 vs H). As expected, fold changes of creatinine were more prominent in plasma than in urine, where they are a reflection of daily generation. In an attempt to discover early markers for CKD or new uremic retention solutes, restrictions were set on the fold changes based on the relative difference in creatinine between CKD3 patients and healthy controls in plasma (FC = 1.21). Only features with fold changes ≥ 1.5 that showed a significant increase or decrease throughout the different stages of CKD were withdrawn. Several features were found to be significant with the required increasing or decreasing trend throughout CKD as demonstrated in Table IV.3. In urine analyzed in positive ESI, 15 features were going up, while 59 features were decreasing with CKD progression. Negative ESI unraveled 5 features that were upregulated and 31 features that were downregulated with CKD progression in urine. In plasma analyzed in positive ESI, 17 features were increasing, while 1 feature was decreasing with CKD progression. Negative ESI unraveled 40 features that were upregulated in plasma, next to 11 features that were downregulated with CKD progression.

Table IV.4 List of metabolites. Upper part: creatinine and well known uremic retention solutes. Down part: confidently identified metabolites that showed a significant increase or decrease throughout CKD

Analysis	Metabolite	Formula	Mass[a]	RT (min)	Freq H	Freq CKD3	Freq CKD5	q (CKD3 vs H)	FC (CKD3 vs H)	q (CKD5 vs CKD3)	FC (CKD5 vs CKD3)	q (CKD5 vs H)	FC (CKD5 vs H)
1a	Creatinine	$C_4H_7N_3O$	113.0584	12.11	20	20	20	1.38E+00	1.04	3.24E-01	1.06	3.04E-02	1.10
2a			113.0583	12.22	20	20	20	2.15E-03	1.21	4.88E-13	1.59	2.22E-16	1.92
2b	CMPF	$C_{12}H_{16}O_5$	240.1003	1.67	20	20	20	2.36E+00	1.14	2.81E-02	1.76	2.30E-02	2.02
2b	Hippuric acid	$C_9H_9NO_3$	179.0590	2.41	20	20	20	1.72E-01	1.39	9.19E-03	2.22	1.67E-03	3.08
2b	Indoxylsulfate	$C_8H_7NO_4S$	213.0104	1.01	20	20	20	4.55E-04	1.72	2.25E-02	1.45	2.13E-05	2.49
2b	p-Cresylsulfate	$C_7H_8O_4S$	188.0151	0.97	20	20	20	1.15E-02	1.58	5.13E-03	1.62	6.71E-06	2.56
1b	*Glycoursodeoxycholic acid*	*$C_{26}H_{43}NO_5$*	*449.3131*	*10.31*	*20*	*15*	*2*	*1.36E-03*	*-3.35*	*1.39E-03*	*-8.20*	*3.52E-05*	*-26.93*
1b	*2-Hydroxyethane sulfonate*	*$C_2H_6O_4S$*	*125.9992*	*3.92*	*20*	*20*	*20*	*4.51E-02*	*-1.51*	*6.85E-03*	*-2.34*	*2.15E-04*	*-3.49*
2b	Cinnamoylglycine	$C_{11}H_{11}NO_3$	205.0744	4.05	20	20	20	1.27E-02	1.50	3.15E-05	1.90	8.54E-08	2.86
2b	*Pregnenolone sulfate*	*$C_{21}H_{32}O_5S$*	*396.1972*	*1.27*	*20*	*20*	*19*	*7.36E-03*	*-1.80*	*1.90E-02*	*-1.52*	*4.53E-04*	*-2.70*

(1a) urine positive ESI; (1b) urine negative ESI; (2a) plasma positive ESI; (2b) plasma negative ESI. Italics represent downregulated metabolites

[a]Measured mass; RT retention time; Freq frequency; FC fold change; CMPF 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; q FDR corrected p value

IV.4.3. Uremic retention solutes in CKD

Currently, the process of metabolite identification in non-targeted metabolomic studies is a significant bottleneck in deriving biological knowledge from metabolomic studies. The Chemical Analysis Working Group of the Metabolomics Standards Initiative (MSI) [40] has defined four different levels of metabolites identification confidence, with level 1 being the highest confidence level corresponding to confidently identified compounds by comparison of two or more orthogonal properties with an authentic chemical standard analyzed under identical analytical conditions; level 4 on the other hand is the lowest confidence level corresponding to unknown compounds. In the present study, metabolites were identified by commercially available authentic standards, based on retention time and accurate mass, which is in accordance with confidence level 1 defined by the MSI. Confidently identified metabolites are presented in the lower part of Table IV.4. Glycoursodeoxycholic acid (GUDCA) and 2-hydroxyethane sulfonate, which have not been reported in the context of CKD yet, were downregulated in urine. Glycoursodeoxycholic acid is an acyl glycine and a secondary bile acid (BA)-glycine conjugate. Primary BAs are synthesized and conjugated in hepatocytes, followed by excretion into bile and the intestinal tract. Gut microorganisms generate secondary BAs by deconjugation and dehydroxylation. Upon reuptake by intestinal transporters, BAs are re-conjugated in the liver to complete the enterohepatic cycle. BAs can also be filtered in the kidney through the glomerulus, followed by urinary excretion. The solute carrier (SLC) family 10 (SLC10A1 and SLC10A2) is involved in the influx transport of bile acids [41]. It was demonstrated that GUDCA suppresses the production of the proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β and prevents nerve cell death induced by unconjugated bilirubin (UCB) [42,43]. Moreover, GUDCA was shown to have antioxidant properties [44]. Unlike the other metabolites, glycoursodeoxycholic acid was not detected in all subjects, but its fold changes were the highest of the identified metabolites. 2-Hydroxyethane sulfonate (isethionic acid) is a short chain alkane sulfonate involved in the taurine and hypotaurine metabolism [45]. Cinnamoylglycine was upregulated in plasma. Cinnamoylglycine is known as an N-acyl glycine metabolite of cinnamic acid [46]. It allows the transport and elimination of phenylpropanoic acids, a pathway probably similar to the elimination of toluene or benzoic acid as hippuric acid. The accumulation of cinnamoylglycine in CKD has recently been reported in literature [23,29,47]. In a study on colon-derived uremic solutes cinnamoylglycine was shown to be less prominent in patients without a colon than in patients with a colon [23]. Finally, pregnenolone sulfate, which elutes quite early in the chromatographic run, was found to decrease in plasma throughout CKD. Pregnenolone sulfate is a steroid sulfate with a plethora of actions and functions [48], but has not yet been linked to CKD in literature. It is not the final product of pregnenolone being sulfated, but it is also the starting point for subsequent steroid synthesis pathways. For this subsequent synthesis it is indispensible that the substrate enters the

cytosol to come into contact with the cytosolic localized sulfohydrolases. As it is unlikely that pregnenolone sulfate is capable of easily crossing the plasma membrane with its hydrophilic sulfate moiety, transmembrane transport is facilitated by a variety of transporter proteins, such as the organic anion-transporting polypeptide (OATP-B), nowadays classified as solute carrier organic anion transporter (SLCO2B1) [49,50], and the sodium-dependent organic anion transport (SOAT), a member of solute carrier family 10 (SLC10A6), which belongs to the same family as the influx transporters of bile acids [51-53]. As a neurosteroid, pregnenolone sulfate modulates a variety of ion channels, transporters, and enzymes. The negative modulation of γ-aminobutyric acid ($GABA_A$) chloride channels [54,55], the positive modulation of glutamate response by N-methyl-D-aspartate (NMDA) receptors [56] and the activation of melastatin-related transient receptor potential ion channels (TRPM1 and TRPM3) are well established [57,58]. It is interesting to remark that dehydroisoandrosterone sulfate, which was earlier found to decrease in CKD [28,29], is a substrate of the same transporter proteins as pregnenolone sulfate [53,59] and also acts as an inhibitor of the $GABA_A$ receptor [60] and a positive NMDA modulator [61]. Both NMDA activation and GABAergic inhibition have been linked to uremic encephalopathy [62,63].

IV.5. Conclusion

A quality controlled hydrophilic interaction liquid chromatography time-of-flight mass spectrometric (HILIC-TOF MS) platform was developed and applied to discover uremic retention solutes and/or potential biomarkers of CKD in urine and serum. Several metabolites could be identified that showed a significant increase or decrease throughout the different stages of CKD and fold changes of these especially in early CKD were markedly more prominent than those for serum creatinine, a current marker of kidney failure. One of the known problems with serum creatinine as a renal marker is its moderate changes during the early stages of the disease. This handicap could be overcome by using markers found by this study, alone, or even better in combination. The data currently reported should be validated in larger populations.

This discovery study also shows the potential of HILIC-based metabolomics in the study of CKD. More research is needed to definitely label these solutes as uremic toxins and/or biomarkers of CKD.

To reach both aims described above, targeted analytical methods have to be developed, which allow quantification of the identified metabolites. Such quantitative methods will provide concentration ranges for the targeted compounds rather than fold changes, this will then allow the evaluation of the metabolites' biological activity at relevant concentrations for CKD, and the evaluation of their kinetic behaviour in CKD and during renal replacement therapy. Furthermore, quantitative data at the different and preferably earlier stages of CKD will enable the evaluation of the metabolites'

predictive value for the presence of CKD and its relative sensitivity en specificity compared to current markers like creatinine.

IV.6. References

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Chapter 5

A novel UPLC–MS/MS method for simultaneous determination of seven uremic retention toxins with cardiovascular relevance in chronic kidney disease patients*

V.1. Abstract

Chronic kidney disease (CKD) is a devastating illness characterized by accumulation of uremic retention solutes in the body. The objective of this study was to develop and validate a simple, rapid, and robust ultra high performance liquid chromatography-tandem mass spectrometric (UPLC-MS/MS) method for simultaneous determination, in serum, of seven organic acid uremic retention toxins, namely uric acid (UA), hippuric acid (HA), indoxylsulfate (IS), p-cresylglucuronide (pCG), p-cresylsulfate (pCS), indole-3-acetic acid (IAA), and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF). Isotopically labeled internal standards (d_5-HA; 1,3-$^{15}N_2$-UA, and d_5-IAA) were used to correct for variations in sample preparation and system performance. Separation on a C18 column was followed by negative electrospray ionization and tandem mass spectrometric detection. Accuracy was below the 15 % threshold. Within-day precision varied from 0.60 to 4.54 % and between-day precision was below 13.33 % for all compounds. The applicability of the method was evaluated by analyzing 78 serum samples originating both from healthy controls and from patients at different stages of CKD. These results were compared with those obtained by use of conventional high performance liquid chromatography-photodiode array-fluorescence (HPLC-PDA-FLD) methods. A good correlation was obtained between both methods for all compounds.

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V.2. Introduction

Chronic kidney disease (CKD) is a growing global public health problem [1]. Many patients with CKD suffer from progressive loss of kidney function. The kidneys lose the capacity to remove potentially toxic compounds from the bloodstream into the urine, resulting in their accumulation in the body. Because of this accumulation, the compounds are called uremic retention solutes. If these compounds are biologically or biochemically active, they are referred to as uremic toxins. Accumulation of these toxins has a negative effect on many body functions [2-4]. Consequently, CKD is a complex disease that often affects multiple organ systems and coexists with numerous associated conditions, one of which, cardiovascular disease, is highly important [5, 6]. In 2003, the European Uremic Toxin (EUTox) Work Group proposed the classification of 90 identified retention solutes [7]. Recently, the list has been extended with an additional 56 solutes [8]. Toxicity is not yet known for all of these solutes, because both clinical and in vitro studies are required. Deleterious effects have already been described for, among others, uric acid (UA) and the protein-bound compounds hippuric acid (HA), indoxylsulfate (IS), p-cresylglucuronide (pCG), p-cresylsulfate (pCS), indole-3-acetic acid (IAA), and 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF) [9-19], all solutes of patho-physiological relevance. Mounting evidence indicates that UA is a secondary if not a primary contributor to CKD and its progression [9]. UA is likely to be an important mediator in the development of hypertension, a critical risk factor and accelerator of CKD, and may contribute to diabetes and cardiovascular disease [10]. HA may enhance drug toxicity and the toxicity of other protein-bound uremic solutes by competition for protein binding [11]. IS stimulates the progression of CKD by increasing renal expression of transforming growth factor-beta 1 (TGF-β1), tissue inhibitor of metalloproteinases 1 (TIMP-1), and pro-alpha 1 collagen (pro-α1(I)) [12]. IS also induces oxidative stress in tubular and endothelial cells and stimulates aortic calcification in hypertensive rats [13-16]. Hence, it is involved in the progression of CKD, osteodystrophy, and cardiovascular disease. In addition, IS is a potent endogenous agonist for the human aryl hydrocarbon receptor, prolonged activation of which may contribute to uremic toxicity observed in dialysis patients [17]. pCS induces shedding of endothelial microparticles, a marker of cell injury [18]. Moreover, pCS also has a pro-inflammatory effect, as illustrated by the increased oxidative burst activity of unstimulated leukocytes [19]. Therefore, it might contribute to the propensity for vascular damage of renal patients. Although no effect on leukocyte oxidative burst activity has been found for pCG, combination with pCS resulted in a synergistic activating effect [20]. IAA is involved in progression of interstitial renal fibrosis. In vitro, IAA induces free radical production in tubular cells, activates NF-kappa B and PAI-1 promoter, and increases PAI-1 expression [13]. CMPF causes uremic toxicity by inhibition of drug binding to

albumin, of hepatic glutathione S-transferase, of erythropoiesis, of mitochondrial respiration, and of thyroxine hepatocyte transport, thereby leading to inhibition of deiodination of thyroxine (T3) [11]. All these solutes except uric acid are protein-bound, which changes the efficiency of their removal by dialysis compared with non-protein-bound uremic retention solutes of similar molecular weight, for example urea or creatinine. Solute reduction ratios by hemodialysis are 20 % for pCS and 30 % for IS, instead of 70 to 80 % for water-soluble molecules, for example creatinine [21, 22]. CMPF cannot be removed by conventional hemodialysis because it is almost 100 % bound to serum albumin [23]. Because many of these molecules have been linked to cardiovascular morbidity and mortality [3, 21], it seems logical to develop methods to optimize removal. This objective necessitates precise determination of several solutes on a repeated basis during a dialysis session, for calculation of clearance and kinetics, and application of the same methods to compare different removal strategies [24]. Achieving this requires measurement methods which are accurate without being too labor-intensive, time-consuming, or costly. Several recent studies have focused on the quantitative analysis of these solutes [20, 22, 25-33], which are routinely determined by HPLC coupled with UV and fluorescence detection. Because no simultaneous method enabling quantification of all these species was yet available, and to increase sensitivity and specificity and to reduce analysis time, a combined U(H)PLC-MS/MS method was developed for analysis of these seven uremic toxins in the serum of CKD patients.

V.3. Materials and methods

V.3.1. Chemicals

UA, HA, IS, and IAA were obtained from Sigma Chemical (St Louis, MO, USA). The purity of these standards was >98 %. pCS was synthesized, in accordance with Feigenbaum and Neuberg, as the potassium salt [34]. The purity of the synthesized pCS was >99 % (determined by ^{1}H NMR). pCG was synthesized from protected glucuronyltrichloroacetimidate and p-cresol by use of a procedure similar to that described by Van der Eycken et al. [35]. The purity of pCG was >98.5 % (determined by LC–DAD–MS). CMPF (purity 98 %) was a kind gift from H. Liebich (Tuebingen, Germany). 1,3-$^{15}N_2$-uric acid (1,3-$^{15}N_2$-UA, purity ≥98 %) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). N-benzoyl-d_5-glycine (d_5-HA, purity 99.2 %) and indole-2,4,5,6,7-d_5-3-acetic acid (d_5-IAA, purity 98.8 %) were obtained from CDN Isotopes (Point Claire, Quebec, Canada). Methanol and water, both LC–MS grade, were purchased from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate was obtained from Fluka (Bornem, Belgium). Acetic acid was purchased from Panreac (Barcelona, Spain).

V.3.2. Apparatus

The Waters (Milford, MA, USA) UPLC–MS/MS equipment included an Acquity UPLC system and a Quattro Micro triple-quadrupole mass spectrometer. Separation was achieved on a reversed-phase Acquity UPLC BEH C18 column (1.7 µm, 100 mm × 2.1 mm) with an Acquity UPLC BEH C18 VanGuard precolumn (1.7 µm, 2.1 mm × 5 mm). A mobile phase gradient was prepared from 0.1 % acetic acid in methanol (component A) and 0.1 % acetic acid in 5 mmol L^{-1} ammonium acetate (pH 4.3; component B). The initial mobile phase composition of 5 % A was increased linearly, in 2 min, to 20 % A which was held for 3 min, followed by an increase, in 1.5 min, to 100 % A, which was held for 2.5 min before, finally, re-equilibration. The total run time was 15 min. The mobile phase flow was 0.2 mL min^{-1}. Note that the column was also flushed with isopropanol every 15 runs for removal of phospholipids which could accumulate on the precolumn and affect quantitative analysis upon uncontrolled co-elution with solutes of interest in the pg mL^{-1} and low ng mL^{-1} range. The column temperature and the autosampler temperature were 35 °C and 8 °C, respectively. Mass spectral ionization, fragmentation, and acquisition conditions were optimized on the tandem quadrupole mass spectrometer by using electrospray ionization (ESI) in the negative mode (Table V.1). Nitrogen was used as drying gas. The desolvation gas flow was 650 L h^{-1} and the cone gas flow was maintained at 10 L h^{-1}. Desolvation temperature was 350 °C and source temperature was 110 °C. Observed capillary potential was 2870 V. The collision gas (argon) was set at 2.60×10^{-3} Torr. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode with dwell and interscan delay times of 0.2 and 0.1 s, respectively. Data were collected and processed by use of Masslynx software (version 4.0).

V.3.3. Preparation of stock solutions, calibration standards, and quality-control (QC) samples

Stock solutions (1 mg mL^{-1}) of 1,3-$^{15}N_2$-UA and UA were prepared in 0.3 mol L^{-1} potassium hydroxide. Other stock solutions (HA, d_5-HA, IS, IAA, d_5-IAA, pCG, pCS, and CMPF, all 1 mg mL^{-1}) were prepared in 50:50 H_2O–MeOH. An internal standard solution containing 200 µg mL^{-1} 1,3-$^{15}N_2$-UA, 30 µg mL^{-1} d_5-HA, and 10 µg mL^{-1} d_5-IAA in H_2O was obtained from the stock solutions of the isotopically labeled species by dilution with H_2O. This solution was used for spiking all calibration solutes and samples such that they contained final concentrations of 50, 7.5, and 2.5 µg mL^{-1} 1,3-$^{15}N_2$-UA, d_5-HA, and d_5-IAA, respectively, at injection. Calibration curve standards were prepared at 10, 20, 40, 60, 80, 100, and 120 µg mL^{-1} for UA, 0.1, 0.5, 1, 5, 20, 40, and 80 µg mL^{-1} for HA and pCS, 0.5, 1, 2.5, 5, 10, 20, and 40 µg mL^{-1} for pCG, and 0.1, 0.5, 1, 5, 10, 20, and 40 µg mL^{-1} for IS, IAA, and CMPF. Low, medium, and high-concentration quality-control (QC) samples were prepared by combining three samples of serum, chosen because of their low

levels of endogenous uremic toxins, and spiking with appropriate amounts of the uremic toxins, taking into consideration the endogenous baseline level. The low, medium, and high QC samples contained, respectively, 15, 45, and 90 μg mL^{-1} UA; 0.25, 7.5, and 45 μg mL^{-1} HA; 0.25, 7.5, and 15 μg mL^{-1} IS and CMPF; 0.75, 7.5, and 15 μg mL^{-1} pCG; 7.5, 15, and 45 μg mL^{-1} pCS, and 0.25, 0.75, and 7.5 μg mL^{-1} IAA, All calibration standards and QC samples were freshly prepared on the day of the analysis. All stock solutions were stored at -20 °C and were stable under these conditions.

V.3.4. Sample preparation

A procedure comparable with that described by Meert et al. was used [20]. Blood samples were centrifuged after collection, then the serum was collected, frozen, and stored at -80 °C. Serum samples were left to thaw at room temperature and vortex mixed to ensure uniformity. Subsequently, 40 μL internal standard solution (200 μg mL^{-1} 1,3-$^{15}N_2$-UA, 30 μg mL^{-1} d_5-HA, and 10 μg mL^{-1} d_5-IAA in H_2O) was added to 160 μL serum, vortex mixed, and 600 μL water was added. Serum samples were deproteinized by heat denaturation. For this purpose, samples were heated for 30 min at 90 °C. After heating, the samples were placed on ice for 10 min. All serum samples were ultrafiltered by use of Millipore Centrifree ultrafiltration devices (Molecular weight cut-off; MWCO 30,000 Da) at 1,469 × g for 25 min. Subsequently, 600 μL ultrafiltrate was evaporated under nitrogen at room temperature and reconstituted in 120 μL of the initial mobile phase. This was transferred to an autosampler vial and 20 μL thereof was injected on to the column. Samples were prepared on the day of analysis.

V.3.5. Validation

The assay was validated for selectivity, limit of detection (LOD), limit of quantification (LOQ), calibration range, accuracy, precision, recovery, and matrix effects. Selectivity was investigated by comparison with previously developed HPLC-PDA-FLD methods. Limits of detection were determined by the procedure recommended by the Environmental Protection Agency (EPA) [36]. A standard solution containing each analyte at an estimated S/N = 10 level was injected seven times, and the standard deviations of the results were calculated. The limits of detection were calculated by multiplying the standard deviations by 3. The limit of quantification (LOQ) was defined as the lowest concentration for which acceptable precision and accuracy could be guaranteed (<20 %). Seven-point calibration curves were generated for all the compounds (in triplicate) for the concentrations given in the section “Preparation of stock solutions, calibration standards, and quality-control (QC) samples”. Internal standard calibration was used for quantification; 1,3-$^{15}N_2$-UA was used for quantification of UA, d_5-HA served for both HA and IS, and d_5-IAA for IAA, pCG, pCS and CMPF. The accuracy and precision of the method were tested by

analysis of samples spiked at three concentrations. Accuracy was defined as the difference between calculated and the specified amounts, and was expressed as a percentage. Precision was assessed as percentage relative standard deviation (% RSD) for a specific compound concentration. Absolute recovery (%) was determined by comparing peak-area ratios for normal serum samples spiked before heat denaturation with peak-area ratios for normal serum spiked after ultrafiltration. To evaluate the matrix effect, water and six different serum samples were spiked at the low QC level. The matrix effect was determined by comparison of areas obtained for water with those obtained for spiked serum samples.

V.3.6. Application to biological samples

To evaluate the suitability of the method for clinical purposes, 78 samples were analyzed by use of previously described HPLC-PDA-FLD methods [20, 33] and the results were compared with those from the novel UPLC-MS/MS method. All patients gave informed consent for sample collection. Patients were classified according to the current CKD classification system based on glomerular filtration rate (GFR). In this study, estimated GFR (eGFR) was calculated by use of the Chronic Kidney Disease Epidemiology Collaboration formulas for Caucasian men and women developed by Levey et al. [37]. We included 19 healthy controls (12 men and 7 women), 10 CKD-1 patients (4 men, 6 women), 8 CKD-2 patients (2 men, 6 women), 8 CKD-3 patients (5 men, 3 women), 8 CKD-4 patients (6 men, 2 women), 6 non-dialysis CKD-5 patients (CKD-5ND, 2 men and 4 women), and 19 hemodialysis patients (CKD-5HD, 13 men and 6 women).

V.3.7. Statistical analysis

Results are expressed as means ± standard deviations. Statistics were performed by use of GraphPad Prism 5.0 via regression analysis and an unpaired t-test. A probability level of <0.05 was considered statistically significant.

V.4. Results and discussion

The U(H)PLC method was developed on a 10 cm × 2 mm BEH C18 × 1.7 µm particle column. The greater efficiency and higher sample capacity of the 10-cm column compared with the (faster) 5-cm column ensured the development of a method reaching the required limits of quantification with symmetric peak shapes for all solutes, even when injecting up to 20 µL sample. Different mobile phase compositions were compared to achieve retention, separation, symmetric peak shapes, and method robustness. Satisfactory retention of the most polar solute, uric acid, was obtained by using initial mobile phase

conditions comparable with those of Kim et al. [26]. Ammonium acetate buffer solution (5 mmol L^{-1}, pH 4.3) was therefore mixed with 5 % methanol (containing 0.1 % acetic acid) for the initial mobile phase. A gradient of the latter was subsequently used, because it also enabled separation of all the targeted uremic toxins with high peak capacity, high S/N ratios, and high sensitivity. This is apparent from Figure V.1, which shows separation of the uremic toxins (and added internal standards) from the serum of a stage 5 CKD patient.

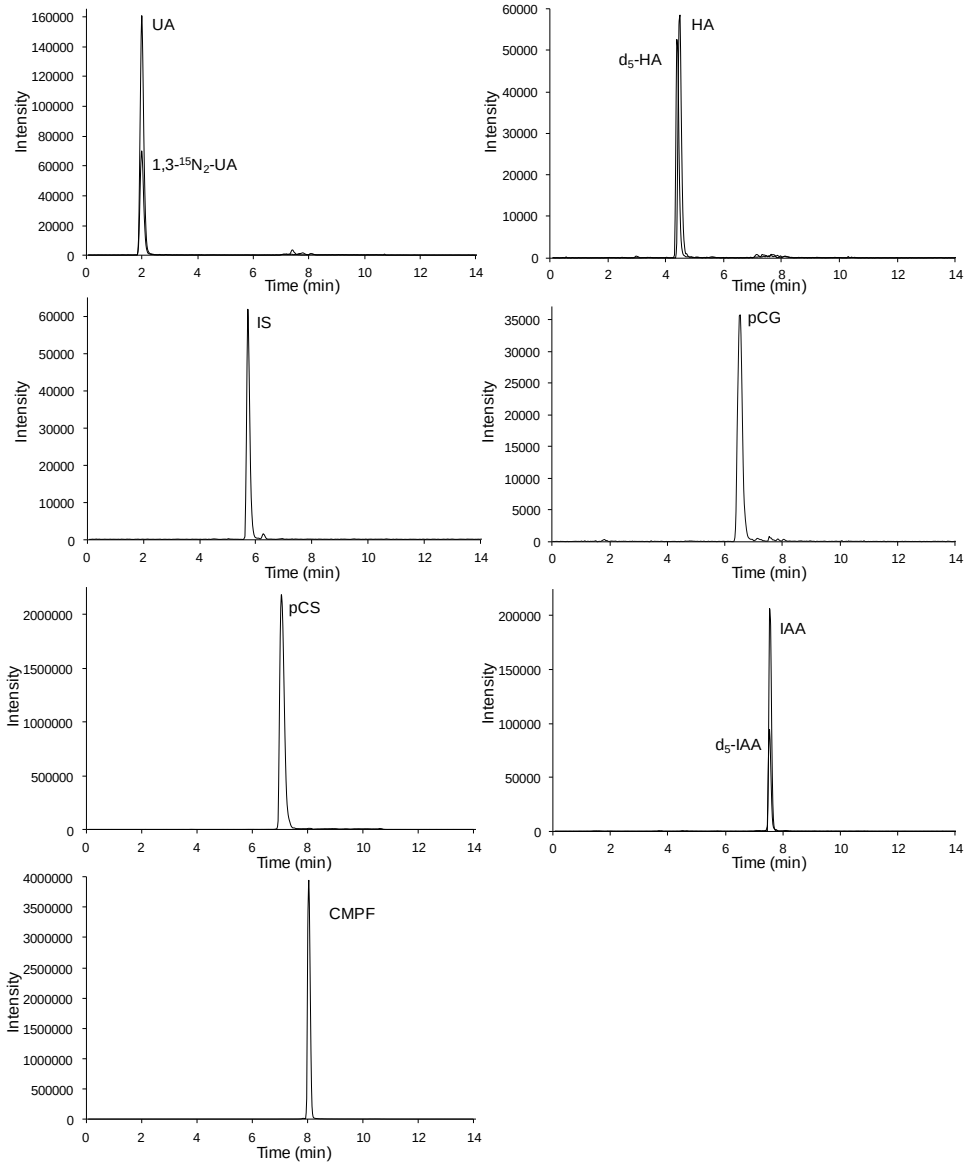


Figure V.1 Representative chromatograms obtained from a patient with CKD stage 5 for the investigated uremic toxins and the internal standards. Abbreviations: UA, uric acid; 1,3-$^{15}N_2$-UA, 1,3-$^{15}N_2$-uric acid; HA, hippuric acid; d_5-HA, N-benzoyl-d_5-glycine; IS, indoxylsulfate; pCG, p-cresylglucuronide; pCS, p-cresylsulfate; IAA, indole-3-acetic acid; d_5-IAA, indole-2,4,5,6,7-d_5-3-acetic acid; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid

Detection was performed by MS/MS operated in multiple reaction monitoring (MRM) mode, whereby only ions with m/z ratios of the solute of interest pass through the first quadrupole and are fragmented in the collision cell, and only characteristic daughter ions thereof are selectively measured in the last quadrupole of the system. Because of the combined selectivity of the compound's specific retention time, molecular weight, and daughter fragment formation, and because of possibility of quantification of several solutes at ng mL^{-1} levels in complex matrixes such as serum, the approach is increasingly implemented in bioanalysis. Although use of positive electrospray ionization enabled sensitive detection of HA, IAA, and CMPF, all seven solutes could only be detected in the negative mode, which was therefore selected, because the instrumentation did not enable use of alternating polarity ionization. Product-scan spectra of the molecular ions of the uremic toxins and of the internal standards are shown in Figure V.2. For each solute the most intense, yet selective, fragment ion was selected for quantification.

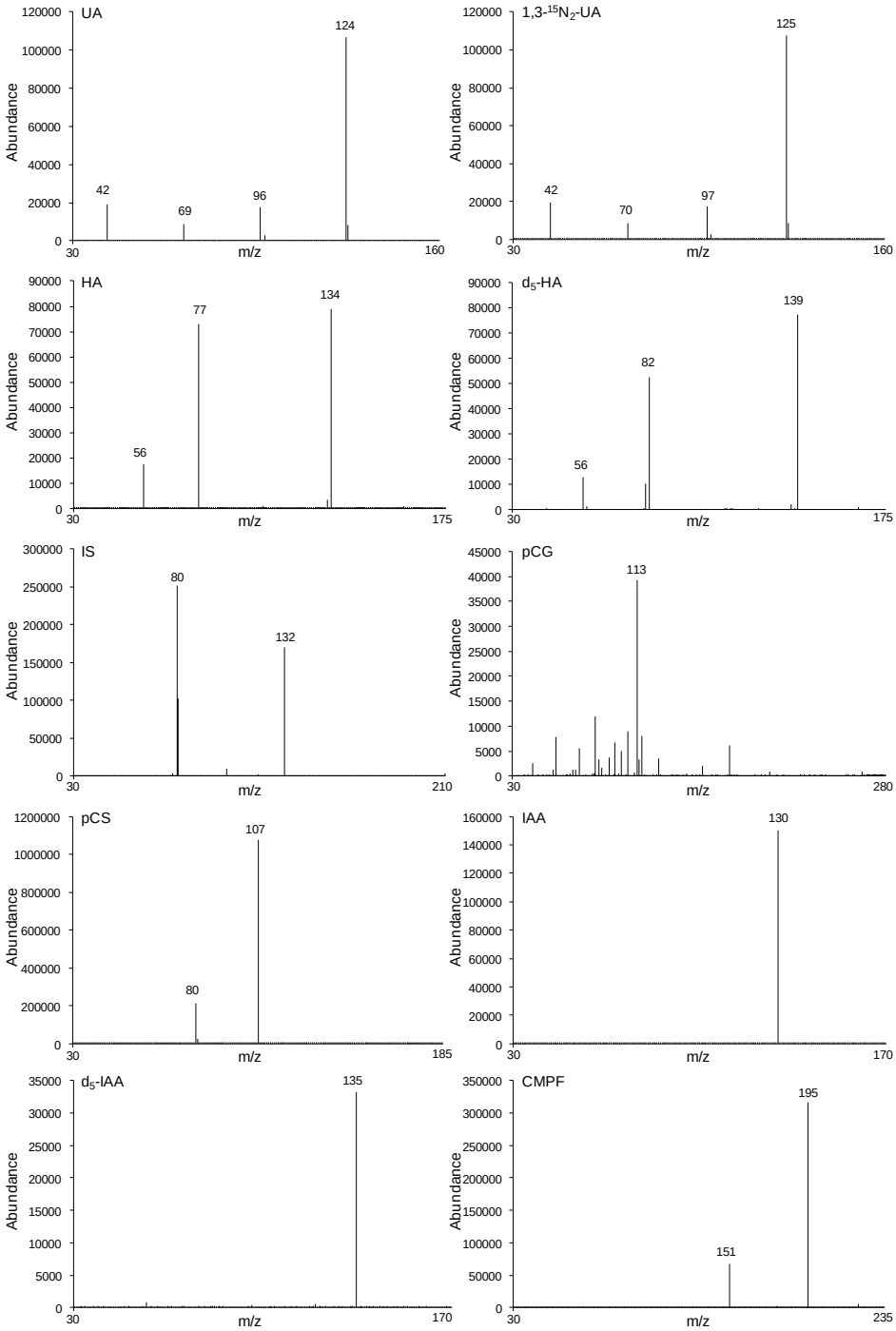
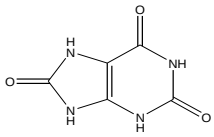
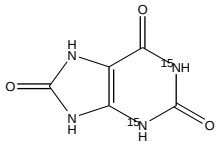
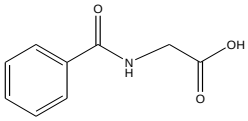
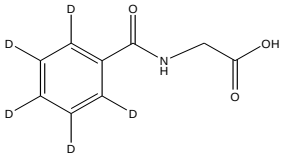
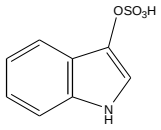
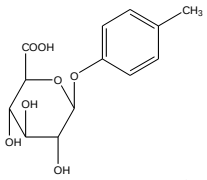
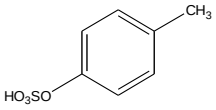
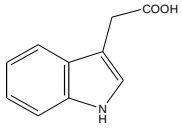
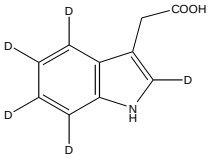
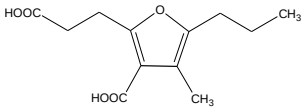


Figure V.2 MS/MS spectra acquired on a Quattro Micro triple-quadrupole mass spectrometer in the negative mode. Abbreviations as for Figure V.1.

The sensitivity for all solutes was further enhanced by adjustment of the cone and collision energy potentials for improved electrospray ionization at the time of elution (Table V.1).

Table V.1 Detection settings for the compounds investigated

Compound	Structure	Transition	Cone (Volts)	Collision Energy (eV)
uric acid (UA)		167 > 124	25	15
1,3-$^{15}N_2$-uric acid (1,3-$^{15}N_2$-UA)		169 > 125	25	15
hippuric acid (HA)		178 > 134	20	15
d_5-hippuric acid (d_5-HA)		183 > 139	20	15
indoxylsulfate (IS)		212 > 80	25	19
p-cresylglucuronide (pCG)		283 > 113	20	15
p-cresylsulfate (pCS)		187 > 107	25	18
indole-3-acetic acid (IAA)		174 > 130	15	10
d_5-indole-3-acetic acid (d_5-IAA)		179 > 135	15	10
3-carboxy-4-methyl-5-propyl-2-furan-propionic acid (CMPF)		239 > 195	20	13

After optimization, the figures of merit of the method were established. LODs and LOQs are listed in Table V.2.

Table V.2 Limits of detection and quantification, accuracy, and within and between-day precision for the quality-control samples

Uremic toxin	LOD (ng/mL)	LOQ (ng/mL)	QC Concentration (µg/mL)	Accuracy (%)	Within-day precision (%)	Between-day precision (%)
			15	3.05	0.66	13.32
UA	11	36	45	-1.60	0.83	5.09
			90	-0.34	1.09	4.87
			0.25	-10.67	2.59	9.61
HA	17	55	7.5	-7.38	1.15	5.68
			45	-0.89	0.93	1.54
			0.25	-4.89	4.05	9.15
IS	23	75	7.5	-1.33	3.68	8.41
			15	0.31	4.00	8.50
			0.75	-11.80	2.07	6.84
pCG	136	500	7.5	-5.91	1.09	4.70
			15	-0.98	2.15	7.79
			7.5	14.00	1.10	7.56
pCS	29	95	15	-1.40	0.60	7.02
			45	-8.35	1.05	4.69
			0.25	-13.33	2.66	4.51
IAA	27	100	0.75	-8.89	3.38	5.51
			7.5	-0.67	2.50	3.36
			0.25	14.84	4.54	4.77
CMPF	23	88	7.5	5.36	1.08	1.69
			15	2.00	0.62	2.94

It is apparent the limits of quantification were below 0.1 µg mL^{-1}, except for p-cresylglucuronide (pCG) for which sensitivity was lower (LOD and a LOQ 0.136 and 0.500 µg mL^{-1}, respectively). Seven-point calibration curves spanning the range of interest for the different CKD stages were constructed with the LOQ as the lowest concentration of the calibration curve for pCG and IAA, and the lowest concentration above the LOQ for all other compounds. After use of a least-squares fit, good linearity ($r^2 \geq 0.99$) was observed for all compounds. None of the calibration curves was forced through the origin, and for the regression calculation a weighing factor of 1/x was used for all data points. Although, ideally, seven isotopically labeled standards should be used, such that each internal standard undergoes the same processing as it native equivalent, this was not possible because of the excessive cost this would involve, because of commercial unavailability, and because of the absence of suitable synthetic procedures for in-

house fabrication. Therefore, three internal standards were used, whereby 1,3-$^{15}N_2$-UA was used for quantification of UA, d_5-HA served for both HA and IS, and d_5-IAA for IAA, pCG, pCS and CMPF, respectively. The choice of the internal standard for correction of each solute was based on closeness of elution. The figures of merit for this approach are reflected in the accuracy and within and between-day precision data summarized in Table V.2. For all compounds deviation of the mean measured concentration from the theoretical concentration was below the acceptable threshold of 15 % [38]. In addition, the within-day precision varied from 0.60 to 4.54 % and between-day precision was below 13.33 % for all compounds. Recovery for the medium QC level (n = 4) was 101.5 ± 4.1 % for UA, 101.9 ± 3.0 % for HA, 91.5 ± 3.9 %. for IS, 99.7 ± 3.1 % for pCG, 98.5 ± 6.3 % for pCS, 100.0 ± 3.5 % for IAA, and 100.5 ± 5.1 % for CMPF. Recovery was similar for the low and high QC levels.

One challenge in bioanalytical LC–MS/MS method development is the need to understand and circumvent matrix effect problems, which are usually caused by co-eluting endogenous compounds from biological matrices. This was evaluated by comparing the peak areas obtained for solutes from serum and water spiked at the low QC level. As shown in Table V.3, the effect of the matrix on signal intensity was always below 15 %, excluding the possibility of occurrence of a significant matrix effect. Moreover, by use of internal standard calibration the effect of this phenomenon was suppressed.

Table V.3 Matrix effect for the uremic toxins investigated

	Serum 1	Serum 2	Serum 3	Serum 4	Serum 5	Serum 6
UA	-10.27	-13.07	-11.83	-13.39	-6.20	-4.53
HA	8.41	4.57	8.74	-11.15	-6.78	-6.45
IS	-2.37	-0.82	-6.96	-3.89	-8.32	-3.88
pCG	-12.97	-9.09	-11.81	-13.32	-6.45	-10.09
pCS	-2.01	-14.63	14.46	3.65	-14.54	-2.06
IAA	-4.49	2.06	4.57	-2.90	-4.76	2.14
CMPF	-10.81	-14.79	-4.29	-13.89	-9.90	-13.95

The method was applied to analyze 78 serum samples from 19 healthy volunteers and 59 patients at CKD stages 1-5. Serum concentrations of the uremic toxins, by CKD stage, are listed in Table V.4. It can be seen that levels are significantly higher from CKD stage 3 on, except for HA and UA, for which an increase was seen at stage 4 only. No significant increase in UA concentration was observed for CKD-5HD patients, however, confirming efficient removal of UA by hemodialysis, because it is a small water-soluble molecule. pCG levels were below the limit of quantification (LOQ) for all controls and CKD-1 and CKD-2 samples.

Table V.4. Serum concentrations of the uremic toxins by CKD stage (µg/mL)

	Control *(n = 19)*	CKD Stage					
		1 *(n = 10)*	**2** *(n = 8)*	**3** *(n = 8)*	**4** *(n = 8)*	**5 ND**[a] *(n = 6)*	**5 HD**[b] *(n = 19)*
UA	59.1 ± 14.5	57.1 ± 11.5	60.3 ± 16.5	71.3 ± 17.0	91.1 ± 30.4**	86.6 ± 19.6**	65.4 ± 12.3
IS	0.5 ± 0.3	0.5 ± 0.3	0.7 ± 0.5	1.7 ± 1.1**	2.7 ± 2.1**	4.6 ± 2.1**	19.2 ± 9.9**
HA	1.3 ± 1.6	0.8 ± 0.5	0.9 ± 0.7	2.0 ± 1.6	3.2 ± 2.0*	6.9 ± 1.6**	34.5 ± 20.0**
pCG	< LOQ[c]	< LOQ[c]	< LOQ[c]	0.7 ± 0.2	0.9 ± 0.3*	1.4 ± 1.5	13.5 ± 10.3**
pCS	6.6 ± 3.7	9.5 ± 4.9	10.3 ± 6.2	20.0 ± 7.3**	21.9 ± 13.7**	26.5 ± 9.8**	55.9 ± 15.1**
IAA	0.5 ± 0.2	0.4 ± 0.2	0.6 ± 0.2	1.0 ± 0.6**	1.0 ± 0.3**	1.0 ± 0.5**	2.2 ± 1.0**
CMPF	1.1 ± 1.1	1.0 ± 0.7	1.4 ± 1.1	3.5 ± 2.6**	3.1 ± 1.6**	5.1 ± 2.4**	5.4 ± 4.0**

[a] ND, non-dialysis; [b] HD, hemodialysis; [c] LOQ, limit of quantitation; * $P < 0.05$ versus control; ** $P < 0.01$ versus control

Although several methods have been used to determine uremic toxin concentrations, to the best of our knowledge no single method enabling determination of these seven uremic toxins had previously been available [20, 22, 25-33]. Our method overcomes several of the disadvantages of previously described approaches. The analysis time of the HPLC-FLD method described by Meert et al. for determination of pCS and pCG is 25 min. Determination of UA, HA, IS, IAA, and CMPF by the method described by Fagugli et al. takes 60 min. Consequently, total analysis time for determination of the seven uremic toxins is 85 min. Hence, the novel UPLC-MS/MS method, with an analysis time of 15 min, is almost a factor of six faster than these commonly used methods. In Figure V.3 the UPLC-MS/MS results are correlated with those obtained by use of the routine methods. The correlation is good, with correlation coefficients (Pearson's r) higher than 0.93 for all compounds.

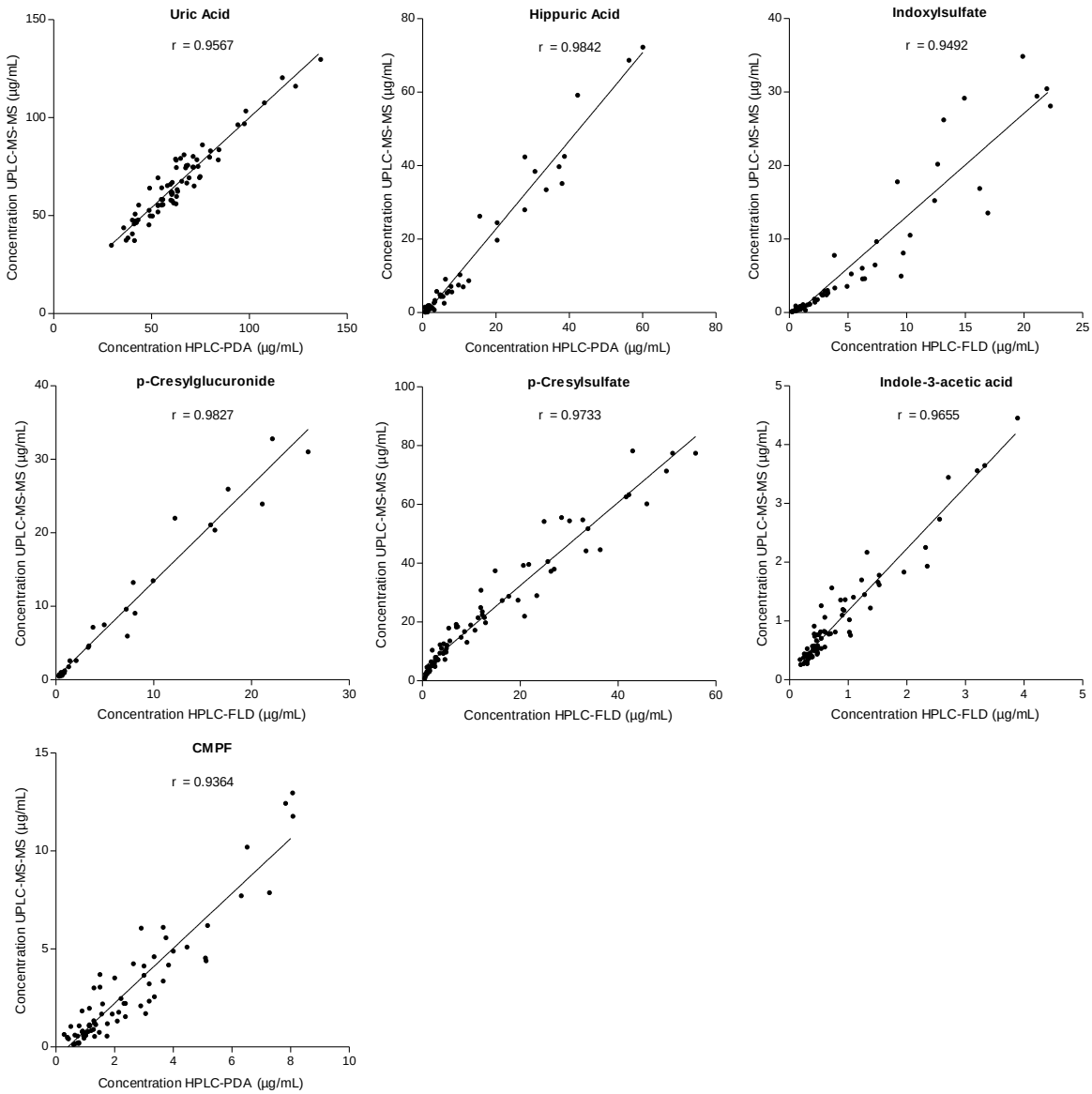


Figure V.3 Correlation of concentrations determined by HPLC–PDA–FLD with those determined by UPLC–MS/MS

V.5. Conclusion

A simple, rapid, and sensitive UPLC-MS/MS method for quantification of seven uremic toxins in serum has been developed and validated. The method was used to quantify the toxins in the serum of patients at all stages of CKD and of healthy volunteers. Good correlation was obtained between these results and those obtained by use of conventional HPLC-PDA-FLD methods. Hence, this method could be of clinical importance for follow-up of CKD patients, for surveillance and improvement of the effectiveness of blood purification during dialysis treatment, and for assessment of cardiovascular disease.

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Chapter 6

Determination of asymmetric and symmetric dimethylarginine in serum from patients with chronic kidney disease: UPLC-MS/MS versus ELISA

VI.1. Abstract

Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide (NO) synthesis, and its structural isomer symmetric dimethylarginine (SDMA) are uremic toxins accumulating in chronic kidney disease (CKD) patients. The objective of this study was to develop and validate a robust UPLC-MS/MS method for the simultaneous determination of ADMA and SDMA in human serum. Chromatographic separation after butylester derivatization was achieved on an Acquity UPLC BEH C18 column, followed by tandem mass spectrometric detection in positive ionization mode. Isotopically labeled ADMA (d_7-ADMA) was used as internal standard. Accuracy (bias) was below 12.35%. Within- and between-day precision ranged from 1.93 to 3.48% and from 5.25 to 10.93%, respectively. The applicability of the method was evaluated by the analysis of serum samples from 10 healthy controls and 77 CKD patients on hemodialysis (CKD5-HD). Both ADMA (0.84 ± 0.19 µM vs. 0.52 ± 0.07 µM) and SDMA concentrations (2.06 ± 0.82 µM vs. 0.59 ± 0.13 µM) were significantly ($p<0.001$) elevated in CKD-5HD patients compared to healthy controls. In addition, an established commercially available ELISA assay was utilized on the same samples (n=87) to compare values obtained both with ELISA and UPLC-MS/MS. For ADMA we found 0.97 ± 0.23 µM vs. 0.49 ± 0.06 µM ($p<0.001$) and for SDMA 2.09 ± 0.59 µM vs. 0.62 ± 0.09 µM ($p<0.001$) in CKD5-HD patients and healthy controls respectively. Correlation between these two methods was significant but weak for both ADMA ($r^2=0.61$) and SDMA ($r^2=0.52$).

VI.2. Introduction

Chronic kidney disease (CKD) is a worldwide public health problem with cardiovascular disease (CVD) as most important and often fatal complication [1,2]. A myriad of toxic solutes, normally cleared by the kidneys, among which asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA), accumulate in the body of CKD patients [3]. Both originate from proteolysis of methylated proteins [4]. Arginine residues within proteins can be post-translationally methylated by a class of enzymes, named protein arginine methyltransferases (PRMTS). Proteolysis of proteins containing methylated arginine releases free methylarginines into the cytosol. Once released, ADMA acts as an endogenous inhibitor of nitric oxide synthase (NOS) by competing with L-arginine as the substrate [5]. Elevated plasma ADMA levels have been associated with endothelial dysfunction [5,6], which is an essential contributing element to vascular disease, and were found in patients with various risk factors for atherosclerosis as well as in CKD [7,8]. Plasma ADMA levels may predict the progression of renal injury in patients with early-stage CKD [9,10], and are an independent risk factor for cardiovascular disease and all-cause mortality in different populations, such as patients with coronary artery disease (CAD) [11,12] and patients with end stage renal disease (ESRD) [13,14]. SDMA, a structural isomer of ADMA, has long been considered biologically inactive [4,5]. Biologic activity was however at first suggested by the finding of a dose-responsive inhibition of NO synthesis by a mechanism different from that elicited by ADMA [15]. Subsequently, SDMA was shown to play a prominent role in leukocyte activation by enhancing generation of radical oxygen species (ROS), which is attributable to increased calcium influx via store-operated Ca^{2+} channels [16] and to activation of NF-κB resulting in cytokine production [17]. In addition, SDMA was proposed as biomarker for renal function outperforming creatinine-based equations for determining estimated glomerular filtration (eGFR) [18,19].

It has been suggested that the removal of ADMA in standard dialysis is completely hampered, eliciting the hypothesis that the compounds are protein bound [20]. Other studies confirmed that dialysance and thus removal of dimethylarginines is lower than would be expected with regard to their molecular weight [21,22]. However, in the latter studies the decrease in ADMA was obviously more substantial than in the one by Kielstein et al [20]. Hence, investigation of the protein-binding of ADMA and SDMA is essential to shed light on these inconsistent results.

Since ADMA shows a very narrow range of normal concentrations, even a small increase in its concentration might be linked to cardiovascular risk. Therefore, high analytical precision is of extreme importance to discriminate between normal and slightly elevated concentrations. Current methods for determination of ADMA and SDMA in biofluids include gas chromatography coupled with mass

spectrometry (GC-MS) [23,24], HPLC with fluorescence detection (HPLC-FLD) after derivatization [25-32], with mass spectrometric detection (LC-MS and LC-MS/MS) underivatized [14,34-41] or after derivatization [42-46], and capillary electrophoresis coupled with ultraviolet (CE-UV) [47] or mass-spectrometric detection (CE-MS) [48]. In addition, an enzyme-linked immunosorbent assay (ELISA) has been developed [49] and several comparisons between this assay and chromatographic methods have been described for ADMA [49-55]. Some comparisons suggest that the ELISA assay for ADMA suffers from matrix effects producing concentration-dependent positive bias compared with other methods [52,54-55]. Moreover, some discrepancies seem to exist between the reported method comparisons. For SDMA no method comparisons have been described yet.

In this study, the primary aim was to develop and validate a robust U(H)PLC-MS/MS method for the simultaneous determination of ADMA and SDMA in human serum. Secondly, we investigated the protein binding of ADMA and SDMA in serum. Finally, the UPLC-MS/MS data were compared with an established ELISA for both ADMA and SDMA.

VI.3. Materials and methods

VI.3.1. Chemicals

Asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) were obtained from Merck (Darmstadt, Germany). The internal standard 2,3,3,4,4,5,5-d_7-ADMA:HCl:H_2O (d_7-ADMA, 98%) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Methanol and water, both LC-MS grade, were purchased from Biosolve (Valkenswaard, The Netherlands). Acetic acid was obtained from Panreac (Barcelona, Spain). Ammonium acetate was purchased from Fluka (Bornem, Belgium). 1-Butanol was obtained from Merck (Darmstadt, Germany) and hydrochloric acid from Fluka (Bornem, Belgium). ELISA kits were purchased from DLD Diagnostika GmbH (Hamburg, Germany).

VI.3.2. UPLC-MS/MS assay

VI.3.2.1. Instrumentation

The Waters UPLC–MS/MS system comprised an Acquity UPLC System and a Quattro Micro triple quadrupole mass spectrometer (Milford, MA, USA). Separation was performed on an Acquity UPLC BEH C18 column (1.7 µm, 2.1 mm x 100 mm) with an Acquity UPLC BEH C18 VanGuard precolumn (1.7 µm, 2.1 mm x 5 mm). The mobile phase consisted of methanol containing 0.1% acetic acid (v/v; mobile phase A) and ammonium acetate buffer (5 mM) containing 0.1% acetic acid (v/v; pH 4.3; mobile phase B). A gradient elution at a flow of 0.25 mL/min was performed with an initial composition of 15% A, which was

held for 3.5 min, followed by an increase in 0.01 min to 100% A (for 1.5 min) and finally a re-equilibration (5 min). The total run time was 10 min. The column was flushed with isopropanol every 15 runs to remove phospholipids which can be a significant source of imprecision in quantitative analyses [56]. The column temperature and the autosampler temperature were kept at 21°C and 8° C, respectively. Mass spectral ionization, fragmentation, and acquisition parameters were optimized on the tandem quadrupole mass spectrometer using electrospray ionization (ESI) in the positive mode (Table VI.1). The ion source temperature and the desolvation temperature were maintained at 120 and 350 °C. Nitrogen was used as nebulizer and desolvation gas. The desolvation gas flow was set at 650 L/h and the cone gas flow was 10 L/h. Capillary voltage was 3260 V. The collision gas (argon, purity 99.999%) was set at 2.58 x10−3 Torr. Quantification was performed in the multiple reaction monitoring (MRM) mode with dwell and interscan delay times of 0.2 and 0.1 s, respectively. Data were acquired and processed using Masslynx software (version 4.0).

VI.2.2.1. Preparation of stock solutions, calibration standards and quality control (QC) samples

Stock solutions of ADMA (1.24 mM), SDMA (1.24 mM) and d_7-ADMA (4.78 mM) were prepared in H_2O. An internal standard solution containing 2.39 µM d_7-ADMA was obtained from the stock solution by dilution with H_2O. This solution was further used for spiking all calibration solutes and the samples such that they contained a final concentration of 0.60 µM d_7-ADMA, at injection. Calibration curve standards were prepared at 0.1, 0.25, 0.49, 0.74, 0.99, 1.24 and 1.48 µM for ADMA and at 0.1, 0.25, 0.49, 1.24, 2.47, 3.71 and 4.94 µM for SDMA. Low, medium, and high-concentration quality-control (QC) samples were prepared by combining three samples of normal serum, selected because of their low levels of endogenous uremic toxins, and spiking them with appropriate amounts of dimethylarginines, taking into account the endogenous baseline level. The low, medium, and high QC samples were spiked with, respectively, 0.25, 0.49 and 0.99 µM ADMA and 0.25, 1.24 and 3.71 µM SDMA. All calibration standards and QC samples were freshly prepared on the day of analysis and were run in triplicate. All stock solutions were stored at -20°C and were stable at these conditions.

VI.3.2.2. Sample preparation

The deproteinization procedure described by Meert et al. was slightly adapted [57]. Blood samples were allowed to clot and were subsequently centrifuged. Serum was collected, frozen and stored at -80°C. Serum samples were thawed at room temperature and vortex mixed to ensure homogeneity. Subsequently, 40 µL of internal standard solution (2.39 µM of d_7-ADMA) was added to 160 µL of serum, vortex mixed and 600 µL of water was added. To determine the total concentration, serum samples were

first deproteinized by heat denaturation. To this end, samples were heated for 30 minutes at 90°C. After heating, the samples were placed on ice for 10 minutes. All serum samples were then ultrafiltered using Millipore Centrifree ultrafiltration devices (MWCO 30,000 Da) at 1,469 x *g* for 25 minutes. To determine the free fraction, serum samples were filtered through Millipore Centrifree ultrafiltration devices prior to heating. Subsequently, 600 µL of ultrafiltrate was dried under nitrogen at room temperature. ADMA, SDMA and the internal standard were analyzed as their butyl ester derivatives. Derivatization step was performed by dissolving the dried extract in 500 µL of a freshly prepared 1M HCl in 1-butanol solution. After 2 min vortexing, the solution was kept at 70°C for 20 min. The solvent was subsequently removed by evaporation under nitrogen. The derivatized samples were reconstituted in 120 µL of the initial mobile phase and were transferred to an autosampler vial (glass insert P/N WAT094171). Afterwards, 20 µL was injected on the column. Samples were prepared on the day of analysis.

VI.3.2.3. Validation

The tested validation parameters were selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, precision, recovery, and matrix effects. The limits of detection were determined according to the EPA recommended procedure [58]. A standard solution containing each dimethylarginine at the estimated S/N=10 level was injected seven times, and the standard deviations of the peak areas (and of the corresponding concentrations) were calculated. The limits of detection were calculated by multiplying the standard deviations by three. The limit of quantification (LOQ) was calculated as three times the LOD. Seven point calibration curves were generated with **a**forementioned concentrations (Section IV.2.2.1)**.** Quantification was carried out by internal standard calibration with d_7-ADMA as internal standard for both ADMA and SDMA. The accuracy and precision of the method were evaluated by the analysis of spiked samples at three QC levels (n=5). Accuracy was defined as the difference between the calculated and the specified amount for the selected compound and expressed as a percentage. Precision was obtained as the percentage relative standard deviation (% RSD) for a selected compound and level. The relative recovery (%) was determined by comparing the peak area ratios of ADMA and SDMA in normal serum samples spiked before heat denaturation to the peak area ratios of normal serum spiked after ultrafiltration, respectively. To evaluate matrix effects, we spiked water and six different serum samples at the low QC level and compared the areas obtained in water with those of the spiked serum samples.

VI.3.2.4. Application to biological samples

The described method was applied to serum samples from healthy controls (n=10) and from CKD patients on hemodialysis (CKD-5HD, n=77). In order to establish protein binding, both total and free concentrations were determined for the healthy control group and for 20 randomly selected CKD-5HD patients. All patients gave informed consent for sample collection.

VI.3.3. ELISA assay

Two established competitive ELISA's were used for measuring ADMA and SDMA according to manufacturer's guidelines. Briefly, serum samples were acylated before adding to the microtiter plate. Overnight, acylated ADMA or SDMA competes with the solid phase bound ADMA or SDMA for a fixed number of rabbit anti-ADMA or anti-SDMA antiserum binding sites. After equilibration, antibody bound to the solid phase ADMA or SDMA is detected by the reaction of anti-rabbit peroxidase and the substrate TMB (3,3',5,5'-Tetramethylbenzidine). Samples were analyzed using the EL808 Ultra Microplate Reader from Bio-Tek Instruments (Winooski, VT) at 450 nm (reference wavelength of 650 nm) using the KC4V3.0 Analysis Software. The amount of antibody measured is inversely proportional to the ADMA or SDMA concentration.

VI.3.4. Nephelometric assay

A1-acid glycoprotein (AAG), an acute phase protein with a MW of 43 kDa, was determined by nephelometry on a Behring Nephelometer (Siemens).

VI.3.5. Statistical Analysis

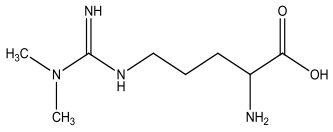
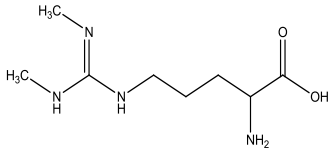
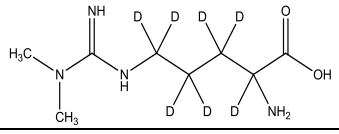
Results are expressed as means ± standard deviations. Statistics were performed using GraphPad Prism 6.0 via regression analysis, unpaired t-test and Bland-Altman plots. A p-value of < 0.05 was considered as statistically significant.

VI.4. Results and discussion

To promote retention on the Acquity UPLC BEH C18 column and to improve sensitivity, the dimethylarginines were derivatized to their butyl ester analogues. This derivatization was based on the method described by Schwedhelm et al [46]. Different mobile phase compositions were compared to achieve retention, separation, symmetric peak shapes, method robustness and fast analysis. Ammonium acetate buffer solution (5 mM, pH 4.3) was mixed with 15% methanol (containing 0.1% acetic acid) as initial mobile phase. Detection was performed by tandem mass spectrometry operated in multiple

reaction monitoring (MRM) mode, which is characterized by its sensitivity and selectivity and therefore widely implemented in bioanalysis. ADMA and SDMA exhibit the same protonated molecular ion with m/z 259 and have their most intense mass transition in common (259→70). Next to this transition they have unique mass transitions, which have lower intensity. ADMA fragments by loss of 45 (corresponding to dimethylamine) and SDMA by loss of 31 (corresponding to methylamine). Although ADMA and SDMA were almost baseline separated and it was therefore not absolutely necessary to distinguish them by their different fragmentation pattern, we chose to monitor these unique transitions because of the unequivocal selectivity towards the parent compounds. The sensitivity for the dimethylarginines was further optimized by adjustment of the cone and collision energy potentials (Table VI.1).

Table VI.1 Detection settings for the compounds investigated

Compound	Structure	t_R (min)	Transition (after derivatization)	Cone (V)	Collision Energy (eV)
ADMA		3.7	259 > 214	27	15
SDMA		3.9	259 > 228	27	15
2,3,3,4,4,5,5-d_7-ADMA		3.7	266 > 221	27	15

Representative MRM chromatograms obtained from a CKD stage 5 patient are depicted in Figure VI.1. No interferences from other endogenous substances were apparent.

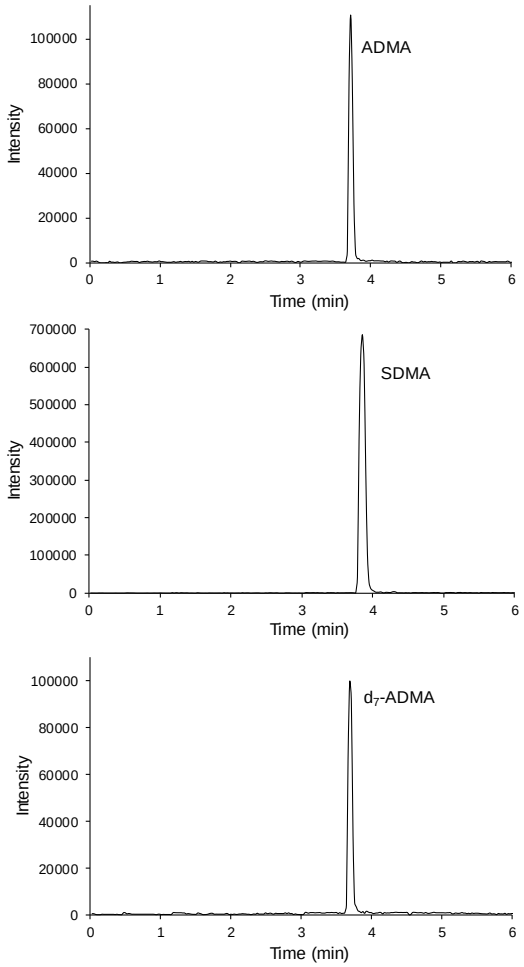


Figure VI.1 Representative chromatograms obtained from a patient with CKD stage 5 for the investigated dimethylarginines and for the internal standard

After optimization, the figures of merit of the method were established. The results of the accuracy, within- and between-day precision, recovery, LOD and LOQ tests are summarized in Table VI.2.

Table VI.2 Figures of merit

Uremic toxin	LOD (nM)	LOQ (nM)	QC Concentration added (µM)	Accuracy (%)	Within-day precision (%)	Between-day precision (%)	Recovery (%)
			0.25	-9.65	2.00	7.79	100.5 ± 3.3
ADMA	7.9	23.7	0.49	-8.14	3.48	6.13	102.2 ± 3.2
			0.99	-4.87	2.56	5.25	98.7 ± 2.5
			0.25	-12.35	3.34	9.92	96.2 ± 4.8
SDMA	6.4	19.2	1.24	-6.83	2.31	10.93	94.6 ± 5.5
			3.71	11.69	1.93	8.61	97.4 ± 4.1

The LOD and LOQ were below the lowest calibration point of the seven-point calibration curves (i.e. below 0.1 µM). Good linearity ($r^2 \geq 0.99$) was observed for both dimethylarginines using a least square fit. Isotopically labeled ADMA served as internal standard for both ADMA and SDMA. The deviation of the mean measured concentration from the theoretical concentration was below 12.35%. Within- and between-day precision were below 3.48% and 10.93%, respectively. Recoveries were high and more importantly reproducible. The effect of the matrix on signal intensity was below 15% for all 6 serum samples. The occurrence of a significant matrix effect could therefore be excluded. Moreover, the use of the isotopically labeled internal standard, the gradient to 100% A, and the additional isopropanol wash after every 15 injections were all measures reducing the risk for such effect.

Figure VI.2 displays the dimethylarginine serum concentrations determined by UPLC-MS/MS in healthy controls and CKD-5HD patients. ADMA and SDMA concentrations are both significantly elevated in CKD patients compared to healthy controls ($p<0.001$). For ADMA, we found 0.52 ± 0.07 µM in healthy controls and 0.84 ± 0.19 µM in CKD-5HD patients. For SDMA, mean normal concentration is 0.59 ± 0.13 µM and mean concentration in CKD-5HD patients is 2.06 ± 0.82 µM. The control values are within the previously reported ranges for ADMA [54] and slightly higher for SDMA [26,36]. Our reported concentrations for CKD patients are also consistent with published values [26,59].

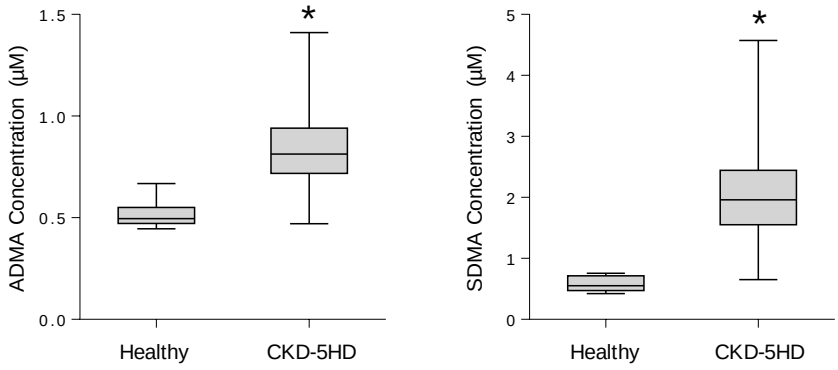


Figure VI.2 ADMA and SDMA serum concentrations determined by UPLC-MS/MS in healthy controls (n=10) and CKD-5HD patients (n=77). * p < 0.001 versus control

In general, low degrees of protein binding were found for both ADMA and SDMA. Protein binding of ADMA is 6.53 ± 4.93 % in healthy controls and 4.01 ± 2.90 % in CKD patients. For SDMA protein binding is 12.02 ± 8.73 % in healthy controls and 10.36 ± 9.74 % in CKD patients. It was, however, not possible to determine the phenomenon accurately as most of the concentration differences were smaller than the error margins of the method. An unpaired t-test showed that the total concentrations were significantly

higher ($p<0.0001$) than the free concentrations in all conditions. A significant increase in ADMA and SDMA was also seen in CKD patients versus healthy controls for both total and free concentrations. For SDMA, however, quite high interindividual variability in protein binding was encountered. Basic compounds such as dimethylarginines not only bind to albumin, but can also bind to a1-acid glycoprotein (AAG), an acute phase protein that is often elevated in CKD. In order to obtain more information on the interindividual differences, the correlation between protein binding and AAG concentration was investigated. However, no correlation between these variables was found. Nevertheless, we can conclude that the ADMA and SDMA are only minimally protein bound in contrast to what is described in literature [20] and that therefore, it is more likely that the removal of ADMA in standard dialysis is hampered because of complex kinetics and distribution instead of protein binding.

Next to the developed UPLC-MS/MS method, a commercially available ELISA assay was utilized to determine ADMA and SDMA in the same 87 serum samples. This assay provided mean normal concentrations of 0.49 ± 0.06 µM for ADMA and 0.62 ± 0.09 µM for SDMA. Mean concentrations of 0.97 ± 0.23 µM for ADMA and 2.09 ± 0.59 µM for SDMA were found in CKD-5HD patients.

UPLC-MS/MS and ELISA results showed only weak correlation, with r^2 of 0.61 for ADMA and 0.52 for SDMA. In literature different method comparisons have been described for ADMA determination. Schulze et al. reported for the first time on the ELISA assay for ADMA [49]. To assess the analytical performance of the assay, ELISA was compared with a GC-MS and LC-MS/MS method [49]. Good correlations were found for both GC-MS (r^2=0.98, n=9) and LC-MS/MS (r^2=0.97, n=29). However, in three out of the nine samples submitted to the GC-MS method, serum was spiked with ADMA concentrations exceeding the concentrations found in human serum of CKD patients. In spite of the good correlation, an overestimation of ~20% was observed for the serum ADMA concentrations determined by ELISA compared with LC-MS/MS. Subsequently, several independent groups also compared their methods with the commercially available ELISA for ADMA. Valtonen et al. found no correlation (r^2=0.10) between serum ADMA concentrations determined by HPLC-FLD (ortho-phthaldialdehyde; OPA-derivatization) and the ELISA assay [50]. However, Schulze et al. signaled that the ELISA kit controls were outside the given range in two out of three ELISA kits [51]. Martens-Lobenhoffer et al. found considerable disagreement in the Bland-Altman plot between LC-MS (OPA-derivatization) and ELISA for ADMA concentrations in plasma from healthy and diseased individuals [52]. The ELISA assay appeared to overestimate the ADMA concentrations by a factor of about two. The matrix dependence of the ELISA was suggested as cause of this overestimation. Široká et al. found good correlation (r^2=0.89) for plasma ADMA concentrations between HPLC-FLD (OPA-dervatization) and the ELISA assay [53]. However, the ELISA assay provided

about two-fold higher ADMA concentrations than HPLC-FLD. Horowitz and Heresztyn found some correlation (r^2=0.69) between ADMA concentrations (serum and plasma) determined by HPLC-FLD (AccQ-Fluor™ derivatization) and ELISA [54]. However, the difference between the two methods increased with increasing ADMA concentration, as was also the case in the study by Martens-Lobenhoffer et al. [53] Recently, Pecchini et al. compared plasma ADMA concentrations determined by LC-MS and ELISA (r^2=0.48) and again an increasingly pronounced overestimation in ADMA levels by ELISA was found with increasing ADMA concentration [55]. From the Bland-Altman plot based on our own data (Figure VI.3) an overestimation in ADMA concentration by ELISA is observed. Moreover, the difference between the two methods tends to increase with increasing ADMA concentration, which is in agreement with previous findings. For SDMA no method comparisons have been described yet and no reference frame is therefore available. The regression line (solid line) intersects the identity line (dashed line). From the Bland-Altman plot a slightly inversed trend seems visible for SDMA, but no fixed bias could be shown.

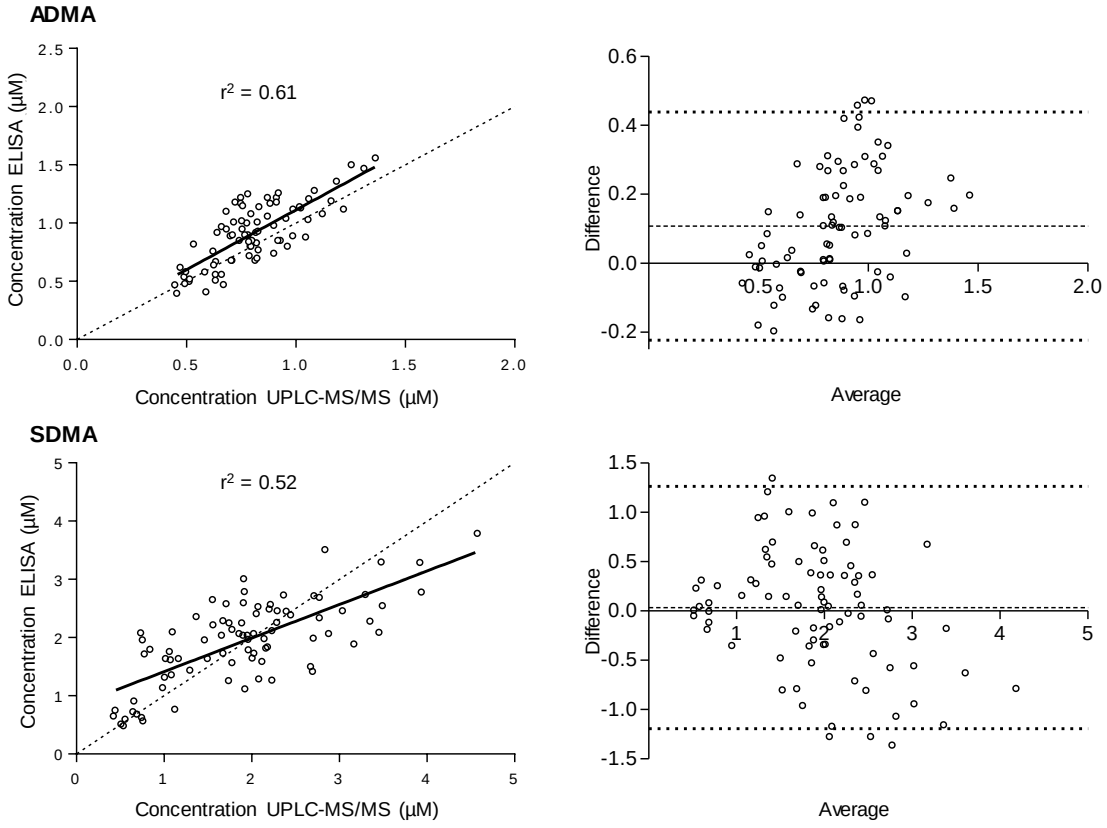


Figure VI.3 Correlation between concentrations determined by ELISA and UPLC-MS/MS (left). The dashed line is the identity line. Bland-Altman plots for ADMA and SDMA: comparison of ELISA and UPLC-MS/MS assay (right)

By comparison the new proposed UPLC-MS/MS method, which allows simultaneous determination of both arginine derivatives over CKD levels while ensuring robustness due to the chromatographic

separation, is shown to be efficient and applicable in CKD research. The observed and reported discrepancies related to the ELISA assay warrant further study.

VI.5. Conclusion

In conclusion, a robust UPLC-MS/MS has been developed and validated for the simultaneous determination of ADMA and SDMA in serum. The method has been applied to analyze serum from healthy controls and CKD patients on hemodialysis. A significant increase in serum concentrations was found in CKD patients. Protein binding of both ADMA and SDMA has been investigated and low protein binding was suggested. Comparison between the developed UPLC-MS/MS method and the commercially available ELISA showed a modest correlation. Accuracy and precision testing confirm the effectiveness of the UPLC-MS/MS methodology.

VI.6. References

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Chapter 7

Summary, conclusions and future perspectives

Chronic kidney disease (CKD) is often characterized by a progressive loss in renal function over a period of months to years. Uremic retention solutes, which are normally secreted by the healthy kidneys into the urine, accumulate in the body. If these solutes have a negative impact on the body, they are called uremic toxins. Approximately 150 uremic retention solutes are known to date, but numerous solutes are not identified yet.

Serum creatinine is a biomarker of CKD that is in widespread clinical use. Biomarkers are measurable characteristics that reflect the severity or presence of a disease state and are therefore used for disease stratification and diagnosis. Serum creatinine is an unsatisfactory biomarker because of its lack of specificity and slow response to alterations in disease severity or treatment. Hence, there is a clear need for novel biomarkers of CKD.

Chapter 1 situates CKD and describes the following aspects: uremic retention solutes and their classification according to physicochemical characteristics, renal replacement therapy such as transplantation and dialysis, current biomarkers of CKD and their limitations. Furthermore, the outline and aims are formulated in this chapter, i.e. the set-up and use of metabolic analytical platforms to identify new uremic retention solutes and/or potential biomarkers of CKD and the acceleration and the broadening of analytical methods that are used for the determination of uremic toxins.

Chapter 2 summarizes the terminology specific to the metabolomics field. Next, the workflow in metabolomics studies is discussed in more detail. Sampling, storage and transfer of the samples, sample preparation, analytical determination, data-treatment, statistical analysis, metabolite identification, and

biological interpretation are described. In the last part of this chapter, a literature survey of metabolomics studies in the context of CKD is presented.

The experimental section of this thesis can be divided into 2 parts. On the one hand, there is a non-targeted part zooming in on the search for novel uremic retention solutes and/or potential biomarkers of CKD (chapters 3 and 4). On the other hand, we distinguish a targeted part focusing on the quantification of known uremic toxins (chapter 5 en 6).

In **chapter 3**, a metabolomics platform, based on a combination of reversed-phase liquid chromatography (RPLC) coupled to high-resolution quadrupole-time-of-flight mass spectrometry (Q-TOF MS) in both negative and positive ionization mode and gas chromatography (GC) coupled to quadrupole MS, has been evaluated as discovery tool to reveal potential uremic toxins and biomarkers of CKD. Serum samples of a healthy control group and of two patient groups suffering from different stages of CKD, i.e. CKD stage 3 (CKD3) and CKD stage 5 on haemodialysis (CKD5HD) were analyzed. Protein precipitation was carried out with methanol and the two-step derivatization before GC-MS analysis consisted of methoxyamination and trimethylsilylation. The quality of analysis was monitored by means of quality control samples. A substantial portion of the serum metabolome was covered. To reveal new uremic retention solutes, restrictions were set on the fold change between different sample groups in accordance with the relative differences uncovered for creatinine, a current marker. Our identification strategy fully exploited the features of the high-resolution Q-TOF MS system. Next to 43 already described uremic retention solutes and/or toxins, 42 yet unknown metabolites could be related to CKD progression. Thirty-one unique metabolites were revealed which were increasing significantly throughout CKD progression. High fold changes were found for aminohydroxyhippuric acid, 2-hydroxyhippuric acid glucuronide, 2-/3-hydroxyhippuric acid sulphate, hydroxypyridine, methoxy-hydroxyphenylglycol glucuronide, lactose, a tetrasaccharide, and trihydroxypentenoic acid. Hydroxyhippurate compounds, here detected as conjugates, are among the most discriminating metabolites, emphasizing their relevance in CKD. Furthermore, 11 metabolites, mainly fatty acids, showed a decreasing trend in function of disease.

The RPLC-MS-based platform is extended by hydrophilic interaction liquid chromatography (HILIC) in **chapter 4**. Polar metabolites, being mainly primary metabolites, elute in the column void or early in the chromatographic run in RPLC. The latter species often represent metabolite classes of high significance and are thus important for the diagnosis of diseases. HILIC has become increasingly popular for the analysis of polar metabolites. However, no HILIC-based metabolomics studies have been reported yet in

the study of CKD. Urine and plasma samples of a healthy control group and of two patient groups suffering from different stages of CKD, i.e. CKD3 and CKD5 not receiving dialysis were analyzed on the HILIC-TOF platform. Again, attention was paid to the quality of analysis by utilizing quality control samples. Four metabolites, exhibiting higher fold changes than creatinine, could be identified by the use of authentic chemical standards. Glycoursodeoxycholic acid en 2-hydroxyethane sulfonate were downregulated in urine of CKD patients. Cinnamoylglycine, which was earlier reported to be accumulate in CKD, was shown to increase in plasma, while pregnenolone sulphate was found to decrease in plasma throughout CKD.

In this thesis, we aimed at fully exploiting the arsenal of analytical techniques to cover the broad metabolic picture. In this context, capillary electrophoresis coupled to mass spectrometry (CE-MS) could be added to the applied techniques. Furthermore, a lipidomics approach could shed light on disturbances in lipid metabolism in CKD.

Metabolomics is an emerging field, which is reflected by a steep increase in the number of published papers. Improvements in technology and lessons from genomics, transcriptomics, and proteomics -other broad, data-intensive disciplines of biological science - have fueled the pace of discovery. In the future we expect further developments such as the extension of databases for metabolite identification, synthesis of metabolite standards, improvements in data-processing software,… However, an increasingly recognized pitfall of the metabolomics approach, is that it tends to flood the literature with studies publishing biomarkers that show potential but which are never submitted to confirmatory validations studies. Therefore, it is important to emphasize that the potential biomarkers of CKD that were revealed by the discovery studies described in chapter 3 and 4 have to be validated in future research by targeted methods studying more extensive study populations. Quantitative data will allow the evaluation of the metabolites' disease related predictive value. Potential biomarkers should also be evaluated in combination, since a panel of biomarkers often improves the predictive performance. Furthermore, in vitro and in vivo tests at, for CKD, relevant concentrations will reveal the metabolites' biological activity and their possible role in inflammation and cardiovascular pathophysiology of CKD. Finally, quantitative data will facilitate the evaluation of their kinetics in CKD and during renal replacement therapy. In the long run, validated biomarkers might help in developing tailor-made treatments.

Uric acid (UA) and the protein-bound compounds hippuric acid (HA), indoxylsulfate (IS), p-cresylglucuronide (pCG), p-cresylsulfate (pCS), indole-3-acetic acid (IAA) and 3-carboxy-4-methyl-5-

propyl-2-furanpropionic acid (CMPF) are uremic toxins, for which deleterious effects have already been described. The development and validation of an UPLC-MS/MS method for the simultaneous determination of theses 7 uremic retention toxins in serum is described in **chapter 5**. Samples were prepared by heat denaturation followed by ultrafiltration. Isotopically labelled internal standards were used to correct for sample preparation and system performance variations. Separation of the uremic toxins was performed on a reversed-phase LC column, followed by negative electrospray ionization and tandem mass spectrometric detection. The figures of merit were determined. The applicability of the method was evaluated by analyzing 78 serum samples originating from healthy controls as well as from patients at different stages of CKD. A good correlation was found between these results and those of thus far used HPLC methods with photodiode array (PDA) and fluorescence detection (FLD). Hence, this method, which is faster than previously described methods, can be of clinical importance for the follow-up of CKD patients, the surveillance and improvement of blood purification effectiveness during dialysis treatment.

Chapter 6 focused on the development and validation of an UPLC-MS/MS method for the simultaneous determination of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS), and its structural isomer symmetric dimethylarginine (SDMA) in serum. These dimethylarginines accumulate in CKD and are linked to cardiovascular disease. After deproteinization, a butylester derivatization was performed. The derivatives were separated on a reversed-phase LC column, followed by positive electrospray ionization and tandem mass spectrometric detection. Isotopically labeled ADMA was used as internal standard. Both free fraction and total concentration were determined. The figures of merit were ascertained. The applicability of the method was evaluated by the analysis of serum samples from 10 healthy controls and 77 CKD patients on hemodialysis. Both ADMA and SDMA concentrations were significantly elevated in CKD5HD patients compared to healthy controls. Our data suggested low protein binding. In addition, an established commercially available enzyme-linked immuno sorbent assay (ELISA) was utilized to determine ADMA and SDMA concentrations in the serum samples. Correlation between these methods was modest for both ADMA and SDMA.

Thus, both targeted methods add novel analytical methods to the already available arsenal. The first method allows the simultaneous measurement of a large array of mainly protein-bound molecules. A single run is performed over a shorter period compared to methods described HPLC-PDA-FLD methods, resulting in a gain of time for high throughput analysis. The second method allows the simultaneous determination of the structural isomers ADMA and SDMA. U(H)PLC-MS/MS is used as the technique of

choice for bioanalysis of small molecules as it has many advantages in terms of resolution, speed and sensitivity. Of note, all solutes determined here have been shown to have an important biological and clinical impact in uremia, especially with regards to cardiovascular disease.

In summary, this thesis focused on (1) the identification of potential uremic toxins and/or biomarkers of CKD demonstrating the potential of comprehensive metabolic analytical platforms in the study of CKD, and (2) the development, validation and application of UPLC-MS/MS methods for the determination of known uremic toxins.

Samenvatting, conclusies en toekomstperspectieven

Chronisch nierfalen wordt vaak gekenmerkt door een progressief verlies in nierfunctie over een periode van maanden tot jaren. Uremische retentiestoffen, die onder normale condities het lichaam verlaten via de urine, zullen zich opstapelen in het lichaam. Indien deze retentiestoffen een negatieve impact hebben op het lichaam, worden ze uremische toxines genoemd. Tot op vandaag zijn een 150-tal uremische retentiestoffen gekend, maar velen zijn nog niet geïdentificeerd.
In de klinische praktijk wordt serum creatinine vaak als biomerker gebruikt voor chronisch nierfalen. Biomarkers zijn karakteristieke stoffen in het lichaam die de toestand van een ziekte weergeven. Ze kunnen gemeten en gekwantificeerd worden en daarom worden ze gebruikt bij de diagnose en het bepalen van de stadia van ziekten. Serum creatinine is echter een verre van ideale biomerker door zijn gebrek aan specificiteit en trage respons op veranderingen in ziekte en behandeling. Er is dus nood aan nieuwe biomerkers voor chronisch nierfalen.

In **hoofdstuk 1** wordt chronisch nierfalen gesitueerd en wordt dieper ingegaan op volgende aspecten ervan: uremische retentiestoffen en hun indeling op basis van fysicochemische eigenschappen, niervervangende therapieën zoals transplantatie en dialyse, huidige biomerkers en hun tekortkomingen. Verder worden de doelstellingen van deze thesis geformuleerd, namelijk het ontwikkelen en gebruiken van metabolome analytische platforms om nieuwe uremische retentiestoffen en/of potentiële biomerkers voor CKD te identificeren en het versnellen en verbreden van analytische methodes voor de bepaling van gekende uremische toxines.

In **hoofdstuk 2** worden definities gegeven van veelgebruikte termen in het metabolomics veld. Vervolgens wordt de workflow van metaboloom studies in detail besproken. Hierbij worden het collecteren van monsters, het bewaren en het transfereren ervan, de monstervoorbereiding, de analytische bepaling, de databehandeling, de statistische analyse, de identificatie en biologische interpretatie behandeld. Op het einde van dit hoofdstuk wordt een literatuuroverzicht gegeven van metaboloom studies uitgevoerd in het kader van chronisch nierfalen, waaruit blijkt dat er de laatste jaren veel onderzoek werd verricht in dit gebied.

Het experimentele gedeelte van deze thesis kan onderverdeeld worden in 2 grote luiken. We onderscheiden enerzijds een niet-gericht luik waarin op zoek gegaan wordt naar nieuwe uremische retentiestoffen en/of potentiële biomerkers voor chronisch nierfalen (hoofdstukken 3 en 4) en anderzijds een gericht luik waarin gekende uremische toxines gekwantificeerd worden (hoofdstukken 5 en 6).

In **hoofdstuk 3** wordt een metabolomics platform, gebaseerd op een combinatie van omkeerfase chromatografie gekoppeld met quadrupool-time-of-flight massaspectrometrie (RPLC-Q-TOF MS) in positieve en negatieve ionisatie mode en gaschromatografie gekoppeld met quadrupool massaspectrometrie (GC-MS), geëvalueerd als 'discovery tool' om uremische toxines en potentiële biomerkers voor chronisch nierfalen te identificeren. Hiertoe werd serum van een gezonde controle groep en van 2 patiënten groepen in verschillende CKD stadia, namelijk stadium 3 (CKD3) en stadium 5 aan hemodialyse (CKD5HD), geanalyseerd. Proteïnen werden geprecipiteerd met behulp van methanol en de derivatisatie bestond voor de GC-monsters uit methoximatie en trimethylsilylering. De kwaliteit van de analyses werd nagegaan met behulp van kwaliteitscontrole monsters. Een substantieel deel van het serum metaboloom werd teruggevonden. Voor de nieuwe uremische retentiestoffen werden restricties gezet op de relatieve veranderingen tussen de verschillende groepen gebaseerd op de relatieve verschillen die voor de huidige merker creatinine werden teruggevonden. De identificatie-strategie benutte de eigenschappen van het hoge resolutie Q-TOF MS systeem ten volle. Naast 43 metabolieten die reeds beschreven waren in de literatuur, vonden wij 42 metabolieten terug die voor het eerst gerelateerd konden worden met de progressie van chronisch nierfalen. Eenendertig metabolieten stegen significant in CKD. Een aantal metabolieten hadden hoge relatieve veranderingen, namelijk aminohydroxyhippuurzuur, 2-hydroxyhippuurzuur glucuronide, 2-/3-hydroxyhippuurzuur sulfaat, hydroxypyridine, methoxy-hydroxyphenylglycol glucuronide, lactose, een tetrasaccharide, en trihydroxypenteenzuur. De aanwezigheid van verschillende hydroxyhippuurzuur conjugaten wijst op het belang van deze metabolieten in chronisch nierfalen. Verder werden 11 metabolieten, voornamelijk vetzuren, gevonden die een dalende trend vertoonden in functie van de ziekte.

In **hoofdstuk 4** wordt het RPLC-MS-gebaseerde platform uitgebreid met hydrofiele interactie vloeistofchromatografie (HILIC). Polaire metabolieten elueren in omkeerfase vloeistofchromatografie met het dood volume of vroeg in de chromatografische run. Deze polaire metabolieten maken vaak deel uit van belangrijke metabolietklassen die diagnostisch kunnen zijn voor een ziekte. Hoewel de HILIC-mode steeds meer toepassingen kent, werd ze nog niet eerder gebruikt in de studie van chronisch nierfalen. Urine en plasma metabolietprofielen van 20 gezonde controles, 20 CKD3 patiënten en 20 CKD5 patiënten die geen dialysetherapie ontvingen werden op het HILIC-TOF platform opgenomen. Ook hier werd opnieuw veel aandacht besteed aan de kwaliteit van de data. Vier metabolieten, die een hogere relatieve verandering vertoonden dan creatinine, konden met behulp van standaarden geïdentificeerd worden. Zowel glycoursodeoxygalzuur als 2-hydroxyethaan sulfonaat dalen in urine van CKD patiënten.

Verder stijgt cinnamoylglycine, dat reeds gekend is als uremische retentiestof, in plasma, terwijl pregnenolone sulfaat daalt in plasma in chronisch nierfalen.

In deze thesis beogen we om het arsenaal aan analytische technieken ten volle te benutten om een zo breed mogelijk spectrum aan metabolieten te detecteren. In deze context zou capillaire elektroforese gekoppeld met massaspectrometrie (CE-MS) nog kunnen toegevoegd worden aan de gebruikte technieken. Verder zou een lipidomics aanpak een licht kunnen werpen op de verstoring in lipidensamenstelling in chronisch nierfalen.
Metabolomics is een relatief nieuw veld dat nog steeds in ontwikkeling is, wat zich uit in de sterke stijging in het aantal publicaties de laatste jaren. Vooruitgang in technologie en lessen geleerd van genomics, transcriptomics and proteomics hebben ontdekkingen versneld. Bovendien verwachten wij in de toekomst verdere ontwikkelingen zoals de uitbreiding van databanken voor identificatie, synthese van metabolietstandaarden, verbeteringen in data-processing software,… Een mogelijke valkuil is dat metabolomics studies de literatuur zouden overspoelen met biomerkers die potentieel vertonen maar nooit het validatiestadium bereiken. Het is dan ook belangrijk om te benadrukken dat potentiële biomerkers voor chronisch nierfalen die geïdentificeerd werden in de 'discovery studies' beschreven in hoofdstuk 3 en 4 in de toekomst dienen gevalideerd te worden in grotere studiepopulaties met behulp van gerichte methodes. Kwantificatie van deze metabolieten over de verschillende stadia van CKD zal toelaten om hun diagnostische waarde te evalueren. Deze metabolieten dienen ook samen geëvalueerd te worden aangezien een panel van biomarkers vaak toelaat om betere en snellere diagnose te stellen dan één enkele biomerker. Verder zullen in vitro en in vivo tests met relevante metabolietconcentraties uitwijzen of het om uremische toxines gaat en wat hun rol is in inflammatie en hart- en vaatziekten in patiënten met chronisch nierfalen. Kwantitatieve data zullen ook toelaten om de kinetiek van deze uremische retentiestoffen te evalueren in chronisch nierfalen en tijdens nierfunctie vervangende therapie. In een later stadium zouden gevalideerde biomarkers gebruikt kunnen worden om behandelingen op maat van de patiënt te ontwikkelen.

Urinezuur (UA), hippuurzuur (HA), indoxylsulfaat (IS), p-cresylglucuronide (pCG), p-cresylsulfaat (pCS), indool azijnzuur (IAA) en 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF) zijn uremische toxines waarvoor reeds schadelijke effecten zijn aangetoond. In **hoofdstuk 5** wordt een ultra hoge performantie vloeistofchromatografie tandem massaspectrometrie (UPLC-MS/MS) methode beschreven die ontwikkeld en gevalideerd werd voor de simultane bepaling van deze 7 uremische toxines in serum. De monstervoorbereiding bestond uit hittedenaturatie gevolgd door ultrafiltratie. Isotopisch gemerkte

interne standaarden werden aangewend om te corrigeren voor variatie tijdens monstervoorbereiding en tijdens de analyses. De uremische toxines werden gescheiden op een omkeerfase kolom gevolgd door negatieve electrospray ionisatie en tandem massaspectrometrische detectie. De 'figures of merit' werden bepaald. De toepasbaarheid van de nieuwe methode werd getest door 78 serummonsters te analyseren van gezonde controles en patiënten in alle CKD stadia, die ook met eerder beschreven HPLC met fotodiode array (PDA) en fluorescentie detectie (FLD) methodes geanalyseerd waren. Er werd een goede correlatie teruggevonden tussen de concentraties bepaald met de verschillende methodes. Bijgevolg kan deze methode, die sneller is dan eerder beschreven methodes, van klinisch belang zijn voor onder meer de opvolging van CKD patiënten en het nagaan en verbeteren van de bloedzuivering tijdens dialysebehandeling.

Hoofdstuk 6 beschrijft de ontwikkeling en de validatie van een UPLC-MS/MS methode voor de simultane bepaling van asymmetrisch dimethylarginine (ADMA), een endogene inhibitor van stikstofoxide synthase (NOS), en zijn structuurisomeer symmetrisch dimethylarginine (SDMA) in serum. Deze dimethylarginines accumuleren in chronisch nierfalen en werden geassocieerd met cardiovasculair lijden. Na deproteïnisatie, werd een butylesterderivatisatie uitgevoerd. De ADMA en SDMA derivaten konden gescheiden worden op een omkeerfasekolom, gevolgd door positieve electrospray ionisatie en detectie met behulp van tandem massaspectrometrie. Hier werd gebruik gemaakt van gedeutereerd ADMA als interne standaard. Zowel de vrije fractie als de totale concentratie werden bepaald. De 'figures of merit' werden nagegaan. De toepasbaarheid van de methode werd geëvalueerd door 10 serummonsters van gezonde controles en 77 serummonsters van CKD patiënten die behandeld werden met hemodialyse te analyseren. Zowel ADMA als SDMA concentraties waren significant hoger in CKD5 patiënten dan in gezonde controles. Onze data suggereren dat ADMA en SDMA nagenoeg niet proteïne gebonden zijn. De resultaten van de UPLC-MS/MS werden vervolgens vergeleken met deze bekomen met een commercieel verkrijgbare enzyme-linked immuno sorbent assay (ELISA). De correlatie tussen de beide resultaten was slechts matig.

Voorgaande gerichte methods zijn dus een uitbreiding van reeds bestaande methods. De eerste methode laat de simultane bepaling toe van een hele reeks hoofdzakelijk proteïne-gebonden moleculen. De tijd voor één run is korter vergeleken met reeds beschreven HPLC-PDA-FLD methodes, wat resulteert in tijdswinst voor high-throughput analyse. De tweede methode laat de simultane bepaling toe van de structuurisomeren ADMA en SDMA. U(H)PLC-MS/MS wordt gebruikt als de techniek van keuze voor de bioanalyse van kleine molecules, aangezien het vele voordelen biedt in termen van resolutie, snelheid en

gevoeligheid voor analytische toepassingen. Verder werd eerder aangetoond dat alle molecule die met bovenstaande methodes bepaald werden een belangrijke biologische en klinische impact hebben op uremie, in het bijzonder met betrekking tot cardiovasculair lijden.

Samenvattend werden in deze doctoraatsthesis (1) nieuwe potentiële uremische toxines en/of biomerkers voor chronisch nierfalen geïdentificeerd waarmee ook het potentieel van comprehensieve metabolome analytische platforms in de studie van chronisch nierfalen werd aangetoond en (2) UPLC-MS/MS methodes ontwikkeld, gevalideerd en toegepast voor de bepaling van reeds gekende uremische toxines.

"*Not everything that can be counted counts, and not everything that counts can be counted*" - Albert Einstein

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Een doctoraat is niet de verdienste van één enkele persoon, maar een proces, een project, waaraan velen zowel rechtstreeks als onrechtstreeks bijdragen. Daarom een oprecht dankwoord aan iedereen die heeft bijgedragen aan de verwezenlijking van dit doctoraat.

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Dank jullie wel, van harte,

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EDUCATION

Oct 2009-present	**PhD in Chemistry**, Ghent University, Belgium *Development of metabolic analytical platforms for the characterization of uremic retention compounds* Promotor: prof. dr. R. Vanholder, co-promotor: prof. dr. em. P. Sandra
2004-2009	**Master in Chemistry**, Ghent University, Belgium, cum laude Master thesis: *Quantitative analysis of cytostatics in urine: development of a screening system* Promotor: prof. dr. P. Sandra
1998-2004	Sciences-mathematics, Sint-Franciscusinstituut, Melle, Belgium

COURSES

2009-2013

Specialist course doctoral schools Ghent University

- Systems biology of the metabolism
- Multivariate statistics in R
- The omics (r)evolution

Transferable skills doctoral schools Ghent University

- Advanced academic English: writing skills
- Personal effectiveness
- Research and legal rights
- Project management
- Applying for a postdoctoral job

A1 PUBLICATIONS

- Boelaert J, Lynen F, Glorieux G, Eloot S, Van Landschoot M, Waterloos MA, Sandra P, Vanholder R (2013) A novel UPLC-MS-MS method for simultaneous determination of seven uremic retention toxins with cardiovascular relevance in chronic kidney disease patients. Analytical and Bioanalytical Chemistry 405:1937-1947
- Neirynck N, Glorieux G, Boelaert J, Schepers E, Liabeuf S, Dhondt A, Massy Z, Vanholder R (2013) Uremia-Related Oxidative Stress in Leukocytes Is Not Triggered by beta2-Microglobulin. Journal of Renal Nutrition 23:456-463
- Boelaert J, t'Kindt R, Schepers E, Jorge L, Glorieux G, Neirynck N, Lynen F, Sandra P, Vanholder R, Sandra K (2014) State-of-the-art non-targeted metabolomics in the study of chronic kidney disease. Metabolomics 10:425-442

- Boelaert J, Schepers E, Glorieux G, Lynen F, Vanholder R, Determination of asymmetric and symmetric dimethylarginine in serum from patients with chronic kidney disease: UPLC-MS/MS versus ELISA. Submitted
- Boelaert J, Lynen F, Schepers E, Glorieux G, Neirynck N, Vanholder R, Non-targeted metabolic profiling of human plasma and urine by ultra-performance hydrophilic interaction chromatography coupled with time-of-flight mass spectrometry in the study of chronic kidney disease. Submitted
- Boelaert J, Lynen F, Glorieux G, Vanholder R, Non-targeted metabolomics in chronic kidney disease: a review. In preparation

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POSTER PRESENTATIONS

- Boelaert J, Lynen F, Eloot S, Glorieux G, Van Landschoot M, Waterloos MA, Vanholder R, Development of an UPLC-MS/MS method for the determination of seven uremic retention toxins in serum of chronic kidney disease patients. Poster presented at the 12th International Symposium on Hyphenated Techniques in Chromatography and Hyphenated Chromatographic Analyzers, Bruges, Belgium, February 1-3, 2012
- Boelaert J, Persyn D, Lynen F, Vanholder R, Metabolic profiling of serum from patients with chronic kidney disease by GC-MS. Poster presented at the 12th International Symposium on Hyphenated Techniques in Chromatography and Hyphenated Chromatographic Analyzers, Bruges, Belgium, February 1-3, 2012
- Boelaert J, Persyn D, Lynen F, Vanholder R, Metabolic profiling of serum from patients with chronic kidney disease by GC-MS. Poster presented at the 11th Chemistry Conference for Young Scientists. Blankenberge, Belgium, March 1-2, 2012
- Boelaert J, Lynen F, Eloot S, Glorieux G, Van Landschoot M, Waterloos MA, Vanholder R, Development of an UPLC-MS/MS method for the determination of seven uremic retention toxins in serum of chronic kidney disease patients Poster presented at the Symposium Wetenschapsdag Faculteit Geneeskunde en Gezondheidswetenschappen, Ghent, Belgium, March 14, 2012
- Boelaert J, Persyn D, Lynen F, Vanholder R, GC-MS-based metabolic profiling of serum from patients with chronic kidney disease. Poster presented at the 36th International Symposium on Capillary Chromatography, Riva del Garda, Italy, May 27-June 1, 2012
- Boelaert J, Lynen F, Eloot S, Glorieux G, Van Landschoot M, Waterloos MA, Vanholder R, Determination of seven uremic retention toxins in serum of chronic kidney disease patients by UPLC-MS/MS. Poster presented at the 36th International Symposium on Capillary Chromatography, Riva del Garda, Italy, May 27-June 1, 2012
- Boelaert J, t'Kindt R, Glorieux G, Schepers E, Jorge L, Neirynck N, Lynen F, Sandra P, Sandra K, Vanholder R, Non-targeted metabolomics in the study of chronic kidney disease. Poster presented at the 50th Congress of the European Renal Association - European Dialysis and Transplant Association, Istanbul, Turkey, May 18-21, 2013
- Boelaert J, t'Kindt R, Jorge L, Glorieux G, Schepers E, Neirynck N, Vanholder R, Lynen F, Sandra P, Sandra K, LC- and GC-MS-based non-targeted metabolomics in the study of chronic kidney disease. Poster presented at the 39th International Symposium on High

Performance Liquid Phase Separations and Related Techniques, Amsterdam, The Netherlands, June 16-20, 2013

- Boelaert J, Glorieux G, Schepers E, Neirynck N, Vanholder R, Lynen F, Metabolic profiling by HILIC-TOF MS in the study of chronic kidney disease. Poster presented at the 39th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Amsterdam, The Netherlands, June 16-20, 2013
- Boelaert J, Schepers E, Glorieux G, Vanholder R, Sandra P, Lynen F, Determination of asymmetric and symmetric dimethylarginine in serum from patients with chronic kidney disease: UPLC-MS/MS versus ELISA. Poster presented at the 39th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Amsterdam, The Netherlands, June 16-20, 2013
- Boelaert J, Lynen F, Glorieux G, Schepers E, Neirynck N, Vanholder R, Metabolic profiling of human plasma and urine in chronic kidney disease by hydrophilic interaction liquid chromatography coupled with time-of-flight mass spectrometry: a pilot study. Poster presented at the 13th International Symposium on Hyphenated Techniques in Chromatography and Hyphenated Chromatographic Analyzers, Bruges, Belgium, January 28-31, 2014

ORAL PRESENTATIONS

- Boelaert J, Schepers E, Glorieux G, Neirynck N, Lynen F, Sandra P, Vanholder R, Metabolomics in chronic kidney disease. Oral presentation at the EuTox research meeting, Montpellier, France, March 18, 2011
- Boelaert J, t'Kindt R, Schepers E, Jorge L, Glorieux G, Neirynck N, Lynen F, Sandra P, Vanholder R, Sandra K, Development of metabolic analytical platforms for the characterization of uremic retention compounds. Oral presentation at the EuTox Continuing Medical Education course in collaboration with the European Renal Association - European Dialysis and Transplant Association and European Society for Artificial Organs, Malaga, Spain, March 16, 2012
- Boelaert J, t'Kindt R, Schepers E, Jorge L, Glorieux G, Neirynck N, Lynen F, Sandra P, Vanholder R, Sandra K, State-of-the art non-targeted metabolomics in the study of chronic kidney disease. Oral presentation at the Symposium Wetenschapsdag Faculteit Geneeskunde en Gezondheidswetenschappen, Ghent, Belgium, March 13, 2013

TEACHING

Academic year	Tutor of master student Dries Persyn
2010-2011	Master thesis: *Total metabolite profiling of serum from kidney dialysis patients by gas chromatography coupled to mass spectrometry*
2009-2013	Supervising practicals · Chemistry, society and ethics (1st Bachelor in Chemistry) · Analytical separation methods (3rd Bachelor in Chemistry) · Advanced separation science and organic mass spectrometry (1st and 2nd Master in Chemistry)
