# Tackling the complexity of CKD-associated cardiovascular disease

# Tackling the Complexity of CKD-associated Cardiovascular Disease

## From Small Molecules to Proteins

VALERIA SAAR-KOVROV

Author: Valeria Saar-Kovrov
Cover design: Valeria Saar-Kovrov
Layout & figure design: Valeria Saar-Kovrov
Printed by: Gildeprint
ISBN: 978-94-6496-111-9

# Tackling the Complexity of CKD-associated Cardiovascular Disease

## From Small Molecules to Proteins

DISSERTATION

to obtain the degree of Doctor at Maastricht University,
on the authority of the Rector Magnificus, Prof.dr. Pamela Habibović
in accordance with the decision of the Board of Deans,
to be defended in public

on Thursday, 16th of May 2024 at 13.00 hours

by

Valeria Saar-Kovrov

**Supervisors:**
Prof.dr. E.A.L. Biessen
Prof.dr. J. Jankowski (RWTH Aachen University, Germany)

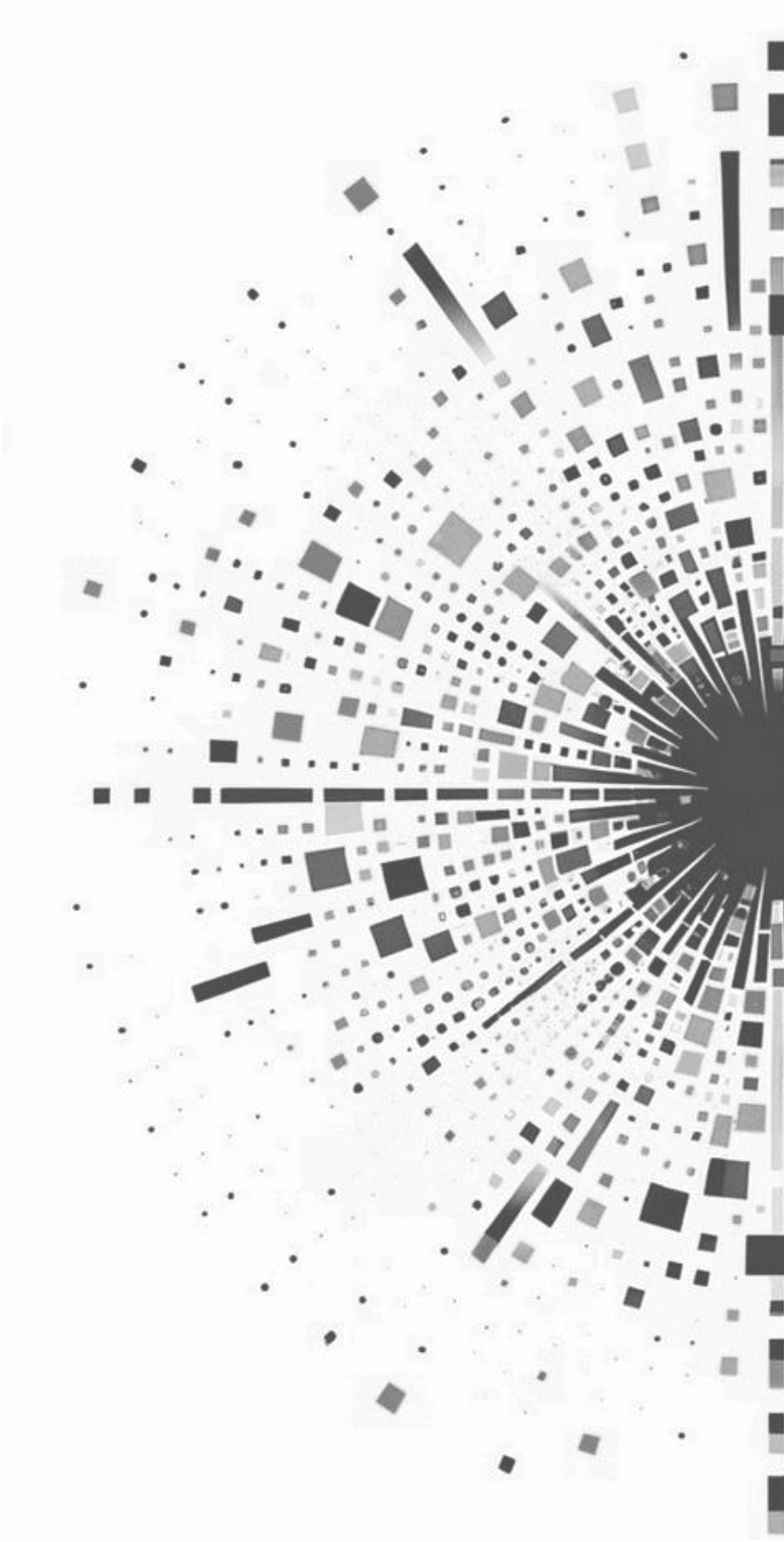
**Co-supervisors:**
Dr. M.M.P.C. Donners
Dr. P. Goossens

**Assessment Committee:**
Prof.dr. C. Schalkwijk (chair)
Dr. M. Christiaans
Prof.dr. M. van Eck (Leiden University, the Netherlands)
Prof.dr. W. Jahnen-Dechent (RWTH Aachen University, Germany)

The research presented in this dissertation was funded with a grant from European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant (agreement No 764474), CaReSyAn.

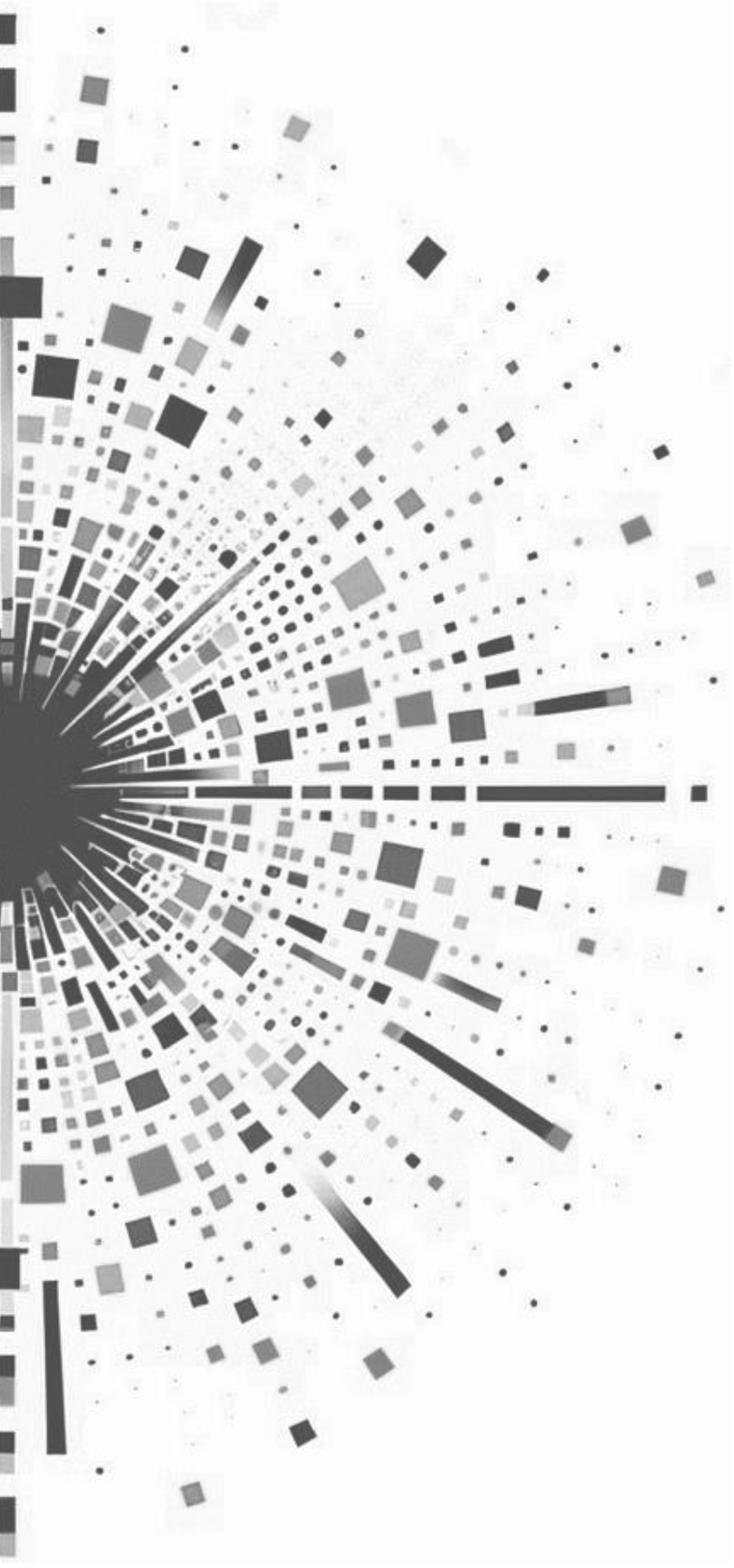
Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.

# Table of contents



# Chapter 1

General Introduction



## Cardiovascular Disease and Atherosclerosis

Cardiovascular disease (CVD) is a general term describing conditions affecting heart and blood vessels. There are 4 main manifestations of CVD: coronary artery disease, cerebrovascular disease, peripheral artery disease and aortic atherosclerosis[1]. CVD is a leading cause of death world-wide with most common major adverse cardiovascular events being myocardial infarction, stroke, unstable angina, heart failure and cardiovascular mortality[2].

Atherosclerosis is the most common form of CVD and a major cause for myocardial infarction, heart failure and stroke. It is a chronic inflammatory condition characterized by thickening of arterial wall due to accumulation of lipids, cholesterol, inflammatory cells, and other components in the subendothelial space[3]. Atherosclerotic plaque can be classified into 8 types based on the progression of the disease: intimal thickening, intimal xanthoma (or "fatty streak"), pathological intimal thickening, thick fibrous cap atheroma, thin fibrous cap atheroma (vulnerable to plaque rupture), calcified nodule, fibrous or fibrocalcific plaque, intraplaque hemorrhage, and plaque erosion with a luminal thrombus[4].

One of the starting mechanisms of atherosclerosis development is activation of endothelial cells that leads to expression of adhesion molecules (e.g. vascular cell adhesion molecule-1, VCAM-1) and causes mononuclear leukocytes, such as monocytes and T-cells, to attach and penetrate into the intima[5]. In the intima, monocytes encounter factors such as macrophage colony-stimulating factor (M-CSF) and differentiate into macrophages. Low density lipoprotein (LDL) particles from the circulation are small enough to penetrate the endothelial layer and leak into the intima at the earliest stages of atherosclerosis where they can bind to the proteoglycan matrix and undergo further modification, e.g. oxidation, forming oxLDL[6]. Differentiated macrophages come in contact and take up modified LDL particles through scavenger receptors or phagocytosis of aggregated particles that gives rise to cholesterol-laden macrophages, otherwise known as foam cells. Often, foam cells lose the ability of cholesterol efflux and undergo apoptosis or necrosis contributing to the formation of a necrotic core. This way necrotic core of the plaque accumulates esterified cholesterol, cholesterol crystals, and cell debris and can decrease plaque stability, i.e. a higher risk of rupture[3]. Additionally, oxLDL particles activate inflammatory response and induce reactive oxygen species (ROS) production by the macrophages that further contribute to the plaque vulnerability[7].

## Cardiorenal Syndrome

As defined by Kidney Disease: Improving Global Outcomes (KDIGO) organization, chronic kidney disease (CKD) is characterized by structural or functional abnormalities of the kidneys that have been present for more than three months and have a particular impact on health[8]. CKD constitutes a huge medical and financial burden for society with the estimated prevalence of 13.4% globally[9]. It is a "silent" disease that is often left unrecognized until later stages. Assessment of the severity of CKD condition is based on the estimated glomerular filtration

rate (eGFR; mL/min per 1.73 $m^2$) and is classified into six stages: G1 (eGFR≥ 90), G2 (eGFR= 60 – 89), G3A (eGFR= 45 – 59), G3B (eGFR= 30 – 44), G4 (eGFR 15 – 29), and G5 (eGFR< 15)[10]. All-cause mortality rates are shown to increase with eGFR decline with relative death odds ratios reaching 4.4[11]. A clear nonlinear relation between the eGFR and the risks of cardiovascular events was demonstrated by Go and colleagues[12]. The relation was especially evident for patients with stage 3b – 4 renal disease with the number of cardiovascular events reaching 36.6 per 100 persons per year. A study conducted on 462 293 adults in Taiwan demonstrated a significant association of chronic kidney disease with all cause and cardiovascular mortality with CKD patients showing 100% higher mortality rate from cardiovascular diseases compared to normal kidney function population [13]. Later, collaborative meta-analysis studies confirmed independent association of reduced kidney function and proteinuria with increased all-cause and cardiovascular mortality risks and identified adjusted hazard ratio of 3.1 for all-cause mortality in patients with severe CKD (eGFR= 15) compared to those with normal eGFR [14,15]. Thus, it might be claimed CKD to be assessed as one of the strongest risk factors for CVD development[16].

The relation between kidney disease and CVDs described above is cumulatively known as cardiorenal syndrome (CRS). There are five types of CRS described depending on the initiation of the disease[17]. Under the scope of this thesis, we focus on type 4 or Chronic renocardiac syndrome, which is characterized by increased cardiovascular morbidity and mortality in patients with CKD.

Postmortem studies have shown increased coronary artery disease in kidney insufficiency patients compared to non-CKD with greater media thickening and presence of medial calcification[18–20]. In living patients, angiographic studies confirmed that reduced kidney function significantly associates with vascular disease severity independently of other risk factors[21–23]. Additionally, coronary artery calcium score increases with the decline of eGFR[23,24] and significantly associates with obstructive atherosclerosis[25]. Plaques of patients with CKD also exhibit higher lipid index, prevalence of calcium, cholesterol crystals, and disruption compared to non-CKD population[26]. Finally, NEFRONA study that involved 2445 patients with eGFR <60 and 559 with normal kidney function confirmed higher prevalence of atherosclerotic disease in CKD population that correlated with the CKD severity[27].

Described underlying mechanisms of kidney disease associated with CVD include neurohormonal dysregulation (e.g. renin–angiotensin–aldosterone system and sympathetic nervous system activation), anemia, oxidative stress, chronic inflammation, increase in uremic toxins and protein-bound toxins[28]. These factors, in turn, lead to cardiac hypertrophy and dysfunction, endothelial dysfunction, increased inflammation, fibrosis, apoptosis, vascular calcification, protein damage and, as a result, increased atherosclerosis and plaque instability.

## Atherosclerosis-associated Factors in CKD

There is a number of factors that contribute to the elevated risk of atherosclerosis in patients with CKD. These include, but are not limited to, abnormalities in lipid particles, increased inflammation, calcification, rise in uremic toxins blood levels, etc. (**Figure 1**) and are described in more detail below.

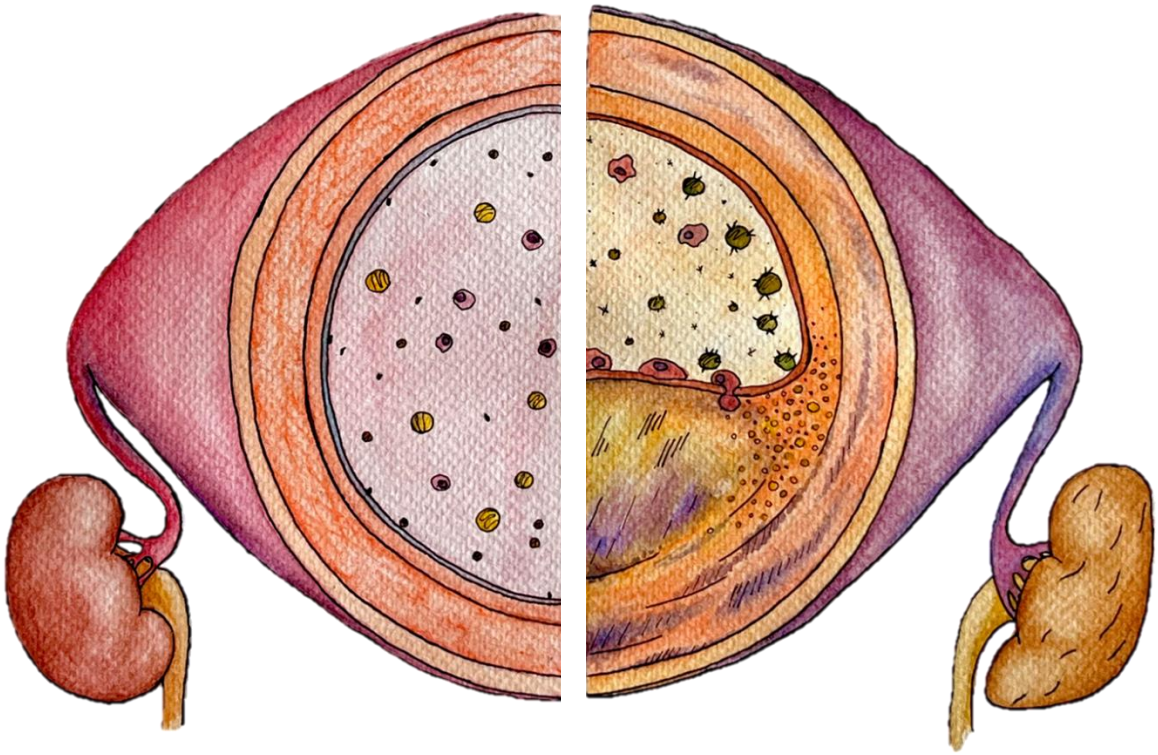


**Figure 1.** Simplified representation of a healthy artery wall (left image) in case of normally functioning kidneys and an artery affected by atherosclerosis (right image) in case of kidney insufficiency. Depicted are healthy (yellow) and modified (green) lipid particles; healthy proteins (e.g. Klotho) interacting with artery epithelium (black dots, left); monocytes (left), activated macrophages (right), uremic toxins (black specs, right); as well as calcification (purple), lipid core (yellow) and foam cells (circles) within the atherosclerotic plaque (right image).

### *Plasma lipids*

It is worth mentioning that CKD patients do not exhibit particular pro-atherogenic changes in their blood lipid levels, while LDL cholesterol levels were in fact a lower cardiovascular risk predictor in CKD compared to general population[29]. However, structural and chemical changes of the plasma components in CKD condition might explain the higher atherogenic profile in this population. For instance, kidney function decrease was shown to affect LDL and HDL particle size and lipid composition with increased triglycerides and decreased cholesterol content[30]. HDL particles in CKD condition lose their antioxidative, anti-inflammatory, and vasoprotective properties and show decreased ability to promote cholesterol efflux from

macrophages (reviewed[31]). Moreover, proteins of the lipid particles are subjected to numerous post-translational modifications in patients with CKD, such as chlorination, oxidation, and carbamylation, which renders them dysfunctional and can cause undesirable effects on cellular homeostasis, e.g. induce inflammatory response, cell proliferation, foam cell formation and ROS production[7,32–35] (**Chapter 3**).

### *Inflammation and oxidative stress*

Besides augmented plasma lipid profile, patients with CKD also demonstrate higher levels of systemic inflammation with increased number of activated monocytes (CD14+CD16+) in the circulation, increase in inflammation-triggered reactive oxygen species, and higher levels of circulating pro-inflammatory cytokines (e.g. interleukin 6 and 18) and c-reactive protein (CRP)[36–41]. CRP, in turn, was associated with the presence of atherosclerotic plaques in this population[27]. Moreover, increased levels and oxidative activity of myeloperoxidase (MPO) in CKD patients was shown to lead to increased protein damage and oxidative stress facilitating development of vascular disease[39,42].

Additionally, the disruption of the anti-inflammatory and anti-oxidative mechanisms, e.g. decrease in superoxide dismutases and glutathione peroxidases activity and loss of soluble Klotho protein, seen in the CKD population significantly contributes to the development of CVD in these patients (reviewed[43] as well as discussed in **Chapter 4**).

### *Calcification*

Vascular calcification is known to be more progressive and severe in patients with CKD compared to non-CKD[44–46]. Patients with CKD show signs of both medial and intimal calcification. Medial arterial calcification is characterized by accumulation of calcium phosphate deposits along the tunica media layer of the arterial wall. It is associated with elastin degradation, extracellular matrix remodeling events, and, as a result, increased arterial stiffening which is a marker and contributor to cardiovascular disease[47]. On the other hand, intimal calcification takes place at the tunica intima of the vessel wall and is associated with disrupted lipid metabolism, chronic inflammation, vascular smooth muscle cells (VSMC) remodeling and, consequentially, with atherosclerosis[44]. Intimal calcification can be further classified into different types according to its location and size, which show different effects on plaque stability. For instance, microcalcification (0.5–50 μm crystals), depending on its localization, might induce plaque vulnerability and rupture[48,49].

In case of CKD, there are multiple factors that trigger initiation of vascular calcification such as inflammatory cytokines, uremic toxins, and disruptions in calcium-phosphate balance[50]. In addition, as already mentioned, loss of protective factors and rise in calcification facilitating factors seen in CKD patients also contributes to augmented vascular calcification. For example, soluble Klotho levels decrease already at the early stages of CKD which causes an increase in circulating phosphate levels and thereby enhances vascular calcification (discussed in **Chapter 4**, this thesis). Another example is increased expression of cyclin-dependent kinase 5 (CDK5)

observed in kidneys of patients with diabetic nephropathy, a leading cause of CKD[51]. CDK5 was shown to promote renal fibrosis as well as inflammation[52,53] and, as demonstrated in **Chapter 5** of this thesis, exacerbate plaque calcification and severity in mice.

### *Uremic toxins*

Uremic toxins, by definition, are molecules that are normally excreted by the kidneys, their concentration is elevated in case of kidney insufficiency, and there is a clear demonstrated relationship between their levels and any manifestations of the uremic syndrome[54,55]. There are currently more than 150 known uremic toxins, which are generally divided into three groups, based on European Uremic Toxins Work Group (EUTox)[56] classification, according to their physicochemical characteristics: small water-soluble compounds (e.g. urea); middle sized molecules (e.g. β2-microglobulin); and protein-bound uremic toxins (PBUTs; e.g. indoxyl sulfate)[57]. Certain uremic toxins, such as indoxyl sulfate and *p*-cresyl sulfate, demonstrate clear profibrotic, proinflammatory and oxidative stress-inducing properties linking them to the development of atherosclerosis[58–60]. They are also shown to be associated with increased risk for cardiovascular and all-cause mortality in CKD patients in several clinical trials[61–63]. A huge fraction of these compounds is protein-bound which renders them especially difficult to remove by conventional dialysis techniques. PBUTs are further discussed in **Chapter 2** of this thesis.

Urea, a byproduct of protein degradation normally excreted by kidneys, is another prominent uremic toxin which blood levels are dramatically increased, up to 10-fold, in patients with late-stage kidney disease[64]. In a recent study, even moderately elevated blood urea levels were shown to significantly increase adverse cardiovascular outcomes independently of other known factors[65]. Multiple *in vitro* studies show direct cellular toxicity of increased urea levels which induce oxidative stress, apoptosis, monocyte adhesion molecules expression by the endothelial cells, and accumulation of advanced glycation end-products[64,66]. Urea is also known to induce protein damage by causing post-translational modifications, e.g. carbamylation (further explained below), which, in turn, increases the risks of cardiac mortality in patients with CKD[67,68].

## Post-translational Protein Modifications

Post-translational modifications (PTMs) are reversible or irreversible covalent chemical modifications of proteins that take place after protein translation. PTMs include protein truncation as well as phosphorylation, acetylation, succinylation, methylation and many others. There are currently more than 600 known modifications[69]. In this way, it can be said that all of the synthesized proteins in the cell are modified. PTMs are crucial for proteins' localization, folding, protein-protein interactions, transport, and function[70].

PTMs can be enzymatic, that is catalyzed by an enzyme, e.g. kinases, or non-enzymatic that happen without involvement of enzyme proteins. Phosphorylation, addition of a phosphate group to an amino acid, is one of the most studied enzymatic protein modifications. It most

commonly occurs on serine, threonine, and tyrosine residues. It is an enzymatic reversible modification which is mediated by a number of protein kinases and phosphatases[71]. Phosphorylation is a necessary modification for many proteins that allows them to maintain their function, e.g. p53 requires phosphorylation to gain its cell cycle regulating properties[72].

Non-enzymatic PTM, on the other hand, can occur when a nucleophilic or redox-sensitive amino acid side chain of a protein encounters a reactive metabolite[73]. Non-enzymatic modifications are common in conditions such as diabetes and chronic kidney disease when increased concentrations of reactive compounds in the circulation lead to glycation, oxidation or carbamylation of plasma proteins. This, in turn, can have devastating effects of vascular system and promote cardiovascular disease[74]. Oxidized LDL is an example of a non-enzymatic modifications that results from reaction between reactive oxygen species in the environment and amino acid residues of the apolipoprotein B (ApoB) on LDL particles[75]. As known from the oxLDL example, these modifications can have detrimental consequences for the protein functions and metabolism. Thus, oxLDL is known to induce endothelial disfunction, apoptosis, expression of monocyte-adhesive molecules, cell proliferation, inflammasome activation, as well as foam cell and cholesterol crystals formation[76].

## Carbamylation

Carbamylation, or carbamoylation, is a non-enzymatic, presumably irreversible, PTM that results from a reaction between cyanate $[OCN]^-$ and free amino groups of protein lysines, arginines or N-terminal domain. During this reaction, a carbamoyl moiety $[-CONH_2]$ is added to the protein sequence which can cause its structural and functional changes[77] (**Figure 2**). The most substantial chemical effect of carbamylation is neutralization of the positive charge of the amino groups which might result in conformational changes of the protein and alter its ionic interaction properties. Lysine is the most common site of carbamylation that leads to formation of carbamyl-lysine, or homocitrulline, which is commonly used as a biomarker for carbamylation.

There are two main sources of cyanate in the organism described so far. One is deamination of urea, which naturally occurs with time and gives rise to isocyanic acid, while cyanate is its active form. The other route of cyanate formation is through oxidation of thiocyanate, ubiquitously found in mammalian organism[78], by MPO which is activated at the sites of inflammation, including atherosclerotic plaque, or due to the environmental factors such as air pollution and smoking[79,80]. Normally, healthy individuals have blood levels of isocyanic acid of about 45 nmol/l, while this number significantly rises in patients with uremia, up to 140 nmol/l[81]. Owing to this rise, protein carbamylation rate in the circulation of CKD patients is drastically increased as well[67,79,82]. Even though the fraction of the carbamylated plasma proteins, such as haemoglobin and albumin, is quite low (generally <2%) it is nonetheless shown to be significantly associated with increased risk of cardiovascular events, all-cause and cardiovascular mortality[67,79,83,84].

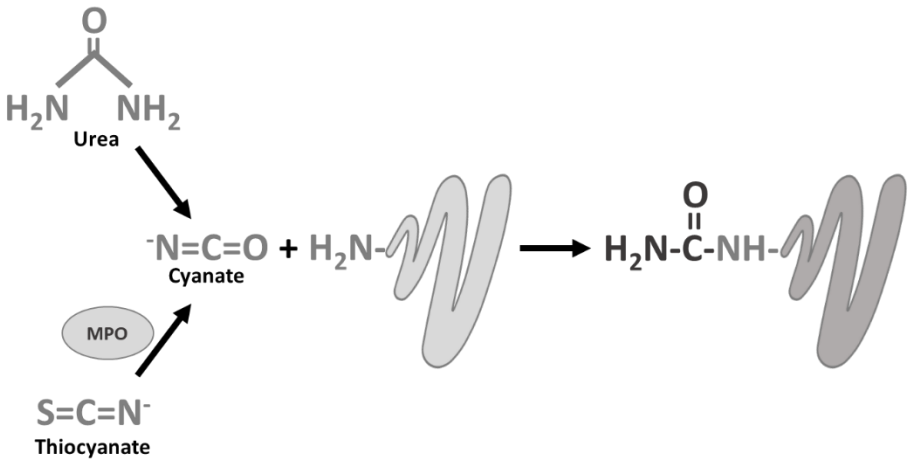



**Figure 2**. Carbamylation reaction between cyanate and amino group of a protein. MPO – myeloperoxidase.

There is a plethora of evidence coming from *in vitro* experiments that demonstrates detrimental effects of carbamylation causing changes in protein conformation[85,86], stability[87], binding properties[88–90], enzyme and hormone activity[91–95], receptor-drug interaction[96,97], and cellular responses[98–104]. Carbamylation is a relatively stable, irreversible modification which can accumulate over time in extracellular matrix (ECM) proteins with a long half-life and impair their functions[77]. For example, type I collagen of the ECM exhibited decreased ability to polymerize into regular fibrils due to carbamylation-induced destabilization at specific regions of its triple helix. This, in turn, affects the capacity of collagen to stimulate oxidative functions of neutrophils disturbing the interaction between the cells and ECM[105]. Additionally, it was shown that aortic elastin carbamylation increases its lamellae stiffness on molecular level and associates with increased pulse wave velocity in NaCNO treated ApoE-deficient mice[106].

Relevance of protein carbamylation was also demonstrated in the pathogenesis of atherosclerosis. Carbamylated LDL (carbLDL) was shown to induce endothelial cell proliferation and death[107] as well as endothelial dysfunction by activation of the lectin-like oxidized LDL receptor-1 (LOX-1) and subsequent activation of the pro-inflammatory NF-κB signaling pathway[33]. In another study, carbLDL treatment led to striking proliferation of the vascular smooth muscle cells and increased expression of adhesion molecules ICAM-1 and VCAM-1[108]. Increase in these molecules was also demonstrated in the endothelial cells treated with carbLDL which resulted in enhanced monocyte adhesion[109]. As mentioned before, monocyte adhesion to the endothelial layer and subsequent transmigration has a key role in initiating and promoting atherogenesis. carbLDL is also effectively taken up by the macrophages inducing cholesterol accumulation and, therefore, foam cell formation[34,79] (**Chapter 3**). Carbamylation was shown to affect high density lipoproteins (HDL) as well attenuating their anti-oxidative and anti-inflammatory properties[110].

Taken together, these data highlight the importance of protein carbamylation in the pathogenesis of cardiovascular disease. However, the exact mechanisms of origins and consequences of it are far from being understood. For instance, it is still unclear if

carbamylation is a solely undesirable product of spontaneous reactions in the organism, or it might also have a functional purpose and thus being regulated by the cell and even reversed as suggested in one study by Joshi et al.[111]. No mechanisms of carbamylation reversion have been elucidated thus far.

This thesis is aimed at shedding light on molecular processes of cardiorenal syndrome bringing together the pathological mechanisms of kidney disease, such as uremic toxins, protein modifications, and vascular calcification, and their role in cardiovascular condition.

## Thesis Outline

To summarize all of the above, chronic kidney disease constitutes a huge societal burden significantly contributing to the pool of cardiovascular morbidity and mortality. Its complications have a broad influence on the organism through various pathways, including effects on blood lipid composition, uremic retention solutes, oxidative stress and inflammation, disbalance in calcification regulation and protein modifications. Thus, I sought to elucidate further the link between the mechanisms associated with kidney insufficiency, such as increase in uremic toxins blood levels, vascular calcification, and protein modifications, and their effects on cardiovascular system and association with atherosclerosis.

In **Chapter 2**, I will discuss the problem of increased levels of protein-bound uremic toxins due to kidney failure and the difficulties in their removal with conventional methods. For this purpose, I conducted a systematic review searching through two scientific literature databases to retrieve and analyze all reported currently available uremic toxins removal techniques and describing their efficacy against all known PBUTs with the aim to find the most effective method.

In **Chapter 3**, I will focus on carbamylation in atherosclerotic plaques of CKD and non-CKD patients. I will describe our findings demonstrating the association between the total carbamylation levels and the progression of atherosclerotic plaque, which, at least partially, can be explained by the increase in foamy macrophages containing carbamylated LDL particles.

In **Chapter 4**, I will touch upon another mechanism associated with uremia such as vascular calcification. Soluble Klotho, an important calcification regulator, was shown to be significantly decreased in patients with kidney insufficiency. Thus, in this review, we will discuss the mechanisms underlying the shedding of Klotho and the functional effects of soluble Klotho in CKD as well as in association with cardiovascular complications.

In **Chapter 5**, I will describe the importance of cyclin-dependent kinase 5 (CDK5), another factor affected by kidney disease, in atherosclerosis development and calcification. In this paper we demonstrate that CDK5 plays a crucial role in macrophage inflammatory processes and cytokine regulation and, as a consequence, atherosclerotic plaque progression and calcification, which makes it a good target for drug development.

Finally, **Chapter 6** will summarize general findings of the thesis and discuss future perspectives.

# References

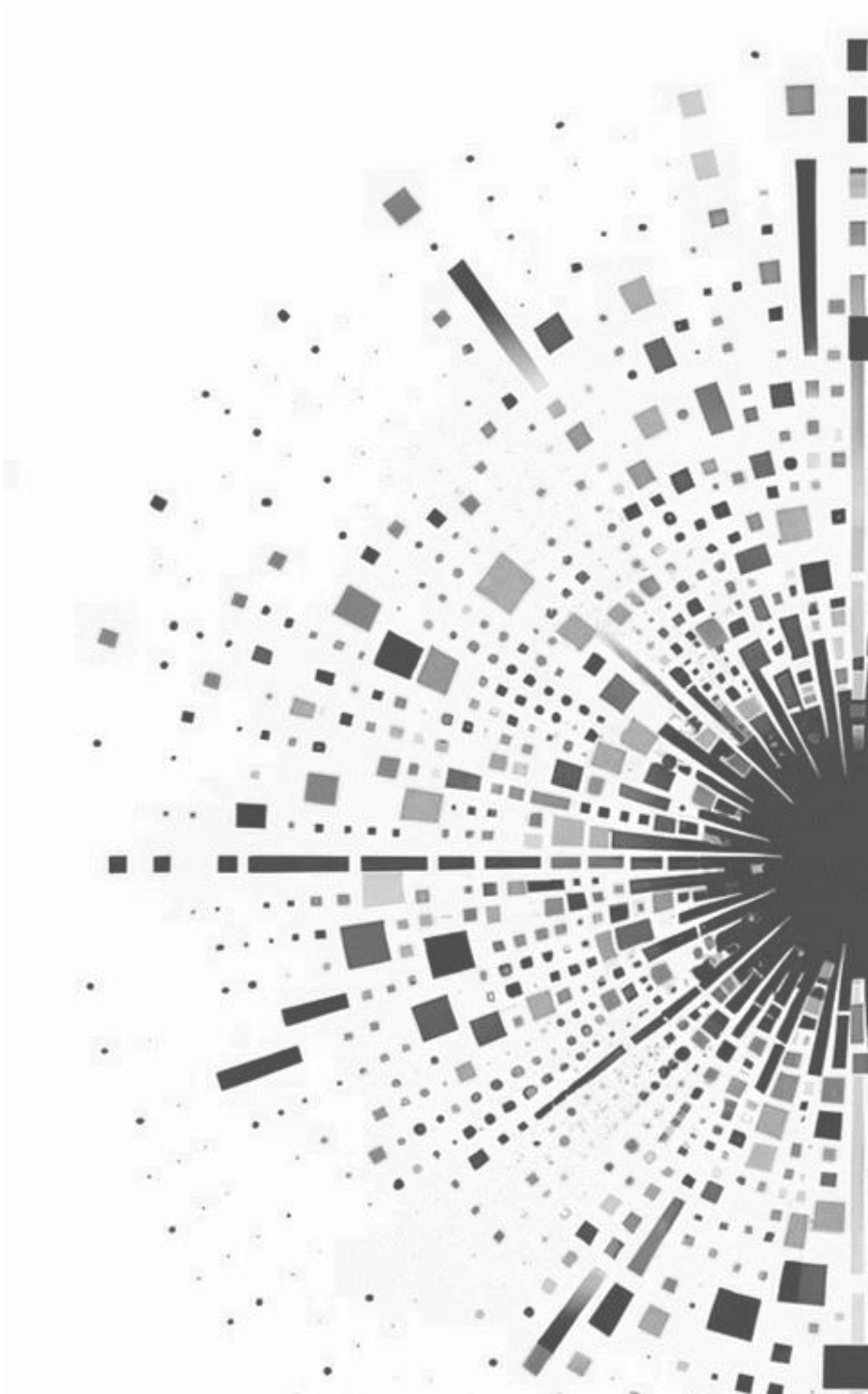

1. Benjamin, E. J. *et al.* Heart Disease and Stroke Statistics—2018 Update: A Report From the American Heart Association. *Circulation* **137**, E67–E492 (2018).
2. Bosco, E., Hsueh, L., McConeghy, K. W., Gravenstein, S. & Saade, E. Major adverse cardiovascular event definitions used in observational analysis of administrative databases: a systematic review. *BMC Med Res Methodol* **21**, 1–18 (2021).
3. Björkegren, J. L. M. & Lusis, A. J. Atherosclerosis: Recent developments. *Cell* **185**, 1630–1645 (2022).
4. Bentzon, J. F., Otsuka, F., Virmani, R. & Falk, E. Mechanisms of Plaque Formation and Rupture. *Circ Res* **114**, 1852–1866 (2014).
5. Frostegård, J. *Immunity, atherosclerosis and cardiovascular disease*. *BMC Medicine* vol. 11 (BioMed Central, 2013).
6. Leiva, E. *et al.* Role of Oxidized LDL in Atherosclerosis. *Adv Exp Med Biol* **285**, 353–365 (2015).
7. Lara-Guzmán, O. J. *et al.* Oxidized LDL triggers changes in oxidative stress and inflammatory biomarkers in human macrophages. *Redox Biol* **15**, 1–11 (2018).
8. Supplements, K. I. Official Journal of the international Society of nephrology KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. *IFAC Proceedings Volumes (IFAC-PapersOnline)* **3**, 30–130 (2013).
9. Hill, N. R. *et al.* Global prevalence of chronic kidney disease - A systematic review and meta-analysis. *PLoS One* **11**, (2016).
10. Eckardt, K. U. *et al.* Evolving importance of kidney disease: from subspecialty to global health burden. *Lancet* **382**, 158–169 (2013).
11. Thompson, S. *et al.* Cause of death in patients with reduced kidney function. *Journal of the American Society of Nephrology* **26**, 2504–2511 (2015).
12. Go, A. S., Chertow, G. M., Fan, D., McCulloch, C. E. & Hsu, C. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med* **351**, 1296–305 (2004).
13. Wen, C. P. *et al.* All-cause mortality attributable to chronic kidney disease: a prospective cohort study based on 462 293 adults in Taiwan. *The Lancet* **371**, 2173–2182 (2008).
14. Matsushita, K. *et al.* Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. *The Lancet* **375**, 2073–2081 (2010).
15. Van Der Velde, M. *et al.* Lower estimated glomerular filtration rate and higher albuminuria are associated with all-cause and cardiovascular mortality. A collaborative meta-analysis of high-risk population cohorts. *Kidney Int* **79**, 1341–1352 (2011).
16. Gansevoort, R. T. *et al.* Chronic kidney disease and cardiovascular risk: Epidemiology, mechanisms, and prevention. *The Lancet* **382**, 339–352 (2013).
17. Rangaswami, J. *et al.* Cardiorenal Syndrome: Classification, Pathophysiology, Diagnosis, and Treatment Strategies: A Scientific Statement From the American Heart Association. *Circulation* **139**, E840–E878 (2019).
18. Schwarz, U. *et al.* Morphology of coronary atherosclerotic lesions in patients with end-stage renal failure. *Nephrology Dialysis Transplantation* **15**, 218–223 (2000).
19. Gross, M. L. *et al.* Calcification of coronary intima and media: Immunohistochemistry, backscatter imaging, and x-ray analysis in renal and nonrenal patients. *Clinical Journal of the American Society of Nephrology* **2**, 121–134 (2007).
20. Nakano, T. *et al.* Association of Kidney Function With Coronary Atherosclerosis and Calcification in Autopsy Samples From Japanese Elders: The Hisayama Study. *American Journal of Kidney Diseases* **55**, 21–30 (2010).
21. Davoodi, G. *et al.* Glomerular filtration rate is related to severity of obstructive coronary artery disease in patients undergoing coronary angiography. *Int Urol Nephrol* **44**, 1161–1168 (2012).
22. Khalique, O. *et al.* Relation of Moderate or Severe Reduction in Glomerular Filtration Rate to Number of Coronary Arteries Narrowed >50% in Patients Undergoing Coronary Angiography for Suspected Coronary Artery Disease. *American Journal of Cardiology* **100**, 415–416 (2007).
23. Kramer, H., Toto, R., Peshock, R., Cooper, R. & Victor, R. Association between chronic kidney disease and coronary artery calcification: The Dallas heart study. *Journal of the American Society of Nephrology* **16**, 507–513 (2005).

24. Raggi, P. *et al.* Cardiac calcification in adult hemodialysis patients: A link between end-stage renal disease and cardiovascular disease? *J Am Coll Cardiol* **39**, 695–701 (2002).
25. Haydar, A. A. *et al.* Coronary artery calcification is related to coronary atherosclerosis in chronic renal disease patients: a study comparing EBCT-generated coronary artery calcium scores and coronary angiography. *Nephrology Dialysis Transplantation* **19**, 2307–2312 (2004).
26. Kato, K. *et al.* Nonculprit coronary plaque characteristics of chronic kidney disease. *Circ Cardiovasc Imaging* **6**, 448–456 (2013).
27. Betriu, A. *et al.* Prevalence of subclinical atheromatosis and associated risk factors in chronic kidney disease: the NEFRONA study. *Nephrology Dialysis Transplantation* **29**, 1415–1422 (2014).
28. Kumar, U., Wettersten, N. & Garimella, P. S. Cardiorenal syndrome-Pathophysiology. *Cardiol Clin* **37**, 251 (2019).
29. Tonelli, M. *et al.* Association between LDL-C and risk of myocardial infarction in CKD. *Journal of the American Society of Nephrology* **24**, 979–986 (2013).
30. Bermudez-Lopez, M. *et al.* An in-depth analysis shows a hidden atherogenic lipoprotein profile in non-diabetic chronic kidney disease patients. *https://doi.org/10.1080/14728222.2019.1620206* **23**, 619–630 (2019).
31. Rysz, J., Gluba-Brzózka, A., Rysz-Górzyńska, M. & Franczyk, B. The Role and Function of HDL in Patients with Chronic Kidney Disease and the Risk of Cardiovascular Disease. *International Journal of Molecular Sciences 2020, Vol. 21, Page 601* **21**, 601 (2020).
32. Holzer, M. *et al.* Myeloperoxidase-derived chlorinating species induce protein carbamylation through decomposition of thiocyanate and urea: Novel pathways generating dysfunctional high-density lipoprotein. *Antioxid Redox Signal* **17**, 1043–1052 (2012).
33. Speer, T. *et al.* Carbamylated low-density lipoprotein induces endothelial dysfunction. *Eur Heart J* **35**, 3021–3032 (2014).
34. Apostolov, E. O. *et al.* Carbamylated-oxidized LDL: proatherosclerotic effects on endothelial cells and macrophages. *J Atheroscler Thromb* **20**, 878–892 (2013).
35. Himmelfarb, J., McMenamin, M. E., Loseto, G. & Heinecke, J. W. Myeloperoxidase-catalyzed 3-chlorotyrosine formation in dialysis patients. *Free Radic Biol Med* **31**, 1163–1169 (2001).
36. Zimmermann, J., Herrlinger, S., Pruy, A., Metzger, T. & Wanner, C. Inflammation enhances cardiovascular risk and mortality in hemodialysis patients. *Kidney Int* **55**, 648–658 (1999).
37. Pecoits-Filho, R., Bárány, P., Lindholm, B., Heimbürger, O. & Stenvinkel, P. Interleukin-6 is an independent predictor of mortality in patients starting dialysis treatment. *Nephrology Dialysis Transplantation* **17**, 1684–1688 (2002).
38. Porazko, T. *et al.* IL-18 is involved in vascular injury in end-stage renal disease patients. *Nephrology Dialysis Transplantation* **24**, 589–596 (2009).
39. Kalantar-Zadeh, K., Brennan, M. L. & Hazen, S. L. Serum Myeloperoxidase and Mortality in Maintenance Hemodialysis Patients. *American Journal of Kidney Diseases* **48**, 59–68 (2006).
40. Merino, A. *et al.* Senescent CD14+CD16+ Monocytes Exhibit Proinflammatory and Proatherosclerotic Activity. *The Journal of Immunology* **186**, 1809–1815 (2011).
41. Rapa, S. F., Di Iorio, B. R., Campiglia, P., Heidland, A. & Marzocco, S. Inflammation and Oxidative Stress in Chronic Kidney Disease—Potential Therapeutic Role of Minerals, Vitamins and Plant-Derived Metabolites. *Int J Mol Sci* **21**, (2020).
42. Kisic, B., Miric, D., Dragojevic, I., Rasic, J. & Popovic, L. Role of Myeloperoxidase in Patients with Chronic Kidney Disease. *Oxid Med Cell Longev* **2016**, (2016).
43. Podkowińska, A. & Formanowicz, D. Chronic Kidney Disease as Oxidative Stress- and Inflammatory-Mediated Cardiovascular Disease. *Antioxidants 2020, Vol. 9, Page 752* **9**, 752 (2020).
44. Dube, P. *et al.* Vascular Calcification in Chronic Kidney Disease: Diversity in the Vessel Wall. *Biomedicines* **9**, (2021).
45. Jagieła, J., Bartnicki, P. & Rysz, J. Selected cardiovascular risk factors in early stages of chronic kidney disease. *Int Urol Nephrol* **52**, 303–314 (2020).
46. Nakanishi, T., Nanami, M. & Kuragano, T. The pathogenesis of CKD complications; Attack of dysregulated iron and phosphate metabolism. *Free Radic Biol Med* **157**, 55–62 (2020).
47. Zieman, S. J., Melenovsky, V. & Kass, D. A. Mechanisms, Pathophysiology, and Therapy of Arterial Stiffness. *Arterioscler Thromb Vasc Biol* **25**, 932–943 (2005).
48. Waring, O. J., Skenteris, N. T., Biessen, E. A. L. & Donners, M. M. P. C. Two-faced Janus: the dual role of macrophages in atherosclerotic calcification. *Cardiovasc Res* **118**, 2768 (2022).

49. Kelly-Arnold, A. *et al.* Revised microcalcification hypothesis for fibrous cap rupture in human coronary arteries. *Proc Natl Acad Sci U S A* **110**, 10741–10746 (2013).
50. Paloian, N. J. & Giachelli, C. M. A current understanding of vascular calcification in CKD. *Am J Physiol Renal Physiol* **307**, F891–F900 (2014).
51. Bai, X., Hou, X., Tian, J., Geng, J. & Li, X. CDK5 promotes renal tubulointerstitial fibrosis in diabetic nephropathy via ERK1/2/PPARγ pathway. *Oncotarget* **7**, 36510 (2016).
52. Cao, S. L. *et al.* TFP5-Mediated CDK5 Activity Inhibition Improves Diabetic Nephropathy via NGF/Sirt1 Regulating Axis. *Front Cell Dev Biol* **10**, 1271 (2022).
53. Pfänder, P., Eiers, A. K., Burret, U. & Vettorazzi, S. Deletion of cdk5 in macrophages ameliorates anti-inflammatory response during endotoxemia through induction of c-maf and il-10. *Int J Mol Sci* **22**, 9648 (2021).
54. Massry, S. G. Is Parathyroid Hormone a Uremic Toxin? *Nephron* **19**, 125–130 (1977).
55. Glassock, R. J. Uremic Toxins: What Are They? An Integrated Overview of Pathobiology and Classification. *Journal of Renal Nutrition* **18**, 2–6 (2008).
56. Vanholder, R. *et al.* A history of uraemic toxicity and of the European Uraemic Toxin Work Group (EUTox). *Clin Kidney J* **14**, 1514–1523 (2021).
57. Duranton, F. *et al.* Normal and Pathologic Concentrations of Uremic Toxins. **23**, 1258–1270 (2012).
58. Dou, L. *et al.* The uremic solutes p-cresol and indoxyl sulfate inhibit endothelial proliferation and wound repair. *Kidney Int* **65**, 442–451 (2004).
59. Sun, C. Y., Chang, S. C. & Wu, M. S. Uremic toxins induce kidney fibrosis by activating intrarenal renin-angiotensin-aldosterone system associated epithelial-to-mesenchymal transition. *PLoS One* **7**, e34026 (2012).
60. Yu, M., Kim, Y. J. & Kang, D. H. Indoxyl sulfate-induced endothelial dysfunction in patients with chronic kidney disease via an induction of oxidative stress. *Clinical Journal of the American Society of Nephrology* **6**, 30–39 (2011).
61. Bammens, B., Evenepoel, P., Keuleers, H., Verbeke, K. & Vanrenterghem, Y. Free serum concentrations of the protein-bound retention solute p-cresol predict mortality in hemodialysis patients. *Kidney Int* **69**, 1081–1087 (2006).
62. Wu, I. W. *et al.* Serum free p -cresyl sulfate levels predict cardiovascular and all-cause mortality in elderly hemodialysis patients—a prospective cohort study. *Nephrology Dialysis Transplantation* **27**, 1169–1175 (2012).
63. Liabeuf, S. *et al.* Free p-cresylsulphate is a predictor of mortality in patients at different stages of chronic kidney disease. *Nephrology Dialysis Transplantation* **25**, 1183–1191 (2010).
64. Vanholder, R., Gryp, T. & Glorieux, G. Urea and chronic kidney disease: the comeback of the century? (in uraemia research). *Nephrology Dialysis Transplantation* **33**, 4–12 (2018).
65. Laville, S. M. *et al.* Urea levels and cardiovascular disease in patients with chronic kidney disease. *Nephrology Dialysis Transplantation* **38**, 184–192 (2023).
66. D'Apolito, M. *et al.* Urea-induced ROS cause endothelial dysfunction in chronic renal failure. *Atherosclerosis* **239**, 393–400 (2015).
67. Berg, A. H. *et al.* Carbamylation of serum albumin as a risk factor for mortality in patients with kidney failure. *Sci Transl Med* **5**, (2013).
68. Velasquez, M. T., Ramezani, A. & Raj, D. S. Urea and protein carbamylation in ESRD: surrogate markers or partners in crime? *Kidney Int* **87**, 1092–1094 (2015).
69. Bradley, D. The evolution of post-translational modifications. *Curr Opin Genet Dev* **76**, 101956 (2022).
70. Karve, T. M. & Cheema, A. K. Small Changes Huge Impact: The Role of Protein Posttranslational Modifications in Cellular Homeostasis and Disease. *J Amino Acids* **2011**, 1–13 (2011).
71. Ardito, F., Giuliani, M., Perrone, D., Troiano, G. & Muzio, L. Lo. The crucial role of protein phosphorylation in cell signalingand its use as targeted therapy (Review). *Int J Mol Med* **40**, 271–280 (2017).
72. Appella, E. & Anderson, C. W. Post-translational modifications and activation of p53 by genotoxic stresses. *Eur J Biochem* **268**, 2764–2772 (2001).
73. Harmel, R. & Fiedler, D. Features and regulation of non-enzymatic post-translational modifications. *Nature Chemical Biology 2018 14:3* **14**, 244–252 (2018).
74. Gajjala, P. R., Fliser, D., Speer, T., Jankowski, V. & Jankowski, J. Emerging role of post-translational modifications in chronic kidney disease and cardiovascular disease. *Nephrology Dialysis Transplantation* **30**, 1814–1824 (2015).
75. Pietzsch, J., Lattke, P. & Julius, U. Oxidation of Apolipoprotein B-100 in Circulating LDL Is Related to LDL Residence Time. *Arterioscler Thromb Vasc Biol* **20**, (2000).

76. Poznyak, A. V. *et al.* Overview of OxLDL and Its Impact on Cardiovascular Health: Focus on Atherosclerosis. *Front Pharmacol* **11**, 2248 (2021).
77. Gorisse, L. *et al.* Protein carbamylation is a hallmark of aging. *Proc Natl Acad Sci U S A* **113**, 1191–1196 (2016).
78. Chandler, J. D. & Day, B. J. Biochemical Mechanisms and Therapeutic Potential of the Pseudohalide Thiocyanate in Human Health. *Free Radic Res* **49**, 695 (2015).
79. Wang, Z. *et al.* Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nat Med* **13**, 1176–1184 (2007).
80. Delanghe, S., Delanghe, J. R., Speeckaert, R., Van Biesen, W. & Speeckaert, M. M. Mechanisms and consequences of carbamoylation. *Nat Rev Nephrol* **13**, 580–593 (2017).
81. Nilsson, L., Lundquist, P., Kågedal, B. & Larsson, R. Plasma cyanate concentrations in chronic renal failure. *Clin Chem* **42**, 482–483 (1996).
82. Davenport, A., Jones, S., Goel, S., Astley, J. P. & Feest, T. G. Carbamylated hemoglobin: A potential marker for the adequacy of hemodialysis therapy in end-stage renal failure. *Kidney Int* **50**, 1344–1351 (1996).
83. Koeth, R. A. *et al.* Protein carbamylation predicts mortality in ESRD. *Journal of the American Society of Nephrology* **24**, 853–861 (2013).
84. Kalim, S. Protein carbamylation in ESRD: Is there a mortality effect? *Curr Opin Nephrol Hypertens* **27**, 454 (2018).
85. Nowicki, C. & Santomé, J. A. Modification of lysine 69 reactivity in bovine growth hormone by carbamylation of its N-terminal group. *Int J Pept Protein Res* **18**, 52–60 (1981).
86. Beswick, H. T. & Harding, J. J. High-molecular-weight crystallin aggregate formation resulting from non-enzymic carbamylation of lens crystallins: Relevance to cataract formation. *Exp Eye Res* **45**, 569–578 (1987).
87. Fazili, K. M., Mir, M. M. & Qasim, M. A. Changes in protein stability upon chemical modificatlon of lysine residues of bovine serum albumin by different reagents. *Biochem Mol Biol Int* **31**, 807–816 (1993).
88. Weisgraber, K. H., Innerarity, T. L. & Mahley, R. W. Role of lysine residues of plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. *Journal of Biological Chemistry* **253**, 9053–9062 (1978).
89. Lee, T. C. K. & Gibson, Q. H. Allosteric properties of carbamylated hemoglobins. *Journal of Biological Chemistry* **256**, 4570–4577 (1981).
90. Dengler, T. J., Rohertz-Vaupel, G. M. & Dengler, H. J. Albumin binding in uraemia: quantitative assessment of inhibition by endogenous ligands and carbamylation of albumin. *Eur J Clin Pharmacol* **43**, 491–499 (1992).
91. Shaw, D. C., Stein, W. H. & Moore, S. Inactivation of Chymotrypsin by Cyanate. *Journal of Biological Chemistry* **239**, PC671–PC673 (1964).
92. De Furia, F. G., Miller, D. R., Cerami, A. & Manning, J. M. The Effects of Cyanate In Vitro on Red Blood Cell Metabolism and Function in Sickle Cell Anemia. *J Clin Invest* **51**, 566–574 (1972).
93. Van Lente, F., McHugh, A. & Pippenger, C. E. Carbamylation of apo-aspartate aminotransferase: a possible mechanism for enzyme inactivation in uremic patients. *Clin Chem* **32**, 2107–2108 (1986).
94. Veronese, F. M., Piszkiewicz, D. & Smith, E. L. Inactivation of Bovine Glutamate Dehydrogenase by Carbamyl Phosphate and Cyanate. *Journal of Biological Chemistry* **247**, 754–759 (1972).
95. Oimomi, M. *et al.* Carbamylation of Insulin and Its Biological Activity. *Nephron* **46**, 63–66 (1987).
96. Smyth, D. G. Carbamylation of Amino and Tyrosine Hydroxyl Groups. *Journal of Biological Chemistry* **242**, 1579–1591 (1967).
97. Erill, S., Calvo, R. & Carlos, R. Plasma protein carbamylation and decreased acidic drug protein binding in uremia. *Clin Pharmacol Ther* **27**, 612–618 (1980).
98. Ha, E., Bang, J.-H., Son, J. N., Cho, H.-C. & Mun, K.-C. Carbamylated albumin stimulates microRNA-146, which is increased in human renal cell carcinoma. *Mol Med Rep* **3**, 275–279 (2010).
99. Jaisson, S. *et al.* Carbamylated albumin is a potent inhibitor of polymorphonuclear neutrophil respiratory burst. *FEBS Lett* **581**, 1509–1513 (2007).
100. Maddock, A. L. & Westenfelder, C. Urea induces the heat shock response in human neuroblastoma cells. *Journal of the American Society of Nephrology* **7**, 275–282 (1996).
101. Balion, C. M., Draisey, T. F. & Thibert, R. J. Carbamylated hemoglobin and carbamylated plasma protein in hemodialyzed patients. *Kidney Int* **53**, 488–495 (1998).
102. Lane, T. A. & Burka, E. R. Decreased Life Span and Membrane Damage of Carbamylated Erythrocytes In Vitro. *Blood* **47**, 909–917 (1976).
103. Garnotel, R., Sabbah, N., Jaisson, S. & Gillery, P. Enhanced activation of and increased production of matrix metalloproteinase-9 by human blood monocytes upon adhering to carbamylated collagen. *FEBS Lett* **563**, 13–16 (2004).

104. Kalim, S., Karumanchi, S. A., Thadhani, R. I. & Berg, A. H. Protein Carbamylation in kidney disease: Pathogenesis and clinical implications. *American Journal of Kidney Diseases* **64**, 793–803 (2014).
105. Jaisson, S. *et al.* Impact of Carbamylation on Type I Collagen Conformational Structure and Its Ability to Activate Human Polymorphonuclear Neutrophils. *Chem Biol* **13**, 149–159 (2006).
106. Doué, M. *et al.* Carbamylation of elastic fibers is a molecular substratum of aortic stiffness. *Scientific Reports 2021 11:1* **11**, 1–11 (2021).
107. Apostolov, E. O., Basnakian, A. G., Yin, X., Ok, E. & Shah, S. V. Modified LDLs induce proliferation-mediated death of human vascular endothelial cells through MAPK pathway. *Am J Physiol Heart Circ Physiol* **292**, 1836–1846 (2007).
108. Asci, G. *et al.* Carbamylated low-density lipoprotein induces proliferation and increases adhesion molecule expression of human coronary artery smooth muscle cells. *Nephrology (Carlton)* **13**, 480–6 (2008).
109. Apostolov, E. O., Shah, S. V., Ok, E. & Basnakian, A. G. Carbamylated low-density lipoprotein induces monocyte adhesion to endothelial cells through intercellular adhesion molecule-1 and vascular cell adhesion molecule-1. *Arterioscler Thromb Vasc Biol* **27**, 826–832 (2007).
110. Holzer, M. *et al.* Protein carbamylation renders high-density lipoprotein dysfunctional. *Antioxid Redox Signal* **14**, 2337–2346 (2011).
111. Joshi, A. D., Mustafa, M. G., Lichti, C. F. & Elferink, C. J. Homocitrullination Is a Novel Histone H1 Epigenetic Mark Dependent on Aryl Hydrocarbon Receptor Recruitment of Carbamoyl Phosphate Synthase 1. *J Biol Chem* **290**, 27767 (2015).

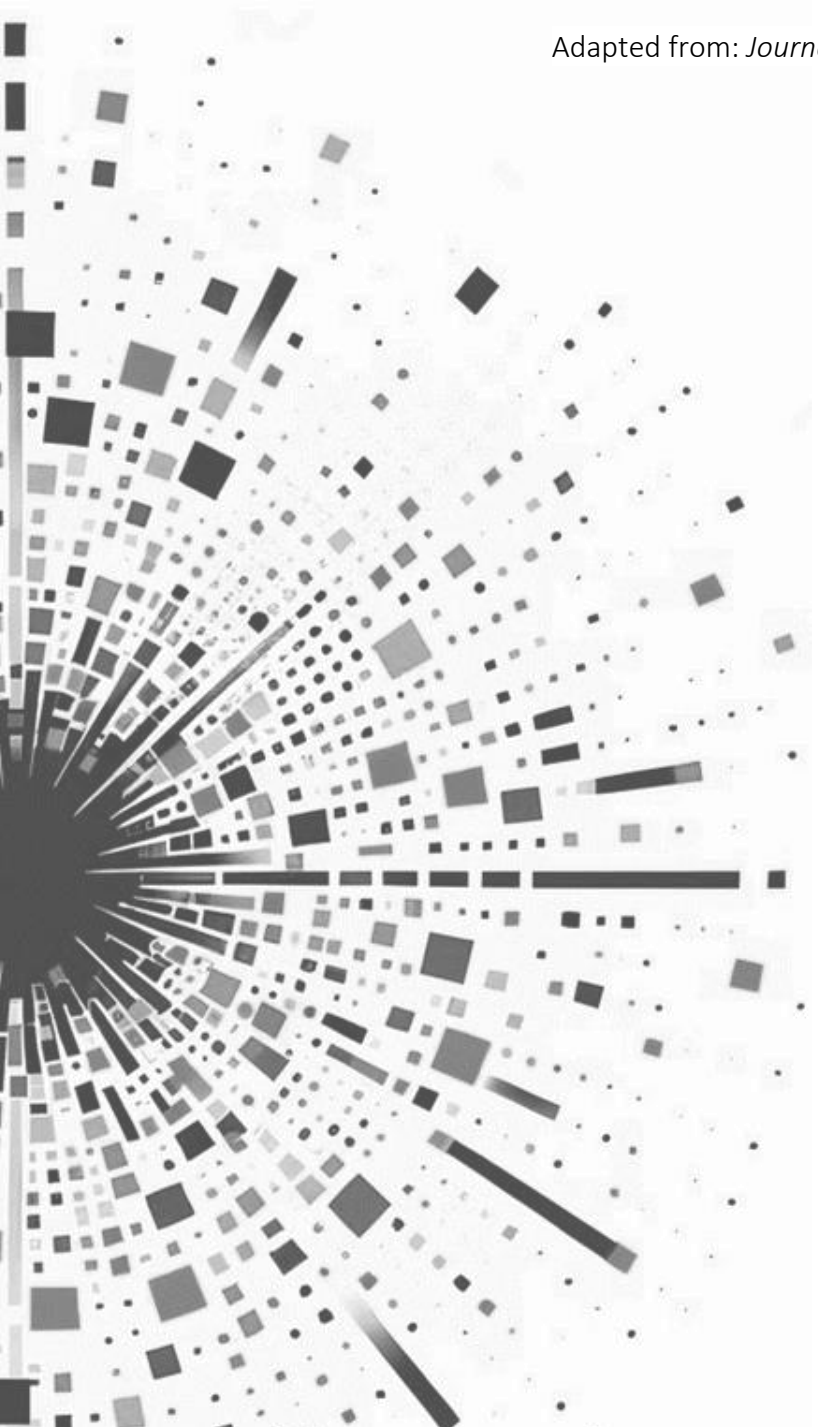


# Chapter 2

## Reduction of protein-bound uremic toxins in plasma of chronic renal failure patients: a systematic review

V Saar-Kovrov, W Zidek, S Orth-Alampour, D Fliser, V Jankowski, EAL Biessen, and J Jankowski

Adapted from: *Journal of Internal Medicine, 2021, 290(3):499-526*



## Abstract

**Background**: Protein-bound uremic toxins (PBUTs) accumulate in patients with chronic kidney disease and impose detrimental effects on the vascular system. However, a unanimous consensus on the most optimum approach for the reduction of plasma PBUTs is still lacking.

**Methods**: In this systematic review, we aimed to identify the most effective clinically available plasma PBUT reduction method reported in the literature between 1980 and 2020. The literature was screened for clinical studies describing approaches to reduce the plasma concentration of known uremic toxins. There were no limits on the number of patients studied, or on the duration, or design of the studies.

**Results**: Out of 1,274 identified publications, 101 studies describing therapeutic options aiming at the reduction of PBUTs in CKD patients were included in this review. We stratified the studies by the PBUTs and the duration of the analysis into acute (data from a single procedure) and longitudinal (several treatment interventions) trials. Reduction ratio (RR) was used as the measure of plasma PBUTs lowering efficacy. For indoxyl sulfate and *p*-cresyl sulfate, the highest RR in the acute studies were demonstrated for fractionated plasma separation, adsorption, and dialysis system. In the longitudinal trials, supplementation of hemodialysis patients with AST-120 (Kremezin®) adsorbent showed the highest RR. However, no superior method for the reduction of all types of PBUTs was identified based on the published studies.

**Conclusions**: Our study shows that there is presently no technique universally suitable for optimum reduction of all PBUTs. There is a clear need for further research in this field.

# Introduction

Chronic kidney disease (CKD) constitutes a huge medical and financial burden for society with an estimated prevalence of 13.4% globally.[1] The causes of CKD vary between countries with diabetes being the most common and accounting for 30-50% of all CKD cases.[2] After diabetes, hypertension and glomerulonephritis constitute substantial risk factors for CKD. Patients with CKD have a dramatically decreased life expectancy, there is an up to 25-year reduction in life expectancy in the later stage of CKD compared to individuals with normal kidney function.[3]

**Table 1. Combined list of protein-bound uremic toxins.**[4–6]

| Compound | Group |
|---|---|
| 3-deoxyglucosone | AGE |
| CMPF | AGE |
| Fructoselysine | AGE |
| Glyoxal | AGE |
| Methylglyoxal | AGE |
| N6-carboxymethyllysine | AGE |
| Pentosidine | AGE |
| Homocysteine | Amino acids |
| Dihydroxyphenylalanine | Catecholamines |
| 4-Hydroxyhippuric acid | Hippurates |
| Hippuric acid | Hippurates |
| p-Hydroxy-hippuric acid | Hippurates |
| Indican | Indoles |
| Indole-3-acetic acid | Indoles |
| Indoxyl sulfate | Indoles |
| Indoxyl-beta-D-glucuronide | Indoles |
| Kinurenine | Indoles |
| Kynurenic acid | Indoles |
| Melatonin | Indoles |
| Quinolinic acid | Indoles |
| Thiocyanate | Other |
| Leptin | Peptides |
| Retinol-binding protein | Peptides |
| 2-Methoxyresorcinol | Phenols |
| Hydroquinone | Phenols |
| p-Cresol | Phenols |
| p-Cresyl sulfate | Phenols |
| Phenol | Phenols |
| Phenylacetic acid | Phenols |
| Putrescine | Polyamines |
| Spermidine | Polyamines |
| Spermine | Polyamines |
| Acrolein | Reactive carbonyl compound |

*Abbreviations: AGE - advanced glycation end-product; and CMPF - 3-Carboxy-4-methyl-5-propyl-2-furanpropionate.*

Impaired kidney function in CKD results in an accumulation of uremic toxins in the circulation. By definition, uremic toxins are solutes excreted by the kidneys, their concentration is elevated in uremia, and there is a demonstrated relationship between their levels and one or more manifestations of the uremic syndrome.[7,8] More than one hundred solutes were identified in the past years[9] and this number is continuously growing. Currently, the European Uremic Toxin Work Group (EUTox; http://www.uremic-toxins.org/) classifies uremic toxins into three categories: small water-soluble compounds with a molecular weight of up to 500 Da (e.g. urea); middle sized molecules (>500 Da; e.g. β2-microglobulin); and protein-bound compounds, with a molecular weight mostly below 500 Da (e.g. indoxyl sulfate)[4]. A combination of listed protein-bound uremic toxins (PBUTs) investigated in three published reviews[4–6] resulted in a total of 33 uremic compounds (**Table 1**.).

In recent years, the interest in PBUTs has gained increasing attention due to their strong association with cardiovascular toxicity, renal fibrosis, and progression of CKD.[10] Extensive *in vitro* as well as *in vivo* studies of indoxyl sulfate and *p*-cresyl sulfate, for instance, have revealed their profibrotic, proinflammatory and oxidative stress-inducing properties.[11–13] Their association with increased risk for cardiovascular and all-cause mortality in CKD patients has also been demonstrated in several clinical trials.[14–16] The protein-bound fraction of these retention solutes in serum is sufficiently high ranging from approximately 30% to 99%,[17] which renders their removal by conventional membrane-based dialysis techniques, challenging. This has spurred the development of more advanced methods to improve the reduction of the PBUTs levels. Consequently, there has been an increase in clinical trials investigating potent high-flux dialyzers, pre- and post-dilution hemodiafiltration systems, as well as intravenous infusion or oral supplementation of agents with various mode of action, e.g. ibuprofen, folates and charcoal adsorbent AST-120 (Kremezin®; Kureha Chemical Industry Co Ltd, Tokyo, Japan). However, a unanimous consensus on the most optimum approach is still lacking.

Common uremic toxins reducing strategies can be generally separated in two classes: those that use diffusion and/ or convection, either extracorporeally or in the peritoneal cavity, for uremic milieu removal; and those that utilize other more specific mechanisms to reduce PBUTs concentrations in plasma. The example of the former can be hemodialysis or peritoneal dialysis, which utilizes diffusion, and hemodiafiltration, in which case both diffusion and convection are used in combination. In the latter case, however, the strategy usually involves use of certain supplements that can be administered to the patients on dialysis. In this work, we describe several of these types of intervention. First, orally administered supplement AST-120 (Kremezin®) acts by adsorbing precursor of indoxyl sulfate, indole, which is produced by intestinal bacteria such as *E. coli* form tryptophan, thus reducing indoxyl sulfate concentration in the blood.[18] Of note, some dialysis systems also use adsorption technique in combination with diffusion to achieve better removal of protein-bound toxin,[19–22] however in this case adsorption takes place in the blood extracorporeally and thus differs in the mode of action from the ingestible adsorbents. Another example of used supplements is synbiotic NATUREN

G® (Farmalabor SRL), which was created to tackle the dysbiosis of gut microbiota in CKD patients and thus decrease the PBUTs production in the intestine.[23] Intravenous infusion of certain supplements, such as ibuprofen, N-acetylcysteine (NAC) or mesna, during the dialysis procedure might also improve removal of several PBUTs.[24–27] This is possible due to the competitive binding of the toxins and supplements to the plasma albumin, by which the supplements can displace the toxins from the protein making them soluble and available for removal by the dialysis. Additionally, plasma levels of some PBUTs can be regulated by targeting their metabolism, as in case of folates, which facilitate homocysteine recycling into methionine,[25,28] and subcutaneous insulin, which can have reduced effects on leptin synthesis by adipocytes comparing to intraperitoneal.[29] Mechanisms of action of the main strategies discussed in this review are summarized in **Table 2**.

**Table 2. Mechanisms of action of PBUTs reduction approaches.**

| Intervention | Type | Mechanism |
|---|---|---|
| Ibuprofen | oral supplement | toxins displacement from plasma protein |
| AST-120 | oral supplement | precursor adsorption in the intestine |
| Folates | intravenous/ oral supplement | enhancement of toxin recycling |
| Mesna | intravenous supplement | toxins displacement from plasma proteins |
| N-acetylcystein | intravenous supplement | toxins displacement from plasma proteins |
| Insulin | intraperitoneal supplement | reduction of leptin production by adipocytes |
| NATUREN G® | oral supplement | reduction of toxins production by gut microbiota |
| Hemodialysis | extracorporeal removal | diffusion |
| Hemodiafiltration | extracorporeal removal | diffusion and convection |
| Hemofiltration | extracorporeal removal | convection |
| Peritoneal dialysis | intracorporeal removal | diffusion |
| Hemoperfusion | extracorporeal removal | adsorption |
| FPAD | extracorporeal removal | diffusion and adsorption |

*FPAD - fractionated plasma separation, adsorption, and dialysis.*

Due to the great variety of kidney replacement therapies as well as additional supplementations for dialysis patients, a comprehensive overview of the techniques available for the reduction of PBUTs plasma levels is of paramount importance for clinicians to facilitate treatment recommendations based on the individual needs of a patient. Therefore, we systematically reviewed clinical studies published between 1980 and 2020 that analyze the

different dialysis therapies with or without supplementations for the lowering of PBUT levels in CKD patients.

The aim of this systematic review is to identify the most effective technique for each type of PBUT on the basis of the reported reduction ratios or change in PBUT plasma levels during the course of the treatment.

## Material and Methods

*Search strategy*

A search was performed in two databases, MEDLINE and Embase (https://pubmed.ncbi.nlm.nih.gov/ and https://www.embase.com/, respectively) for scientific papers published in English during the time period between 01.01.1980 and 01.02.2020. The search terms included the full compound list found in **Table 1**. as well as the following terms: ("renal replacement therapy" OR "hemodialysis" OR "hemofiltration" OR "hemodiafiltration" OR "peritoneal dialysis" OR "adsorption dialysis" OR "renal dialysis" OR "extracorporeal dialysis" OR "haemofiltration") AND ("filtration" OR "adsorption" OR "clearance" OR "separation" OR "removal"). The full search query can be found in the Supplementary materials.

*Eligibility criteria and study selection*

The literature query in the two databases yielded a total of 1,274 articles (710 from MEDLINE and 564 from Embase). After duplicates removal, the titles and abstracts of 805 publications were assessed independently by two reviewers (V.S.K and J.J.), based on the criteria listed in **Figure 1**. Disagreements were resolved by consulting a third reviewer (S.O.A.). Clinical studies comparing different methods or reporting efficacy of uremic solutes plasma levels reduction by any renal replacement therapy (hemodiafiltration (HDF), hemodialysis (HD), hemofiltration (HF), peritoneal dialysis (PD), excluding kidney transplantation) with or without adsorbing or other types of supplements were included. There were no restrictions on sample size, study design or study duration. If authors published more than one paper on the same method, data from the most recent study was used.

The workflow was based on the ´Preferred Reporting Items of Systematic Reviews´ (PRISMA) recommendations [30] and is depicted in **Figure 1** together with the exclusion criteria. The following data was extracted from the papers: (a) name of the first author, (b) year of publication, (c) study design, (d) number of participants in each group, (e) participants' age and gender, (f) duration of the intervention, (g) technique used, (h) studied PBUTs, (i) results in form of either reduction ratio (RR) or PBUT concentrations before and after the treatment, as well as briefly (j) the purpose of the study, (k) methods, (l) conclusion and (m) mentioned limitations, if any. Papers that did not report any information on the change in plasma concentration of the PBUTs studied after the treatments were disregarded. After the full-text analysis, 95 articles were deemed to meet the inclusion criteria. A cross-referencing search yielded additional 15 publications, 6 of which were included in the review, bringing the total number of references to 101.

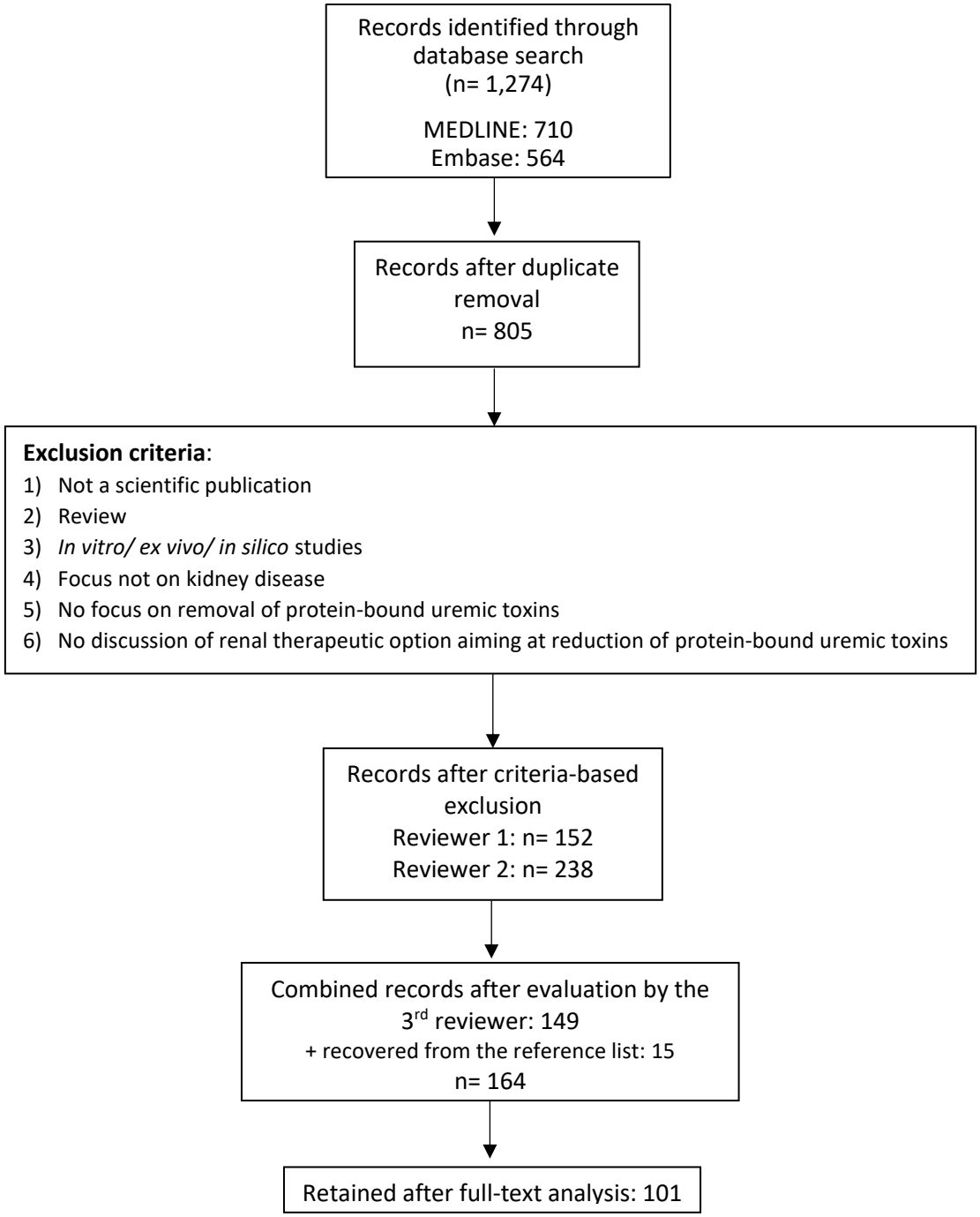



**Figure 1. "Preferred Reporting Items of Systematic Reviews" (PRISMA)[30] flow diagram for study selection for the removal techniques of protein-bound uremic toxins.**

*Data synthesis*

We stratified the studies by the PBUTs studied and according to the time period of the analysis differentiating between *i) acute* (data from a single treatment intervention) and ii) *longitudinal* (three or more treatment interventions) trials. The results were additionally subdivided according to the mode of action of the studied approaches (e.g. diffusion vs intestinal adsorption of the toxins) where possible. Because of the wide range of metrics used

in the studies as an assessment of PTBUTs reduction by any given technique, this systematic review focused on the reported RR of the solutes as the most straightforward and clinically relevant outcome**.** For the acute studies that did not report RR, it was calculated from the total mean (or median) plasma concentrations of the compound before and after the single treatment as follows:

$$((C_{before}-C_{after})/C_{before})*100\%$$

The same approach was applied in regard to the longitudinal studies using mean (median) pre-dialysis concentrations before and at the end of the treatment. In the case of studies reporting on both the immediate and long-term impact of the dialysis technique, the results of the former were disregarded. In cases where PBUT concentration increased after the intervention, the RR was assumed to be zero. In cases where there was no numerical representation of the results in the text of the paper, approximate reduction ratios (approx. RR) were taken from the figures of the corresponding paper. Data is presented as either mean ± standard deviation or median (range), as reported by the authors.

Given the wide range of methods used for the assessment of performance of the dialysis techniques as well as the great variety of study designs, we opted to conduct a narrative synthesis of the evidence instead of a meta-analysis. Since the review does not evaluate the methodological quality of the studies, a risk-of-bias tool was not required.

This systematic review was registered at inception (i.e. at the protocol stage) to help avoid unplanned duplication.

Available from:
http://www.crd.york.ac.uk/PROSPERO/display_record.asp?ID=CRD42015029993

## Results

In this study, we assessed literature on kidney replacement therapies with or without supplementations for PBUTs plasma levels reduction published over the past 40 years, deploying the strategy depicted in **Figure 1**. We identified 1,274 citations in total via database search (MEDLINE, https://www.ncbi.nlm.nih.gov/pubmed; and Embase, https://www.embase.com). Based on the selection criteria, we included 101 studies in this systematic review (**Table 3**).

**Table 3. Summary of the publications included in this review.**

| Author, year | Main technique(s) | Toxins analyzed | Author, year | Main technique(s) | Toxins analyzed |
|---|---|---|---|---|---|
| van Gelder, 2020[31] | OL-HDF vs HD | IS, pCS, IAA, KY, KA, PCG, HA | Ouseph, 2008[32] | HD | leptin |
| Rocchetti, 2020[23] | supplements | IS, pCS | De Smet, 2007[33] | HD | IS, CMPF, IAA, HA, PENT |
| Paats, 2020[34] | HD vs HDF | IS, IAA | Gugliucci, 2007[35] | HD | acrolein |
| Krieter, 2019[36] | HD vs HDF | IS, pCS | Mandolfo, 2006[37] | HD vs HDF | leptin |
| Madero, 2019[24] | supplements | IS, pCS | Panichi, 2006[38] | HDF | leptin |
| Belmouaz, 2019[39] | HD | Hcy, Leptin | Schulman, 2006[40] | AST-120 | IS |
| Snauwaert, 2019[41] | HDF vs HD | IS, pCS, IAA, HA, CMPF, PCG | Testa, 2006[42] | HDF | RBP |
| Lenglet, 2019[43] | supplements | IS, pCS, IAA, HA, KY, CMPF, KA, PCG | Urquhart, 2006[26] | supplements | Hcy |
| Gomolka, 2019[44] | HD vs HDF | IS, pCS, leptin | Agalou, 2005[45] | HD vs PD | CML, GO, MG, 3-DG, PENT |
| Chen, 2020[46] | HFR | IS, pCS | Beerenhout, 2005[47] | HD vs HDF | Hcy, leptin |
| Etinger, 2018[48] | HD | IS, pCS, IAA, HA, KA, KY, PCG | Czupryniak, 2005[49] | PD | Hcy |
| Kalim, 2018[50] | HD | IS, pCS, HA, | Galli, 2005[51] | HD | PENT |
| Ramon, 2018[52] | HDF | RBP | Krieter, 2005[53] | HDF | RBP |
| Yamamoto, 2018[21] | HD | IS, pCS, IAA | Martinez, 2005[54] | HD | pCS, IND |
| Esquivias-Motta, 2017[55] | HDF | IS, pCS | Mudge, 2005[56] | HD | Hcy |
| Krieter, 2017[57] | HD vs HDF | IS, pCS | Santoro, 2005[58] | HDF | leptin, RBP |
| Panichi, 2017[59] | HD vs HDF | IS, pCS | Bammens, 2004[60] | HD vs HDF | p-cresol |
| Sirich, 2017[61] | HD | IS, pCS, HA, | Scholze, 2004[27] | supplements | Hcy |
| Hohmann, 2017[62] | HD | CML, PENT | Splendiani, 2004[63] | HDF | Hcy |
| Abad, 2016[64] | HDF | IS, pCS, Hcy | Tessitore, 2004[65] | HD | PENT |
| Camacho, 2016[66] | HD | IS, pCS | van Tellingen, 2004[67] | HD | leptin |
| Chazot, 2015[68] | HD | Hcy | De Vriese, 2003[69] | HD | Hcy |

| | | | | | |
|---|---|---|---|---|---|
| Cornelis, 2015[70] | HD vs HDF | IS, pCS, CMPF, IAA, HA, PCG, CML, GO, MG, 3-DG, PENT | Galli, 2003[71] | HD | Hcy |
| Eloot, 2015[72] | HD | IS, pCS, IAA, HA, PCG | Opatrná, 2003[73] | PD | leptin |
| Yamamoto, 2015[74] | AST-120 | IS, pCS | Arnadottir, 2002[75] | HD | Hcy |
| Galli, 2014[76] | HD | Hcy | Ducloux, 2002[28] | supplements | Hcy |
| Lee, 2014[77] | AST-120 | IS, pCS | Fagugli, 2002[78] | HD | IS, CMPF, IAA, HA, *p*-cresol |
| Pedrini, 2014[79] | HDF | Hcy | Ludemann, 2001[80] | HD | MEL |
| Riccio, 2014[81] | HDF | *p*-cresol | Van Tellingen, 2001[82] | HD | Hcy |
| Sirich, 2014[83] | HD | IS, pCS, HA | House, 2000[84] | HD | Hcy |
| Brettschneider, 2013[19] | FPAD vs HD | IS, pCS, PAA | Lesaffer, 2000[85] | HD | IS, CMPF, HA, *p*-cresol |
| Kneis, 2013[86] | HD vs HDF | leptin | Nevalainen, 2000[29] | supplements | leptin |
| Sequera, 2013[87] | HDF | RBP | Ducloux, 1999[88] | PD | Hcy |
| Eloot, 2012[89] | HDF | IS, pCS, CMPF, IAA, HA | Jadoul, 1999[90] | HD | PENT |
| Perna, 2012[25] | supplements | Hcy | Kim, 1999[91] | PD | leptin |
| Sirich, 2012[92] | HD | IS, pCS, HA | Biasioli, 1998[93] | HD | Hcy |
| Badiou, 2011[94] | HD vs HDF | Hcy | Wiesholzer, 1998[95] | HD vs HDF | leptin |
| Chen, 2011[22] | HD | leptin | Nakazono, 1998[96] | HD | leptin |
| Meert, 2011[97] | HD vs HDF | IS, pCS, HA, IAA, RBP | Miyata, 1997[98] | HD vs PD | PENT |
| Meijers, 2011[99] | HD | IS, pCS | Friedlander, 1996[100] | HD vs PD | PENT |
| Pedrini, 2011[101] | HD vs HDF | Hcy | Tamura, 1996[102] | HD | Hcy |
| Krieter, 2010[103] | HD vs HDF | IS, pCS | Friedlander, 1995[104] | HD vs PD | PENT |
| Meert, 2010[105] | HDF | IS, pCS, CMPF, IAA, HA | Odetti, 1995[106] | HD vs HDF | PENT |
| Righetti, 2010[107] | HD vs HDF | Hcy | Vaziri, 1993[108] | HD | MEL |
| Luo, 2009[109] | HD | IS, pCS, HA, KA | Vanholder, 1992[110] | HD vs HDF | IS, HA |
| Maduell, 2009[111] | HDF | RBP | Niwa, 1991[18] | AST-120 | IS |
| Meert, 2009[112] | HDF vs HF | IS, pCS, CMPF, IAA, HA | Vanholder, 1988[113] | HD | HA |
| Susantitaphong, 2009[114] | HDF | *p*-cresol | Wichman, 1985[115] | HF | RBP |
| Krieter, 2008[116] | HD vs HDF | RBP | Pahl, 1982[117] | HD | TC |
| Mandolfo, 2008[118] | HD vs HDF | leptin | Röckel, 1982[119] | HF | RBP |
| Meijers, 2008[20] | HD vs HDF | pCS | | | |

*Technique abbreviations: CVVH – continuous venovenous hemofiltration; CVVHD – continuous venovenous hemodialysis; FPAD – fractionated plasma separation, adsorption, and dialysis; HD - hemodialysis; HDF – hemodiafiltration; HF – hemofiltration; MAHD - magnetically-assisted hemodialysis; and PD – peritoneal dialysis. Compounds abbreviations: TC – thiocyanate; RBP - retinol-binding protein; PENT – pentosidine; pCS – p-cresyl sulfate; PCG – p-cresylglucuronide; PAA – phenylacetic acid; MG – methylglyoxal; MEL – melatonin; KY – kynurenine; KA – kynurenic acid; IS – indoyl sulfate; IND – indicant; IAA – Indole-3-acetic acid; Hcy – homocysteine; HA – hippuric acid; GO – glyoxal; CMPF – 3-Carboxy-4-methyl-5-propyl-2-furanpropionate; CML – Ne-(carboxymethyl)lysine; and 3-DG – 3-deoxyglucosone.*

*Extracted data*

We stratified the publications by the retention solutes they were focusing on. The number of studies analyzing reduction techniques for each protein-bound uremic toxin is presented in **Figure 2**. The following approaches were investigated in the included works: hemodialysis (HD);[21,22,32,33,35,48,50,51,54,56,61,62,65–69,71,72,75,76,78,80,82–85,90,92,93,96,99,102,108,109,113,117] hemofiltration (HF);[115,119] dilution techniques, membranes or hemofiltrate reinfusion (HFR) in hemodiafiltration (HDF);[38,42,46,52,53,55,58,63,64,79,81,87,89,105,111,114] continuous ambulatory peritoneal dialysis (CAPD);[49,73,88,91] effects of various adsorbing, displacing or metabolically active supplements (e.g. AST-120 (Kremezin®), ibuprofen or folates) in different dialysis techniques;[18,23–29,40,43,74,77] as well as comparisons of two or more different techniques between each other (**Figure 3**).[19,20,31,34,36,37,39,41,44,45,47,57,59,60,70,86,94,95,97,98,100,101,103,104,106,107,110,112,116,118]

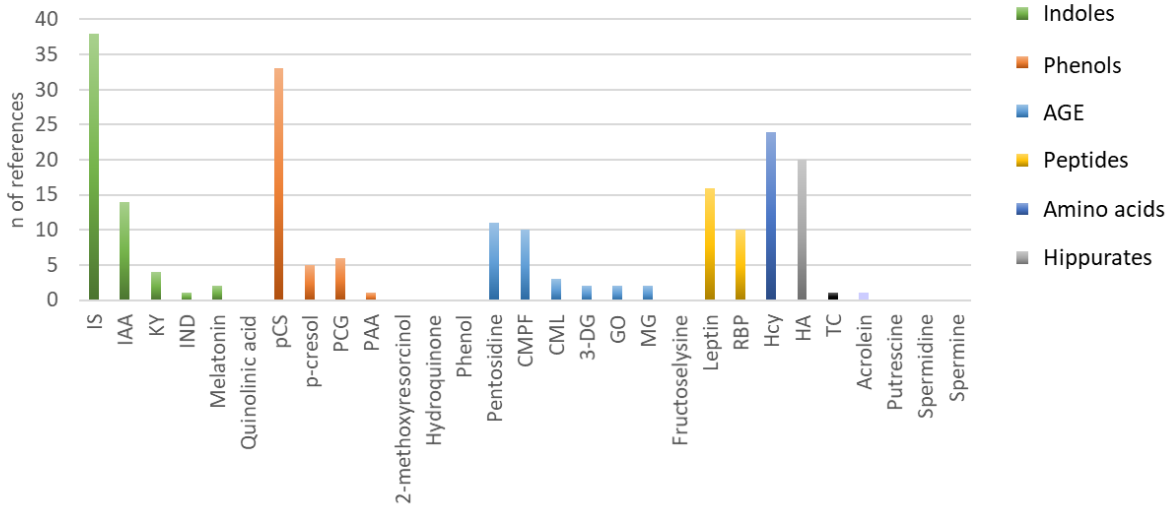



**Figure 2. Number of citations for each protein-bound uremic toxin for the time period between 1980 and 2020 included in this review.** Toxins separated into groups according to their type. Abbreviations: AGE – advanced glycation end-products; IS – indoxyl sulfate, IAA – Indole-3-acetic acid; KY – kynurenine; IND – indicant; pCS - p-cresyl sulfate; PCG – p-cresylglucuronide; PAA – phenylacetic acid; CMPF – 3-Carboxy-4-methyl-5-propyl-2-furanpropionate; CML – Ne-(carboxymethyl)lysine; 3-DG – 3-deoxyglucosone; GO – glyoxal; MG – methylglyoxal; Hcy – homocysteine; HA – hippuric acid; and TC – thiocyanate.

The data on the most-referred compounds are presented below: indoxyl sulfate (**Table 4**), *p*-cresyl sulfate (**Table 5**), homocysteine (**Table 6**), hippuric acid (**Table 7**), leptin (**Table 8**), and indole-3-acetic acid (IAA; **Table 9**). Each technique from each individual study is presented in a separate row. Results for the remaining PBUTs are presented in the Supplementary material. **Figure 4** presents an overview of all PBUTs discussed in this work with their RR from the longitudinal (**Figure 4A**) and acute (**Figure 4B**) trials.

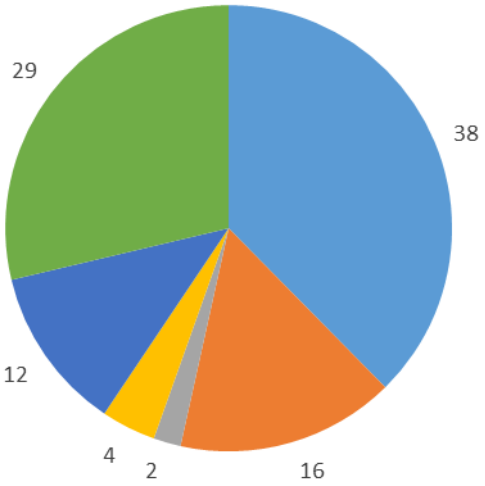



**Figure 3. Number of studies per technique for the time period between 1980 and 2020 included in this review.** HD – hemodialysis (light blue); HDF – hemodiafiltration (orange); HF – hemofiltration (grey); PD – peritoneal dialysis (yellow); supplements (dark blue); and several various techniques (green).

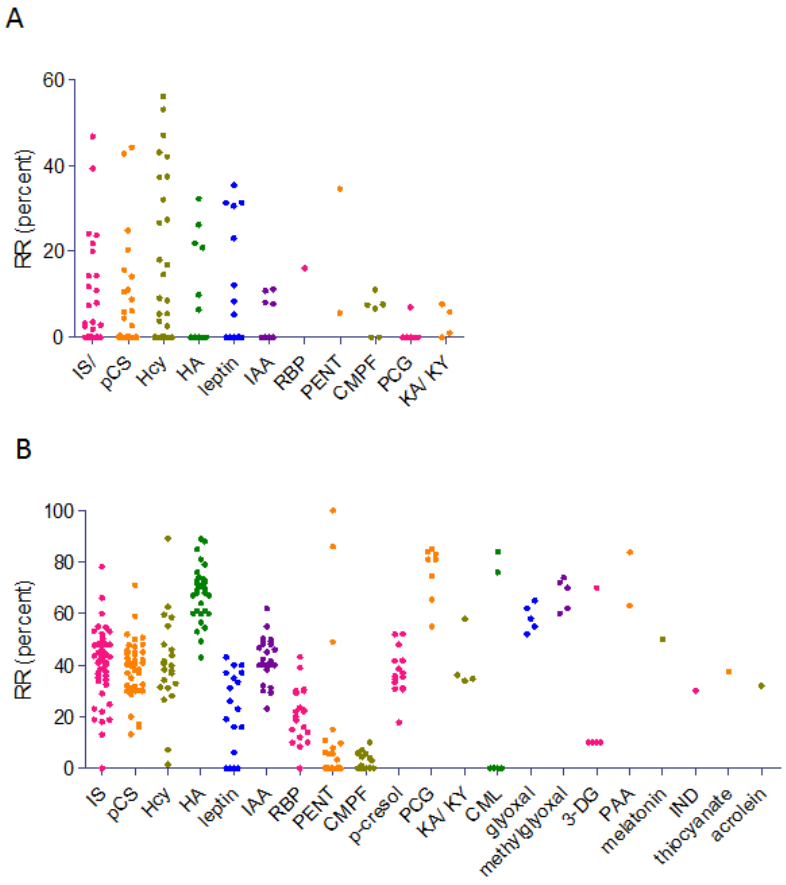



**Figure 4. Overview of PBUTs discussed in this systematic review.** (A) Reduction ratios from the longitudinal trials, (B) reduction ratios from the acute trials. Abbreviations: IS – indoxyl sulfate; pCS - p-cresyl sulfate; Hcy – homocysteine; HA – hippuric acid; IAA – Indole-3-acetic acid; RBP – retinol-binding protein; PENT – pentosidine; CMPF – 3-Carboxy-4-methyl-5-propyl-2-furanpropionate; PCG – p-cresylglucuronide; KA – kynurenic acid; KY – kynurenine; CML – Ne-(carboxymethyl)lysine; 3-DG – 3-deoxyglucosone; PAA – phenylacetic acid; and IND – indicant.

*Indoxyl sulfate*

*i) Longitudinal trials.* Analysis of the longitudinal trials showed that such approaches as supplementation of dialysis patients with AST-120 (Kremezin®) adsorbent[18,40,74,77] as well as use of synbiotic NATUREN G® (Farmalabor SRL) in combination with polyvinylpyrrolidone-coated divinylbenzene (DVB-PVP) cartridge in hemodialysis[23] that act by preventing indoxyl sulfate or its precursor synthesis by gut microbiota were the most effective, with RRs ranging between 20% and 46.7% (**Table 4**). It should be noted, however, that the NATUREN G® symbiotic study only monitored effects of the combined treatment for 1 week, and plasma levels of indoxyl sulphate at the baseline in the NATUREN G® group, even though not significantly, were higher comparing to that in the *placebo* group. Same applies to the results of the *p*-cresyl sulphate reduction (see below).

*ii) Acute trials.* From a total of 23 studies that involved single session analysis (acute studies), reported or estimated RR values varied from 0% to 78.2% with an average of 40.7±13.5%. The most effective technique appeared to be fractionated plasma separation, adsorption, and dialysis (FPAD)[19] representing highest RR (78.2%). From the remaining studies, (a) high and (b) standard mass transfer area coefficient (KoA) and dialysate flow (Qd) in 8-hour nocturnal HD,[70,92] (c) 8-hour post-dilution HDF,[70] and (d) use of PUREMA H+ membrane in HD[103] were the most effective removal methods. Standard nocturnal 7-8-hour long HD was also studied in a longitudinal trial which, however, showed no significant effect on indoxyl sulfate plasma concentration change after one year.[50]

**Table 4. Reduction ratios (RR) of indoxyl sulfate.** RR extracted directly or calculated from the analyzed articles. The colours represent the values of the RR with the highest values in green and lowest in red.

| | | Longitudinal studies | | |
|---|---|---|---|---|
| **First author, year** | **Technique** | **Analysis period** | **Results** | **RR** |
| Yamamoto, 2015[74] | **oral 6g/day AST-120 in HD** | 4 weeks | RR: 45.7%, p< 0.001 | 46.7% |
| Schulman, 2006[40] | **oral 9g/day AST-120 in HD** | 3 months | before 0.84±0.407 mg/dl change -0.33±0.298, p< 0.001; 39.3% decrease | 39.3% |
| Rocchetti, 2020[23] | **synbiotic NATUREN G®(Farmalabor SRL) in HD with polyvinylpyrrolidone coated divinylbenzene (DVB-PVP) cartridge** | 1 week | before 19.9±6 after 15.1±6 µg/ml, p= 0.04 | 24.1% |
| Lee, 2014[77] | **oral 6g/day AST-120 in HD and PD** | 3 months | before 38.4±13.6 mg/l after 29.3±14.3 mg/l, p< 0.01 | 23.7% |
| Niwa, 1991[18] | **oral 6g/day AST-120 in HD** | 3 months | approx. before 3.2 after 2.5 mg/dl, p< 0.05 | 21.9% |
| Schulman, 2006[40] | oral 6.3g/day AST-120 in HD | 3 months | 0.85±0.386 mg/dl, change -0.171±0.321, p= 0.002 | 20.0% |
| Camacho, 2016[66] | high clearance (Qd 800 ml/min, Qb 270 ml/min, F250NR dialyzer) in HD | 2 weeks | before 2.1 (1.4-2.4) after 1.8 (0.9-2.5) mg/dl | 14.3% |
| Panichi, 2017[59] | high-volume post-dilution HDF | 6 months | approx. before 3.5 after 3 mg/dl, p< 0.001 | 14.3% |

| | | | | |
|---|---|---|---|---|
| Vanholder, 1992[110] | canonical HD | 22 weeks | before 34±35 after 30±16 µmol/l, ns | 11.8% |
| Meert, 2010[105] | post-dilution HDF | 9 weeks | before 1.65±0.70 after 1.47±0.73 mg/dl, ns | 10.9% |
| van Gelder, 2020[31] | post-dilution high-flux HDF | 6 months | RR: 8.0 (34.6 to -15.3)%, ns | 8.0% |
| Sirich, 2017[61] | increased frequency HD (6 per week) | at the end of 12 months | before 2.7±1.3 after 2.5±1.0 mg/dl, ns | 7.4% |
| Gomolka, 2019[44] | low-flux HD (Braun LOPS) | 8 weeks | before 25.9 ±11.5 after 25.0±12.6 mg/l, ns | 3.5% |
| Rocchetti, 2020[23] | HD with polyvinylpyrrolidone coated divinylbenzene (DVB-PVP) cartridge | 1 week | approx. before 16 after 15.5 µg/ml, ns | 3.2% |
| Rocchetti, 2020[23] | synbiotic NATUREN G®(Farmalabor SRL) bicarbonate HD (7 weeks) | 7 weeks | before 20.5±8 after 19.9±6, p= 0.02 | 2.9% |
| Schulman, 2006[40] | oral 2.7g AST-120 in HD | 3 months | before 0.78±0.332 mg/dl, change -0.020±0.359, ns | 2.6% |
| Snauwaert, 2019[41] | high-flux HD | 12 months | after 2.06 (1.46; 2.93), change -0.04 (-0.66; 0.48) mg/dl | 1.9% |
| Camacho, 2016[66] | low clearance (Qd 300 ml/min, Qb 350 ml/min, F160NR dialyzer) in HD | 2 weeks | before 1.9 (1.0-2.3) after 2.2 (1.1-2.9) mg/dl | 0.0% |
| Gomolka, 2019[44] | high-flux HD (Braun HIPS) | 8 weeks | before 25.0±12.6 after 25.9±11.2 mg/l, ns | 0.0% |
| Gomolka, 2019[44] | HDF | 8 weeks | before 25.9±11.2 after 26.7±15.8 mg/l, ns | 0.0% |
| Kalim, 2018[50] | canonical HD | 12 months | final-to-baseline ratio: 1.16±0.73, ns | 0.0% |
| Kalim, 2018[50] | nocturnal 7-8-hour HD | 12 months | final-to-baseline ratio: 1.10±0.43, ns | 0.0% |
| Krieter, 2019[36] | HDF | 6 weeks | no significant reduction | 0.0% |
| Krieter, 2019[36] | high-flux HD | 6 weeks | no significant reduction | 0.0% |
| Krieter, 2019[36] | low-flux HD | 6 weeks | no significant reduction | 0.0% |
| Lenglet, 2019[43] | sevelamer-hydrochloride in HD | 24 weeks | before 36.962±24.184 after 38.607±21.869 ng/ml, ns | 0.0% |
| Lenglet, 2019[43] | nicotinamide in HD | 24 weeks | before 38.808±31.206 after 43.168±32.107 ng/ml, ns | 0.0% |
| Panichi, 2017[59] | bicarbonate HD | 6 months | approx. before 0.31 after 0.41±0.17 mg/dl, ns | 0.0% |
| Rocchetti, 2020[23] | bicarbonate HD | 7 weeks | approx. before 15.5 after 16 µg/ml, ns | 0.0% |
| Sirich, 2017[61] | canonical HD | 12 months | before 2.6±0.9 after 2.9±1.1 mg/dl, ns | 0.0% |
| Snauwaert, 2019[41] | post-dilution HDF | 12 months | after 2.11 (1.27; 3.09), change 0.01 (-0.50; 0.59) mg/dl | 0.0% |
| Snauwaert, 2019[41] | low-flux HD | 12 months | after 2.66 (2.13; 3.27), change 0.22 (-0.59; 0.90) mg/dl | 0.0% |
| van Gelder, 2020[31] | low-flux HD | 6 months | RR: -11.9 (15.4 to -31.9)%, increase, ns | 0.0% |

**Acute studies**

| First author, year | Technique | Results | RR |
|---|---|---|---|
| Brettschneider, 2013[19] | **FPAD** | before 275±73 after 60±24 µmol/l | 78.2% |
| Sirich, 2012[92] | **high KoA-Qd (Rexeed 25S dialyzer) in 8-hour nocturnal HD** | RR: 66±6% | 66.0% |
| Cornelis, 2015[70] | **8-hour post-dilution HDF** | approx. RR: 60% | 60.0% |

| | | | |
|---|---|---|---|
| Cornelis, 2015[70] | **8-hour HD** | approx. RR: 55% | 55.0% |
| Krieter, 2010[103] | **PUREMA H+ in HD** | RR: 54.6±8.7% | 54.6% |
| Krieter, 2010[103] | PUREMA H+ in post-dilution HDF | RR: 53.3±8.4% | 53.3% |
| Luo, 2009[109] | high KoA and Qd in HD | before 3.9±1.2 after 1.8±0.5 mg/dl, RR: 53±7% | 53.0% |
| Krieter, 2010[103] | PUREMA H in post-dilution HDF | RR: 52.2±12.2% | 52.2% |
| Krieter, 2010[103] | PUREMA H in HD | RR: 50.4±2.6% | 50.4% |
| Esquivias-Motta, 2017[55] | HFR | RR: 53.7 (48.8–67.3)% | 48.8% |
| Eloot, 2012[89] | post-dilution HDF | RR: 48.7±10.0% | 48.7% |
| Meert, 2009[112] | pre-dilution HDF | RR: 48.5±10.0% | 48.5% |
| Paats, 2020[34] | high Qb (378 ± 30 ml/min) and Qd (793 ± 47 ml/min) in HDF | approx. RR: 48% | 48.0% |
| Meert, 2011[97] | membranes (second- vs first-generation PES) in post-dilution HDF | both membranes approx. RR: 48% | 48.0% |
| Meert, 2011[97] | membranes (second- vs first-generation PES) in pre-dilution HDF | both membranes approx. RR: 48% | 48.0% |
| Abad, 2016[64] | FX-1000 CorDiax dialyzer in post-dilution HDF | RR: 48.7±14.1% | 47.8% |
| Eloot, 2012[89] | mid-dilution HDF | RR: 47.4±8.2% | 47.4% |
| Esquivias-Motta, 2017[55] | HDF | RR: 45.2 (38.3–59.3)% | 45.2% |
| Cornelis, 2015[70] | post-dilution HDF | approx. RR: 45% | 45.0% |
| Meert, 2009[112] | post-dilution HDF | RR: 44.8±12.1% | 44.8% |
| Lesaffer, 2000[85] | high-flux membrane (F60) in HD | before 1.86±0.70 after 1.04±0.46 mg/100ml, p< 0.05 | 44.1% |
| Chen, 2020[46] | HDF with endogenous infusion | RR: 43.64±13.05%, p= 0.0051 | 43.6% |
| Krieter, 2017[57] | pre-dilution HDF-IPIS | before 13.4±4.5 after 7.6±4.1 mg/l | 43.3% |
| Esquivias-Motta, 2017[55] | high-flux HD | RR: 45.9 (43.2–69.6)% | 43.2% |
| Meijers, 2011[99] | nocturnal 8-hour HD | RR: 43% | 43.0% |
| Lesaffer, 2000[85] | low-flux membrane (F8) in HD | before 1.71±0.56 after 0.99±0.44 mg/100ml, p< 0.05 | 42.1% |
| Paats, 2020[34] | medium Qb (306 ± 62 ml/min) and Qd (793 ± 57 ml/min) in HDF | approx. RR: 42% | 42.0% |
| Lesaffer, 2000[85] | HF-CTA membrane (Nissho Nipro) in HD | before 1.69±1.27 after 0.99±0.82 mg/100ml, p< 0.05 | 41.4% |
| Eloot, 2015[72] | canonical HD | RR: 41±11% | 41.0% |
| Sirich, 2012[92] | low KoA-Qd (F160NR dialyzer) in 8-hour nocturnal HD | RR: 41±11% | 41.0% |
| Madero, 2019[24] | oral binding competitor (IBU) in HD | before (median) 4.4 (3.3) after 2.6 (3.1) mg/dl, change -1 (-2.4 to -0.3) p< 0.001 | 40.9% |
| Meert, 2011[97] | membranes (second- vs first-generation PES) in HD | both membranes approx. RR: 40% | 40.0% |
| Brettschneider, 2013[19] | high-flux HD | before 216±8 after 132±54 µmol/l | 38.9% |
| Yamamoto, 2018[21] | adsorbent (HICB) in HD | RR: 38.4±15.7% | 38.4% |
| Krieter, 2017[57] | pre-dilution HDF | before 12.6±5.0 after 7.9±3.6 mg/l | 37.3% |
| Krieter, 2017[57] | canonical HD | before 13.6±6.5 after 8.6±5.0 mg/l | 36.8% |
| Sirich, 2014[83] | canonical HD | RR: 36±13% | 36.0% |
| Luo, 2009[109] | canonical HD | RR: 36±10% | 36.0% |
| Cornelis, 2015[70] | canonical HD | approx. RR: 35% | 35.0% |
| Yamamoto, 2018[21] | canonical HD | RR: 34.4±14.3% | 34.4% |
| Meert, 2009[112] | pre-dilution HF | RR: 33.8±9.9% | 33.8% |
| De Smet, 2007[33] | SF-CTA (Sureflux-150FH) in HD | RR: 32.5±8.3% | 32.5% |

| | | | |
|---|---|---|---|
| Fagugli, 2002[78] | canonical HD | before 3.35±1.68 after 2.38±1.16mg/dl | 29.0% |
| De Smet, 2007[33] | membranes LF-CTA (Sureflux-150L) in HD | RR: 24.8±6.5% | 24.8% |
| Eloot, 2015[72] | MPHD | RR: 23±11% | 23.0% |
| Paats, 2020[34] | low Qb (200 ± 10 ml/min) and Qd (301 ± 11 ml/min) in HD | approx. RR: 22% | 22.0% |
| Fagugli, 2002[78] | DHD | before 2.85±1.08 after 2.31±0.9mg/dl | 18.9% |
| Etinger, 2018[48] | canonical (37 mM buffer bicarbonate) | before 139.42±55.45 after 113.04±48.84 µmol/l, p< 0.001 | 18.9% |
| Etinger, 2018[48] | isohydric dialysis (25 mM buffer bicarbonate) | before 142.18±52.08 after 116.56±44.89 µmol/l, p< 0.001 | 18.0% |
| Vanholder, 1992[110] | HDF | RR: 13±35% | 13.0% |
| Vanholder, 1992[110] | canonical HD | RR: -45±35%, increase | 0.0% |

*Abbreviations: ns – not significant; HD – hemodialysis; HDF – hemodiafiltration; HF – hemofiltration; HFHD – high-flux hemodialysis; HICB – hexadecyl-immobilized cellulose bead; LFHD – low-flux hemodialysis; DHD – daily short hemodialysis; HFR – hemofiltrate-reinfusion; CTA – cellulose triacetate; MAHD - magnetically-assisted hemodialysis; CAPD – continuous ambulatory peritoneal dialysis; IPIS – increased plasma ionic strength; IBU – ibuprofen; PES – polyethersulfone; KoA - mass transfer-area coefficient; Qd - dialysate flow; and Qb - blood flow.*

### *p-Cresyl sulfate*

*i) Longitudinal trials.* Similarly to indoxyl sulfate, oral supplementation with 6 g per day of AST-120 in HD or PD[74,77] patients resulted in a *p*-cresyl sulfate reduction ratio of 43-44% after 1- to 3-month intervention periods (**Table 5**). In addition, the use of synbiotic NATUREN G® (Farmalabor SRL) in combination with polyvinylpyrrolidone-coated divinylbenzene (DVB-PVP) cartridge in hemodialysis patients[23] also demonstrated significant RR. As noted for indoxyl sulphate, however, the study of NATUREN G® combination with DVB-PVP hemodialysis only lasted for one week with some differences in the *p*-cresyl sulphate baseline plasma levels between the groups.[23]

From techniques that remove PBUTs using convection and diffusion, standard and high-volume post-dilution HDF[59,105] were the most effective approaches in the long-term trials.

*ii) Acute trials.* The average of all reported or calculated RRs in the total set of 20 acute studies was 38.4±11.2%. The most effective techniques were among extracorporeally toxins removal techniques, namely (a) fractionated plasma separation and adsorption (FPSA), otherwise called FPAD,[20] with RR of 71%,[19] (b) high KoA and Qd in 8-hour nocturnal HD,[92] (c) 8-hour post-dilution HDF,[70] and (d) HDF with hemofiltrate-reinfusion (HFR).[55] As in the case of indoxyl sulfate, longitudinal studies were not conducted with any of these strategies for *p*-cresyl sulfate, except for standard nocturnal 7-8-hour long hemodialysis, which did not result in any significant impact on *p*-cresyl plasma concentration change after one year of treatment.[50]

Altogether, the studies that analyzed efficacy of AST-120 adsorbent[74,77] showed the highest reduction ratios for both indoxyl sulfate and *p*-cresyl sulfate in the longitudinal trials, while in the acute trials FPAD system[19] was found to be the most effective technique for the removal of these toxins.

**Table 5. Reduction ratios (RR) of *p*-cresyl sulfate.** RR extracted directly or calculated from the analyzed articles. The colors represent the values of the RR with the highest values in green and lowest in red.

**Longitudinal studies**

| First author, year | Technique | Analysis period | Results | RR |
|---|---|---|---|---|
| Lee, 2014[77] | **oral 6g/day AST-120 in HD and PD** | 3 months | before 27.8±16.8 after 15.5±9.9 mg/l after, p< 0.01 | 44.2% |
| Yamamoto, 2015[74] | **oral 6g/day AST-120 in HD** | 4 weeks | before 2.90 (1.59–4.34) after 1.66 mg/dl (0.96–2.77), p= 0.019 | 42.8% |
| Rocchetti, 2020[23] | **synbiotic NATUREN G® (Farmalabor SRL) in HD with polyvinylpyrrolidone coated divinylbenzene (DVB-PVP) cartridge** | 1 week | before 28.9±9 after 21.7±9 µg/ml, p= 0.02 | 24.9% |
| Meert, 2010[105] | **post-dilution HDF** | 9 weeks | before 3.98±1.51 after 3.17±1.77 mg/dl, -20%, p< 0.05 | 20.4% |
| Panichi, 2017[59] | high-volume post-dilution HDF | 6 months | approx. before 7 after 5.9±3.6 mg/l, p< 0.001 | 15.7% |
| Rocchetti, 2020[23] | synbiotic NATUREN G®(Farmalabor SRL) bicarbonate HD (7 weeks) | 7 weeks | before 33.7±18 after 28.9±9 µg/ml, p= 0.04 | 14.2% |
| Gomolka, 2019[44] | HDF | 8 weeks | before 38.1 ±21.5 after 33.9 ±25.1 mg/l, ns | 11.0% |
| Gomolka, 2019[44] | high-flux HD (Braun HIPS) | 8 weeks | before 42.6 ±21.2 after 38.1 ±21.5 mg/l, ns | 10.6% |
| van Gelder, 2020[31] | low-flux HD | 6 months | RR: 8.8 (28.9 to -29.5)%, ns | 8.8% |
| Gomolka, 2019[44] | low-flux HD (Braun LOPS) | 8 weeks | before 45.4 ±18.6 after 42.6 ±21.2 mg/l, ns | 6.2% |
| Sirich, 2017[61] | canonical HD | 12 months | before 3.4 ±1.5 after 3.2 ±1.4 mg/dl, ns | 5.9% |
| Snauwaert, 2019[41] | post-dilution HDF | 12 months | after 1.75 (1.05; 3.49), change -0.08 (-0.95; 0.68) mg/dl | 4.4% |
| van Gelder, 2020[31] | post-dilution high-flux HDF | 6 months | RR: 2.7 (27.4 to -10.2)%, ns | 2.7% |
| Lenglet, 2019[43] | nicotinamide in HD | 24 weeks | before 39.573±26.706 after 39.400±30.803 ng/ml, ns | 0.4% |
| Kalim, 2018[50] | nocturnal 7-8-hour HD | 52 weeks/ 12 months | final-to-baseline ratio: 1.02 ±0.12, ns | 0.0% |
| Kalim, 2018[50] | canonical HD | 52 weeks/ 12 months | final-to-baseline ratio: 1.14 ±0.66, ns | 0.0% |
| Sirich, 2017[61] | increased frequency (6 per week) | At the end of 12 months | before 3.3 ±1.7 after 3.3 ±1.6 mg/dl, ns | 0.0% |
| Camacho, 2016[66] | high clearance (Qd 800 ml/min, Qb 270 ml/min, F250NR dialyzer) in HD | 2 weeks (2 periods x 7 thrice-weekly sessions) | before 4.1 (3.1. 4.8) after 4.1 (2.5. 4.5) mg/dl | 0.0% |
| Snauwaert, 2019[41] | high-flux HD | 12 months | after 2.18 (1.09; 4.02), change 0.10 (-0.68; 0.82) mg/dl | 0.0% |
| Snauwaert, 2019[41] | low-flux HD | 12 months | after 3.01 (1.90; 4.95), change 0.21 (-0.67; 0.88) mg/dl | 0.0% |
| Rocchetti, 2020[23] | HD with polyvinylpyrrolidone coated divinylbenzene (DVB-PVP) cartridge | 1 week | approx. before 15.5 after 18 µg/ml, ns | 0.0% |

| | | | | |
|---|---|---|---|---|
| Rocchetti, 2020[23] | bicarbonate HD | 7 weeks | approx. before 15.5 after 15.5 µg/ml, ns | 0.0% |
| Lenglet, 2019[43] | sevelamer-hydrochloride in HD | 24 weeks | before 45.551±35.660 after 51.012±41.269 ng/ml, ns | 0.0% |
| Camacho, 2016[66] | low clearance (Qd 300 ml/min, Qb 350 ml/min, F160NR dialyzer) | 2 weeks (2 periods x 7 thrice-weekly sessions) | before 3.2 (2.6- 4.0) after 3.8 (3.5- 4.0) mg/dl | 0.0% |
| Krieter, 2019[36] | low-flux HD | 6 weeks | no significant reduction | 0.0% |
| Krieter, 2019[36] | high-flux HD | 6 weeks | no significant reduction | 0.0% |
| Krieter, 2019[36] | HDF | 6 weeks | no significant reduction | 0.0% |
| Panichi, 2017[59] | bicarbonate HD | 6 months | approx. before 0.46 after 0.74±0.38 mg/dl, ns | 0.0% |

**Acute studies**

| First author, year | Technique | Results | RR |
|---|---|---|---|
| Brettschneider, 2013[19] | **FPAD** | before 341 ±70 after 99 ±32 µmol/l | 71.0% |
| Sirich, 2012[92] | **high KoA-Qd (Rexeed 25S dialyzer) in 8-hour nocturnal HD** | RR: 59 ±8% | 59.0% |
| Cornelis, 2015[70] | **8-hour post-dilution HDF** | RR: 52% | 52.0% |
| Esquivias-Motta, 2017[55] | **HFR** | RR: 50.7 (34.1–68.1)% | 50.7% |
| Meijers, 2008[20] | **FPSA** | RR: 50% | 50.0% |
| Luo, 2009[109] | high KoA and Qd in HD | RR: 48 ±7% | 48.0% |
| Krieter, 2010[103] | membranes (PUREMA H) in post-dilution HDF | RR: 47.8±10.3% | 47.8% |
| Krieter, 2010[103] | membranes (PUREMA H+) in HD | RR: 47.3±14.8% | 47.3% |
| Krieter, 2010[103] | membranes (PUREMA H) in HD | RR: 45.6±2.0% | 45.6% |
| Cornelis, 2015[70] | 8-hour HD | RR: 45% | 45.0% |
| Meert, 2011[97] | membranes (second- vs first-generation PES) in pre-dilution HDF | both membranes approx. RR: 45% | 45.0% |
| Meert, 2011[97] | membranes (second- vs first-generation PES) in post-dilution HDF | both membranes approx. RR: 45% | 45.0% |
| Abad, 2016[64] | FX-1000 CorDiax dialyzer in post-dilution HDF | RR: 44.4 ±15.7% | 44.4% |
| Eloot, 2012[89] | post-dilution HDF | RR: 44.0±8.4% | 44.0% |
| Eloot, 2012[89] | mid-dilution HDF | RR: 42.7±7.2% | 42.7% |
| Esquivias-Motta, 2017[55] | high-flux HD | RR: 42.2 (22.7–50.8)% | 42.2% |
| Meert, 2009[112] | pre-dilution HDF | RR: 41.9±6.3% | 41.9% |
| Sirich, 2012[92] | low KoA-Qd (F160NR dialyzer) in 8-hour nocturnal HD | RR: 41 ±11% | 41.0% |
| Chen, 2020[46] | hemodiafiltration with endogenous infusion (HFR) | RR: 40.91 ±11.31%, p= 0.0051 | 40.9% |
| Krieter, 2010[103] | membranes (PUREMA H+) in post-dilution HDF | RR: 40.4±25.3% | 40.4% |
| Meert, 2009[112] | post-dilution HDF | RR: 40.0±8.8% | 40.4% |
| Esquivias-Motta, 2017[55] | HDF | RR: 39.8 (32.9–57.8)% | 39.8% |
| Krieter, 2017[57] | pre-dilution HDF-IPIS | before 21.4 ±9.1 after 13.2 ±7.1 mg/l | 38.3% |
| Cornelis, 2015[70] | post-dilution HDF | RR: 38% | 38.0% |
| Eloot, 2015[72] | canonical HD | RR: 37±9% | 37.0% |
| Meijers, 2011[99] | nocturnal 8-hour HD | RR: 37% | 37.0% |
| Meert, 2011[97] | membranes (second- vs first-generation PES) in HD | both membranes approx. RR: 35% | 35.0% |
| Brettschneider, 2013[19] | high-flux HD | before 692 ±283 after 467 ±174 µmol/l | 32.5% |
| Krieter, 2017[57] | pre-dilution HDF | before 22.1 ±13.7 after 15.0 ±11.0 mg/l | 32.1% |

| | | | |
|---|---|---|---|
| Krieter, 2017[57] | canonical HD | before 21.0 ±10.0 after 14.3 ±7.7 mg/l | 31.9% |
| Sirich, 2014[83] | canonical HD | RR: 31 ±13% | 31.0% |
| Meert, 2009[112] | pre-dilution HF | RR: 30.6±7.3% | 30.6% |
| Cornelis, 2015[70] | canonical HD | RR: 30% | 30.0% |
| Luo, 2009[109] | canonical HD | RR: 30 ±14% | 30.0% |
| Meijers, 2008[20] | high-flux HD | RR: 30% | 30.0% |
| Yamamoto, 2018[21] | canonical HD | approx. RR: 30% | 30.0% |
| Yamamoto, 2018[21] | adsorbent (HICB) in HD | RR: 30% | 30.0% |
| Madero, 2019[24] | oral binding competitor (IBU) in HD | before (median) 1.4 (1.3) after 1.0 (1.1) mg/dl change -0.3 (-0.8 to -0.01), p< 0.001 | 28.6% |
| Martinez, 2005[54] | membrane (Fresenius F70NR kidneys) in HD | RR: 20 ±9%, p<0.05 | 20.0% |
| Etinger, 2018[48] | canonical HD | 258.18±84.71 after 214.14±54.66 µmol/l, p< 0.001 | 17.1% |
| Eloot, 2015[72] | MPHD | RR: 16±15% | 16.0% |
| Etinger, 2018[48] | isohydric dialysis (25 mM buffer bicarbonate) | before 250.98±66.16 after 217.83±60.34 µmol/l, p< 0.001 | 13.2% |

*Abbreviations: ns – not significant; HD – hemodialysis; HDF – hemodiafiltration; HF – hemofiltration; HFHD – high-flux hemodialysis; HICB – hexadecyl-immobilized cellulose bead; LFHD – low-flux hemodialysis; MAHD - magnetically-assisted hemodialysis; FPSA – fractionated plasma separation and adsorption; FPAD – fractionated plasma separation, adsorption, and dialysis; MPHD – multipass hemodialysis; HFR – hemofiltrate-reinfusion; PD – peritoneal dialysis; CAPD – continuous ambulatory peritoneal dialysis; IPIS – increased plasma ionic strength; IBU – ibuprofen; PES – polyethersulfone; KoA - mass transfer-area coefficient; Qd - dialysate flow; and Qb - blood flow.*

### *Homocysteine*

*i) Longitudinal trials.* In case of homocysteine, there was a total number of 14 longitudinal studies (**Table 6**). The most effective treatments were supplementation of HD patients with intravenous folates (5-methyltetrahydrofolate) alone or in combination with N-acetylcysteines, that act by enhancing homocysteine recycling and by toxin displacement form plasma albumin, respectively, which resulted in 53-56% RR after 10 sessions.[25] Another study which tested intravenous or oral folinic or folic acid supplementations in HD also showed significant results.[28]

*ii) Acute trials.* In the 10 acute studies, the highest reduction ratios were also achieved by supplementation of HD patients with N-acetylcysteine intravenously (RR= 89.1%),[27] as well as with intravenous mesna, a thiol-containing drug analogue of taurine, which also acts by displacing homocysteine from plasma albumin.[26] From the extracorporeal techniques, post-dilution HDF with (a) high-flux polysulphone (PS) Xevonta Hi23 (B. Braun Avitum AG),[79] (b) high-flux polyamide Polyflux 210H (Gambro),[79] or (d) FX-1000 CorDiax (FMC®) dialyzers[64] resulted in the highest outcomes. HDF was also studied in a longitudinal trial that lasted 6 months and resulted in an 18% reduction of homocysteine.[101] Overall RR for the acute studies was 40.5±18.4% on average.

**Table 6. Reduction ratios (RR) of homocysteine.** RR extracted directly or calculated from the analyzed articles. The colors represent the values of the RR with the highest values in green and lowest in red.

| | | Longitudinal studies | | |
|---|---|---|---|---|
| First author, year | Technique | Analysis period | Results | RR |
| Perna, 2012[25] | **intravenous folates (5-methyltetrahydrofolate) and NAC in HD** | 10 sessions | before 51.83±7.10 after 22.74±1.17 µmol/l | 56.1% |
| Perna, 2012[25] | **intravenous folates (5-methyltetrahydrofolate) in HD** | 10 sessions | before 52.63±5.33 after 24.75±1.22 µmol/l | 53.0% |
| Ducloux, 2002[28] | **intravenous folinic acid in HD** | 6 months | RR: 47±7% , p< 0.00001 | 47.0% |
| Ducloux, 2002[28] | **oral folinic acid in HD** | 6 months | RR: 43±6%, p< 0.0001 | 43.0% |
| Ducloux, 2002[28] | **oral folic acid in HD** | 6 months | RR: 42%, p< 0.0001 | 42.0% |
| Galli, 2014[76] | DHD | 6 months | before 29.4±17.0 after 18.4±10.3 µmol/l, p< 0.01 | 37.4% |
| Van Tellingen, 2001[82] | super-flux CTA membrane (Tricea 150G) in HD | 3 months | before 24.4±8.7 ater 15.3±3.7 µmol/l, p< 0.008 | 37.3% |
| Galli, 2003[71] | PLD (BK-F) in HD | 6 moths | RR: 32%, p< 0.01 | 32.0% |
| Van Tellingen, 2001[82] | super-flux PS membrane (F 500S) in HD | 3 months | before 29.6±9.9 after 21.5±8.5 µmol/l, p< 0.007 | 27.4% |
| Ducloux, 1999[88] | CAPD | 6 months | before 31.9±9 after 23.4±7.5 µmol/l, p< 0.0005 | 26.6% |
| Pedrini, 2011[101] | HDF | 6 months | after 15.4±5.0 µmol/l, reduction 18% | 18.0% |
| Beerenhout, 2005[47] | pre-dilution OLHF | 6 months | before 25.4±7.4 after 21.1±7.0 µmol/l, p< 0.05 | 16.9% |
| De Vriese, 2003[69] | super-flux CTA membrane (Sureflux-150F) in HD | 4 weeks | RR: 14.6±2.8%, p< 0.001 | 14.6% |
| Beerenhout, 2005[47] | low-flux HD | 6 months | before 29.7±8.2 after 27.0±9.3 (units NA), ns | 9.1% |
| Perna, 2012[25] | canonical HD | 10 sessions | before 47.96±4.61 after 43.88±4.82 µmol/l | 8.5% |
| Mudge, 2005[56] | high-flux HD (HF80S) | 3 months | before 27.43±7.68 after 25.91±5.78 µmol/l, ns | 5.5% |
| House, 2000[84] | high-flux PS membrane (F80) in HD | 3 months | before 18.5 (15.8-22.3) after 17.5 (16.0-19.5) µmol/l, ns | 5.4% |
| Mudge, 2005[56] | high-flux HD (FX80) | 3 months | before 26.0±4.58 after 25.0±6.61 µmol/l, ns | 3.8% |
| Galli, 2003[71] | non-PLD (HF PMMA/ LF CA/ CURAY) in HD | 6 moths | before 26.6±5.0 after 25.9±7.3 µmol/l | 2.6% |
| Chazot, 2015[68] | low-flux PS membranes (FX8 and FX10) in extended HD | 9 months | before 31.0 (13.0–120.0) after 33.0 (14.0–103.0) µmol/l | 0.0% |
| Chazot, 2015[68] | high-flux PS membranes (FX60 and FX80) in extended HD | 9 months | before 31.0 (13.0–120.0) after 31.0 (12.0–83.0) µmol/l | 0.0% |
| Pedrini, 2011[101] | low-flux HD | 6 months | after 18.7 ±8.2 µmol/l, reduction ns | 0.0% |
| Righetti, 2010[107] | low-flux HD | 2x 6 months | before 18.3±0.9 after 23.8±0,5 µmol/l | 0.0% |
| Righetti, 2010[107] | internal HDF | 2x 6 months | before 18.3±0.9 after 21.5±0.5 µmol/l | 0.0% |
| De Vriese, 2003[69] | low-flux CTA membrane (Sureflux-150L) in HD | 4 weeks | no significant reduction | 0.0% |
| De Vriese, 2003[69] | high-flux CTA membrane (FB-150U) in HD | 4 weeks | no significant reduction | 0.0% |
| Van Tellingen, 2001[82] | high-flux PS membrane (F 60) in HD | 3 months | before 23.1±9.7 after 26.7±14.2 µmol/l, ns | 0.0% |
| Belmouaz, 2019[39] | medium cut-off HD (Theranova 500) | 3 months | before 28 (23-40) after 28.6±13.4 µmol/l | 0.0% |

| | | | | |
|---|---|---|---|---|
| Belmouaz, 2019[39] | high-flux HD (Elision 21H) | 3 months | before 28 (23-40) after 30.7±13.6 µmol/l | 0.0% |
| House, 2000[84] | low-flux PS membrane (F8) in HD | 3 months | before 17.5 (13.0-22.5) after 19.0 (14.0-22.5) µmol/l, ns | 0.0% |

**Acute studies**

| First author, year | Technique | Results | RR |
|---|---|---|---|
| Scholze, 2004[27] | **intravenous acetylcystein in HD** | before 20.1±8.5 after 2.2±1.2 µmol/l | 89.1% |
| Pedrini, 2014[79] | **high-flux PS membrane (Xevonta Hi23) in post-dilution HDF** | RR: 62.8±5.6% | 62.8% |
| Pedrini, 2014[79] | **high-flux polyamide membrane (Polyflux 210H) in post-dilution HDF** | RR: 59.6±5.9% | 59.6% |
| Abad, 2016[64] | **FX-1000 CorDiax dialyzer in post-dilution HDF** | RR: 58.6±8.8% | 58.6% |
| Urquhart, 2006[26] | **intravenous mesna 5mg/kg in HD** | before 24.7±8.5 approx. after 11.06 µmol/l; 55.2%±3.9% decrease | 55.2% |
| Urquhart, 2006[26] | intravenous 2.5 mg/kg mesna in HD | baseline: 24.7±8.5 approx. after 12.84 µmol/l; 48.0±2.2% decrease | 48.0% |
| Badiou, 2011[94] | high-flux HDF | RR: 46.0% | 46.0% |
| Splendiani, 2004[63] | HFR in HDF | before 57.6 µmol/l (24.1–119.7) after 35.3 µmol/l (9.9–80.3) µmol/l, p = 0.005; 43.8% (31.9–58.9%) reduction | 43.8% |
| Badiou, 2011[94] | high-flux HD | RR: 41.5% | 41.5% |
| Biasioli, 1998[93] | CURAY membrane in HD | before 152.45±7.15 (outliers) after 90.55±45.04 µmol/l, ns | 40.6% |
| Scholze, 2004[27] | canonical HD | before 19.8±9.2 after 11.9±7.8 µmol/l | 39.9% |
| Biasioli, 1998[93] | HEMO membrane in HD | before 64.88±40.11 after 40.06±31.57 µmol/l, ns | 38.3% |
| Biasioli, 1998[93] | PS+HEMO membrane in HD | before 37.25±10.79 after 23.15±7.52 µmol/l, ns | 37.9% |
| Urquhart, 2006[26] | canonical HD | before 24.7±8.5 approx. after 16.25 µmol/l, 34.2±5.3% decrease and approx. after 14.96 µmol/l, 39.4±7.7% decrease | 36.8% |
| Tamura, 1996[102] | high-flux PS membrane in HD | before 36.8 after 24.2 µmol/l, p< 0.0001 | 34.2% |
| Biasioli, 1998[93] | PMMA membrane in HD | before 32.33±13.71 after 21.68±9.56 µmol/l, ns | 32.9% |
| Biasioli, 1998[93] | AN69 membrane in HD | before 80.86±42.03 after 55.40±36.78 µmol/l, ns | 31.5% |
| Arnadottir, 2002[75] | high-flux membrane (Polyflux 14S) in HD | before 13.3±3.5 after 9.1±2.4 µmol/l, 31±8% decrease, p< 0.0001 | 31.0% |
| Arnadottir, 2002[75] | low-flux membrane (Polyflux 6L) in HD | before 13.3±3.5 after 9.6±2.7 µmol/l, 28±7% decrease, p< 0.0001 | 28.0% |
| Biasioli, 1998[93] | DIAC membrane in HD | before 50.00±29.31 after 36.68±23.48 µmol/l, ns | 26.6% |
| Czupryniak, 2005[49] | CAPD solution (icodextrin) | before 18.2±9.9 after 16.9±8.2 µmol/l, ns | 7.1% |
| Czupryniak, 2005[49] | CAPD solution (glucose) | before 18.8±9.3 after 18.5±9.6 µmol/l, ns | 1.6% |

*Abbreviations: ns – not significant; HD – hemodialysis; HDF – hemodiafiltration; HF – hemofiltration; HFHD – high-flux hemodialysis; LFHD – low-flux hemodialysis; CAPD – continuous ambulatory peritoneal dialysis; NAC - N-acetylcysteine; PES – polyethersulfone; PLD – protein-leaking dialyzer; PS – polysulphone; PMMA – polymethylmethacrylate; DIAC – cellulose diacetate; HEMO – hemophan; and CURAY – cuprammonium rayon.*

## *Hippuric acid*

*i) Longitudinal trials.* Reduction of hippuric acid plasma levels was studied in six longitudinal trials (**Table 7**). Low-flux HD demonstrated the highest RR value (32.3%) in children and young adults in a recent one-year trial.[41] However, the study did not specify the significance of this change. In addition, increasing frequency of the HD procedures to six times per week resulted in a 26.2% reduction at the end of a one-year intervention.[61]

*ii) Acute trials.* In the acute studies, techniques with the highest RR values were (a) 8-hour nocturnal HDF and HD regardless of the KoA or Qd resulting in RR above 80%,[70,92] and (b) high KoA and Qd in standard HD.[109] As with indoxyl sulfate and *p*-cresyl sulfate, nocturnal HD was studied in one longitudinal trial without significant plasma hippuric acid levels changes after a treatment period of one year.[50] The average of all reported or calculated RR from the acute trials was 68.1±10.4%.

**Table 7. Reduction ratios (RR) of hippuric acid.** RR extracted directly or calculated from the analyzed articles. The colors represent the values of the RR with the highest values in green and lowest in red.

| Longitudinal studies | | | | |
|---|---|---|---|---|
| **First author, year** | **Technique** | **Analysis period** | **Results** | **RR** |
| Snauwaert, 2019[41] | **low-flux HD** | 12 months | after 2.47 (1.10- 5.85), change -1.18 (-2.47- 0.14) mg/dl | 32.3% |
| Sirich, 2017[61] | **increased frequency (6 per week) HD** | at the end of 12 months | before 6.5±5.4 after 4.8±3.3 mg/dl | 26.2% |
| van Gelder, 2020[31] | **post-dilution high-flux HDF** | 6 months | RR: 21.9 (47.6 to -42.4)%, ns | 21.9% |
| Lenglet, 2019[43] | **nicotinamide in HD** | 24 weeks | before 57.911±48.018 after 45.824±34.870 ng/ml, ns | 20.9% |
| Snauwaert, 2019[41] | **high-flux HD** | 12 months | after 2.80 (1.41; 5.85), change -0.25 (-1.97; 0.90) mg/dl | 9.9% |
| Meert, 2010[105] | post-dilution HDF | 9 weeks | before 4.07±2.42 after 3.81±2.74 mg/dl, ns | 6.4% |
| Kalim, 2018[50] | nocturnal 7-8-hour HD | 12 months | final-to-baseline ratio: 1.63±1.97, ns | 0.0% |
| Kalim, 2018[50] | canonical HD | 12 months | final-to-baseline ratio: 2.52±6.38, ns | 0.0% |
| Sirich, 2017[61] | canonical HD | 12 months | before 5.1±4.2 after 5.7±4.0 mg/dl | 0.0% |
| van Gelder, 2020[31] | low-flux HD | 6 months | RR: -5.7 (44.6 to -54.5)%, increase, ns | 0.0% |
| Snauwaert, 2019[41] | post-dilution HDF | 12 months | after 2.28 (1.37; 3.28), change 0.34 (-0.44; 1.79) mg/dl | 0.0% |
| Lenglet, 2019[43] | sevelamer-hydrochloride in HD | 24 weeks | before 68.240 (36.256–88.416) after 70.083±51.675 ng/ml, ns | 0.0% |

| Acute studies | | | |
|---|---|---|---|
| **First author, year** | **Technique** | **Results** | **RR** |
| Sirich, 2012[92] | **high KoA-Qd (Rexeed 25S dialyzer) in 8-hour nocturnal HD** | RR: 89±7% | 89.0% |
| Cornelis, 2015[70] | **8-hour post-dilution HDF** | approx. RR: 88% | 88.0% |
| Cornelis, 2015[70] | **8-hour HD** | approx. RR: 85% | 85.0% |
| Sirich, 2012[92] | **low KoA-Qd (F160NR dialyzer) in 8-hour nocturnal HD** | RR: 81±7% | 81.0% |
| Luo, 2009[109] | **high KoA and Qd in HD** | before 7.4±3.3 after 1.5±0.6 mg/dl, RR: 79±4% | 79.0% |

| | | | |
|---|---|---|---|
| Lesaffer, 2000[85] | high-flux CTA membrane (Nissho Nipro) in HD | before 1.84±1.15 after 0.44±0.25 mg/100ml, p< 0.05 | 76.1% |
| Meert, 2009[112] | pre-dilution HDF | RR: 74.3±10.5% | 74.3% |
| Meert, 2009[112] | post-dilution HDF | RR: 73.6±9.5% | 73.6% |
| Lesaffer, 2000[85] | high-flux PS membrane (F60) in HD | before 1.82±1.06 after 0.50±0.30 mg/100ml, p< 0.01 | 72.5% |
| Lesaffer, 2000[85] | low-flux PS membrane (F8) in HD | before 1.71±0.53 after 0.47±0.26 mg/100ml, p< 0.05 | 72.5% |
| Cornelis, 2015[70] | post-dilution HDF | approx. RR: 72% | 72.0% |
| Vanholder, 1992[110] | canonical HD | change: 72±7% | 72.0% |
| Sirich, 2014[83] | canonical HD | RR: 71±5% | 71.0% |
| Eloot, 2012[89] | mid-dilution HDF | RR: 70.6±13.5% | 70.6% |
| Fagugli, 2002[78] | canonical HD | before 6.96±2.83 after 2.06±0.82 mg/dl | 70.4% |
| Eloot, 2012[89] | post-dilution HDF | RR: 70.4±13.5% | 70.4% |
| De Smet, 2007[33] | super-flux CTA membrane (Sureflux-150FH) in HD | RR: 69.7±7.1% | 69.7% |
| De Smet, 2007[33] | low-flux CTA membrane (Sureflux-150L) in HD | RR: 69.0±6.8% | 69.0% |
| Eloot, 2015[72] | canonical HD | RR: 68±10 % | 68.0% |
| Cornelis, 2015[70] | canonical HD | approx. RR: 68% | 68.0% |
| Luo, 2009[109] | canonical HD | RR: 67±8% | 67.0% |
| Vanholder, 1988[113] | PS400 membrane in HD | RR: 0.67±0.10 | 67.0% |
| Vanholder, 1992[110] | HD | before 242±154 after 97±55 µmol/l, change: 64±11% | 64.0% |
| Meert, 2009[112] | pre-dilution HF | RR: 61.1±11.7% | 61.1% |
| Vanholder, 1992[110] | HDF | change: 61±8% | 61.0% |
| Meert, 2011[97] | second- vs first-generation PES membranes in pre-dilution HDF | both membranes approx. RR: 60% | 60.0% |
| Meert, 2011[97] | second- vs first-generation PES membranes in post-dilution HDF | both membranes approx. RR: 60% | 60.0% |
| Meert, 2011[97] | second- vs first-generation PES membranes in HD | both membranes approx. RR: 60% | 60.0% |
| Etinger, 2018[48] | isohydric dialysis (25 mM buffer bicarbonate) | before 280.65±168.45 after 121.85±80.43 µmol/l, p< 0.001 | 56.6% |
| Etinger, 2018[48] | canonical HD | before 212.42±154.92 after 96.67±81.40 µmol/l, p< 0.001 | 54.5% |
| Vanholder, 1988[113] | cuprophan membrane in HD | RR: 0.53±0.09% | 53.0% |
| Fagugli, 2002[78] | DHD | before: 7.12±2.21 after 3.62±1.15 mg/dl | 49.2% |
| Eloot, 2015[72] | MPHD | RR: 43±16% | 43.0% |

*Abbreviations: ns – not significant; HD – hemodialysis; HDF – hemodiafiltration; HF – hemofiltration; MPHD – multipass hemodialysis; DHD – daily short hemodialysis; CAPD – continuous ambulatory peritoneal dialysis; PES – polyethersulfone; PS – polysulphone; HEMO – hemophan; CTA – cellulose triacetate; KoA - mass transfer-area coefficient; Qd - dialysate flow; and Qb - blood flow.*

*Leptin*

*i) Longitudinal trials.* From the longitudinal studies that provided data on change in leptin plasma concentrations (**Table 8**), the highest RR were achieved with substitution of intravenous insulin injection with intraperitoneal in continuous ambulatory peritoneal dialysis (CAPD) for patients with diabetes, which reduced the effects of insulin on increase of leptin production and resulted in 35.4% RR after 3 months of study.[29] From extracorporeal interventions, (a) post-dilution high-flux HDF,[37](b) combination of HD with hemoperfusion,[22] (c) low-flux HD (not significant),[47] and (d) use of super-flux PS membrane (F 500S, Fresenius) in HD[67] showed the highest RR.

*ii) Acute trials.* In the acute studies, the highest RR values were achieved with dialysis techniques (a) mid-dilution HDF (RR= 40 - 43%),[58,118] (b) high-flux HD,[58] (c) high-flux HDF,[95] and (d) use of a high-cut-off membrane in HDF.[86] The average of the reported or calculated RR was 22.4±15.7%. In a longitudinal trial, use of high-flux HD with a different dialyzer than in the acute study (F 60S, Fresenius, and BLS 819, Bellco, respectively) did not result in significant changes in leptin plasma levels after 12 weeks of treatment.[67]

**Table 8. Reduction ratios (RR) of leptin.** RR extracted directly or calculated from the analyzed articles. The colors represent the values of the RR with the highest values in green and lowest in red.

| First author, year | Technique | Longitudinal studies Analysis period | Results | RR |
|---|---|---|---|---|
| Nevalainen, 2000[29] | **intraperitoneal insulin injections (instead of subcutaneous) in CAPD** | 3 months | before 19.8±5.9 after 12.8±6.2 ng/ml, p< 0.001 | 35.4% |
| Mandolfo, 2006[37] | **post-dilution high-flux HDF** | 1 week | RR: 31.4±14.3% | 31.4% |
| Chen, 2011[22] | **HD+HP combination** | 2 years | RR: 31.34% | 31.3% |
| Beerenhout, 2005[47] | **low-flux HD** | 6 months | before 24.9±30.0 after 17.3±24.8 µg/l, ns | 30.5% |
| van Tellingen, 2004[67] | **super-flux PS membrane (F 500S) in HD** | 12 weeks | before 38.3±33.0 after 29.5±31.9 ng/ml, p= 0.02 | 23.0% |
| Mandolfo, 2006[37] | medium-flux HD | 1 week | RR: 12.1±6.4% | 12.1% |
| van Tellingen, 2004[67] | high-flux PS membrane (F 60S) in HD | 12 weeks | before 36.0±31.8 after 33.0± 31.2 ng/ml, ns | 8.3% |
| Panichi, 2006[38] | HDF | 4 months | before 8.6±2.3 after 8.14±2.5 ng/ml, ns | 5.3% |
| Chen, 2011[22] | canonical HD | 2 years | increase 10.04% | 0.0% |
| Beerenhout, 2005[47] | pre-dilution OLHF | 6 months | before 24.4±23.4 after 25.7±28.7 µg/l, ns | 0.0% |
| Kim, 1999[91] | CAPD | 3 months | before 12.85±15.18 after 33.37±42.45 ng/ml | 0.0% |
| Mandolfo, 2006[37] | low-flux HD | 1 week | no reduction | 0.0% |
| Panichi, 2006[38] | HFR | 4 months | before 9.17±2.1 after 9.71±3.1 ng/ml, ns | 0.0% |
| van Tellingen, 2004[67] | low-flux PS membrane (F 6HPS) in HD | 12 weeks | before 30.4±23.0 after 40.5±25.4 ng/ml, p= 0.05 | 0.0% |

| | | | | |
|---|---|---|---|---|
| Belmouaz, 2019[39] | medium cut-off HD (Theranova 500) | 3 months | before 27.9 (10-143) after 29.6 (16–116) ng/ml | 0.0% |
| Belmouaz, 2019[39] | high-flux HD (Elision 21H) | 3 months | before 27.9 (10-143) after 39.7 (18–135) ng/ml | 0.0% |
| Gomolka, 2019[44] | low-flux HD (Braun LOPS) | 8 weeks | before 35.52±38.0 after 36.2±38.9 ng/m, ns | 0.0% |
| Gomolka, 2019[44] | high-flux HD (Braun HIPS) | 8 weeks | before 36.2±38.9 after 36.4±42.2 ng/ml, ns | 0.0% |
| Gomolka, 2019[44] | HDF | 8 weeks | before 36.4±42.2 after 38.7±42.0 ng/ml, ns | 0.0% |
| van Tellingen, 2004[67] | super-flux CTA membrane (Tricea 150G) in HD | 12 weeks | before 29.4±23.7 after 32.0±27.9 ng/ml, ns | 0.0% |

**Acute studies**

| First author, year | Technique | Results | RR |
|---|---|---|---|
| Mandolfo, 2008[118] | **mid-dilution HDF** | RR: 43±16% | 43.0% |
| Santoro, 2005[58] | **mid-dilution HDF** | approx. RR: 40% | 40.0% |
| Santoro, 2005[58] | **high-flux HD** | approx. RR: 40% | 40.0% |
| Wiesholzer, 1998[95] | **high-flux HDF** | before 63.10±47.33 after 39.72±35.41 ng/ml, p= 0.017/ p= 0.001 (after correction) | 37.1% |
| Kneis, 2013[86] | **high-cut-off membrane in HDF** | RR: 37%, p< 0.01 | 37.0% |
| Mandolfo, 2008[118] | high-flux HD | approx. RR: 35% | 35.0% |
| Nakazono, 1998[96] | PS-N dialyzer (Fresenius) in HD | RR: 33.4±21.2% | 33.4% |
| Kneis, 2013[86] | high-cut-off membrane in HD | RR: 31% ml, p< 0.01 | 31.0% |
| Ouseph, 2008[32] | PS membrane (F70NR) in HD | RR: 26±4% | 26.0% |
| Ouseph, 2008[32] | PAES membrane (Polyflux 17S) in HD | RR: 23±11% | 23.0% |
| Kneis, 2013[86] | conventional HD | RR: 19%, p< 0.01 | 19.0% |
| Kneis, 2013[86] | Polyflux 11 S dialyzer in HDF | RR: 16%, p< 0.05 | 16.0% |
| Nakazono, 1998[96] | PS-UW dialyzer (Fresenius) in HD | RR: 16.0±26.4% | 16.0% |
| Wiesholzer, 1998[95] | high-flux HD | before 48.21±51.08 after 45.26±53.67 ng/ml, p= 0.013 (after correction) | 6.1% |
| Opatrná, 2003[73] | CAPD solution (icodextrin) | no change in plasma levels | 0.0% |
| Opatrná, 2003[73] | CAPD solution (glucose) | no change in plasma levels | 0.0% |
| Wiesholzer, 1998[95] | low-flux HD | before 11.94±16.92 after 14.74±20.76 ng/ml; | 0.0% |
| Nakazono, 1998[96] | AM-SD dialyzer (Asahi) in HD | RR: –1.0±24.2%, increase | 0.0% |

*Abbreviations: ns – not significant; HD – hemodialysis; HDF – hemodiafiltration; HF – hemofiltration; OLHF – online hemofiltration; HFR – hemofiltrate-reinfusion; HP – hemoperfusion; CAPD – continuous ambulatory peritoneal dialysis; PES – polyethersulfone; PS – polysulphone; CTA – cellulose triacetate; PAES – polyarylethersulfone; and HCO – high-cut-off.*

### *Indole-3-acetic acid*

*i) Longitudinal trials.* In case of indole-3-acetic acid (IAA), the highest reported RR, 11.1%, was achieved for children and young adults undergoing post-dilution HDF for a period of 12 months (**Table 9**). However, the significance of this reduction in relation to the baseline

concentration of the toxin was not reported in the paper. The other three techniques studied in the longitudinal trials did not result in any significant reduction ratios.

*ii) Acute trials.* Additionally, removal of IAA was studied in 11 acute trials, of which **8**-hour post-dilution HDF and HD procedures showed the highest RRs in a single session (62.0 and 55.0%, respectively).[70] Similar RRs (49.5 to 50.4%), were also reported for standard hours pre- and post-dilution HDF.[70,89,112] The average of reported or calculated RR for the acute studies was 42.3±8.8%.

**Table 9. Reduction ratios (RR) of indole-3-acetic acid (IAA).** RR extracted directly or calculated from the analyzed articles. The colors represent the values of the RR with the highest values in green and lowest in red.

| Longitudinal studies | | | | |
|---|---|---|---|---|
| **First author, year** | **Technique** | **Analysis period** | **Results** | **RR** |
| Snauwaert, 2019[41] | **post-dilution HDF** | 12 months | after 0.16 (0.11; 0.26), change -0.02 (-0.05; 0.04) mg/dl | 11.1% |
| van Gelder, 2020[31] | **post-dilution high-flux HDF** | 6 months | RR: 10.8 (26.0 -14.0)%, ns | 10.8% |
| Lenglet, 2019[43] | **sevelamer-hydrochloride in HD** | 24 weeks | before 1624 ± 1177 after 1492 ± 933 ng/ml, ns | 8.1% |
| Lenglet, 2019[43] | **nicotinamide in HD** | 24 weeks | before 1287 ± 910 after 1186 ± 733 ng/ml, ns | 7.8% |
| Meert, 2010[105] | post-dilution HDF | 9 weeks | before 0.23 ± 0.17 after 0.21 ± 0.13 mg/dl, ns | 0.0% |
| van Gelder, 2020[31] | low-flux HD | 6 months | RR: -9.2 (19.6 -34.9)%, increase, ns | 0.0% |
| Snauwaert, 2019[41] | high-flux HD | 12 months | after 0.19 (0.13; 0.24), change -0.00 (-0.12; 0.04) mg/dl | 0.0% |
| Snauwaert, 2019[41] | low-flux HD | 12 months | after 0.28 (0.16; 0.49), change 0.02 (-0.07; 0.12) mg/dl | 0.0% |

| Acute studies | | | |
|---|---|---|---|
| **First author, year** | **Technique** | **Results** | **RR** |
| Cornelis, 2015[70] | **8-hour post-dilution HDF** | approx. RR: 62% | 62.0% |
| Cornelis, 2015[70] | **8-hour HD** | approx. RR: 55% | 55.0% |
| Meert, 2009[112] | **pre-dilution HDF** | RR: 50.4 ± 11.6% | 50.4% |
| Cornelis, 2015[70] | **post-dilution HDF** | approx. RR: 50% | 50.0% |
| Eloot, 2012[89] | **post-dilution HDF** | RR: 49.5 ± 7.4% | 49.5% |
| Eloot, 2012[89] | mid-dilution HDF | RR: 49.4 ± 6.4% | 49.4% |
| Meert, 2009[112] | post-dilution HDF | RR: 48.0 ± 9.6% | 48.0% |
| Paats, 2020[34] | high Qb (378 ± 30 ml/min) and Qd (793 ± 47 ml/min) in HDF | approx. RR: 48% | 48.0% |
| De Smet, 2007[33] | super-flux CTA (Sureflux-150FH) | RR: 46.8 ± 13.5% | 46.8% |
| Eloot, 2015[72] | canonical HD | RR: 46 ± 8% | 46.0% |
| Paats, 2020[34] | medium Qb (306 ± 62 ml/min) and Qd (793 ± 57 ml/min) in HDF | approx. RR: 45% | 45.0% |
| De Smet, 2007[33] | membranes low-flux CTA (Sureflux-150L) | RR: 42.3 ± 24.1% | 42.3% |
| Meert, 2009[112] | pre-dilution HF | RR: 41.6 ± 8.1% | 41.6% |
| Cornelis, 2015[70] | canonical HD | approx. RR: 40% | 40.0% |
| Meert, 2011[97] | membranes (second- vs first-generation PES) in pre-dilution HDF | both membranes approx. RR: 40% | 40.0% |

| | | | |
|---|---|---|---|
| Meert, 2011[97] | membranes (second- vs first-generation PES) in post-dilution HDF | both membranes approx. RR: 40% | 40.0% |
| Meert, 2011[97] | membranes (second- vs first-generation PES) in HD | both membranes approx. RR: 40% | 40.0% |
| Yamamoto, 2018[21] | adsorbent (HICB) in HD | RR: 40% | 40.0% |
| Yamamoto, 2018[21] | canonical HD | RR: 40% | 40.0% |
| Etinger, 2018[48] | canonical (37 mM buffer bicarbonate) | before 7.76 ± 6.37 after 5.17 ± 4.00 µml/l, p< 0.001 | 38.3% |
| Eloot, 2015[72] | MPHD | RR: 32 ± 8% | 32.0% |
| Fagugli, 2002[78] | canonical HD | before 0.16 ± 0.04 after 0.11 ± 0.03 mg/dl | 31.3% |
| Paats, 2020[34] | low Qb (200 ± 10 ml/min) and Qd (301 ± 11 ml/min) in HD | approx. RR: 30% | 30.0% |
| Etinger, 2018[48] | isohydric dialysis (25 mM buffer bicarbonate) | before 7.94 ± 5.23 after 5.62 ± 3.62 µmol/l, p< 0.001 | 29.2% |
| Fagugli, 2002[78] | DHD | before 0.13 ± 0.03 after 0.10 ± 0.03 mg/dl | 23.1% |

*Abbreviations: ns – not significant; HD – hemodialysis; HDF – hemodiafiltration; HF – hemofiltration; HICB – hexadecyl-immobilized cellulose bead; DHD – daily short hemodialysis; CAPD – continuous ambulatory peritoneal dialysis; CTA – cellulose triacetate; and PES – polyethersulfone.*

### *Other PBUTs*

Besides the aforementioned uremic retention solutes, reduction of retinol-binding protein (RBP), pentosidine, CMPF, *p*-cresol, *p*-cresylglucuronide (PCG), N-carboxymethyllysine (CML), glyoxal, methylglyoxal, 3-deoxyglucosone (3-DG), kynurenic acid, kynurenine, and indican, as well as melatonin, phenylacetic acid, and thiocyanate was also described in the literature (Supplementary tables). The highest RRs achieved for the toxins that were studied in at least three papers included in this review, are presented in **Table 10.**

**Table 10. Reduction ratios from the longitudinal and acute studies for *p*-cresylglucuronide (PCG), *p*-cresol, retinol-binding protein (RBP), total pentosidine, kynurenic acid or kynurenine (KA/ KY), and CMPF.**

| Longitudinal studies | | | | | |
|---|---|---|---|---|---|
| **First author, year** | **PBUT** | **Technique** | **Analysis period** | **Results** | **RR** |
| Galli, 2005[51] | total pentosidine | protein-leaking dialyzer (BF-K) in HD | 6 months | before 23.5 ± 9.3 after 15.4 ± 5.2 pmol/mg prot, p< 0.01 | 34.5% |
| Röckel, 1982[119] | RBP | high-flux HF | 6 months | before 21.8 ± 1.1 after 18.3 ± 2.1 mg/dl, ns | 16.1% |
| Meert, 2010[105] | CMPF | post-dilution HDF | 9 weeks | before 0.72 ± 0.52 after 0.64 ± 0.46 mg/dL, -11%, p < 0.01 | 11.1% |
| van Gelder, 2020[31] | KA/ KY | low-flux HD | 6 months | KY RR: 7.7 (22.6 to -14.5)%, ns; KA RR: increase -5.6 (8.6 to -69.1)%, ns | 7.7% |
| van Gelder, 2020[31] | PCG | low-flux HD | 6 months | RR: 7.0 (38.1 to -69.8)%, ns | 7.0% |

| Acute studies | | | | |
|---|---|---|---|---|
| **First author, year** | **PBUT** | **Technique** | **Results** | **RR** |
| Cornelis, 2015[70] | PCG | 8-hour HD | approx. RR: 85% | 85.0% |
| Luo, 2009[109] | KA/ KY | high KoA and Qd | RR: 58 ± 7% | 58.0% |
| Susantitaphong, 2009[114] | *p*-cresol | mid-dilution in HDF | RR: 52.2 ± 20.5% | 52.2% |
| Sequera, 2013[87] | RBP | FXCorDiax1000 dialyzer in mixed-dilution HDF | RR: 43.2% | 43.2% |
| Odetti, 1995[106] | total pentosidine | Hemofiltration | before 23.94 ± 5.31 after 21.36 ± 3.74 pmol/mg protein, ns | 10.8% (ns) |
| Cornelis, 2015[70] | CMPF | 8-hour HD | approx. RR: 10%, ns | 10.0% (ns) |

*HD – hemodialysis, ns – not significant; KoA - mass transfer-area coefficient; and Qd - dialysate flow.*

## Discussion

Adequate reduction of PBUTs plasma levels is of paramount importance for better cardiovascular outcomes for dialysis patients due to the high toxicity of these substances.[10,120–123] In particular, in clinical trials levels of indoxyl sulfate showed an association with left ventricular dysfunction,[124] coronary atherosclerosis,[125] cardiovascular and heart failure events[126,127], as well as all-cause mortality.[128] *p*-Cresyl sulfate levels, in turn, also demonstrated an association with increased risks for cardiovascular events[129] as well as cardiovascular and all-cause mortality in CKD patients.[14–16] Detrimental effects on vasculature and associations with cardiovascular complications in CKD patients were also demonstrated for other PBUTs such as homocysteine,[130,131] hippuric acid,[132,133] IAA,[132,134] and *p*-cresol.[135,136] However, the benefit of homocysteine lowering on cardiovascular outcomes is debatable due to the lack of strong evidence from randomised control trials.[137] Nevertheless, all together, the evidence emphasises the importance of the protein-bound uremic toxins reduction for improving the prognosis of renal disease patients.

Currently, a variety of methods for reducing uremic toxins have been described in the literature; however, it is still unclear which of these methods are most effective in reducing PBUT in the plasma of CKD patients. Therefore, the aim of this systematic review was to find the techniques with the highest outcomes described in the literature. Thus, we screened literature records up to 2.01.2020 for the effectiveness of protein-bound uremic toxin plasma levels lowering methods with the aim to identify the most appropriate technique available for CKD patients to date. To warrant the broadest possible scope of our analysis, we did not implement any limits on the number of patients studied or design of the studies, thus scoring methods for quality assessment were not applied in this review.

We stratified the techniques extracted from the studies by the uremic retention solute being investigated and duration of the studies. We applied a uniform result metrics to facilitate easier comparison and ranked studies from the highest to the lowest reduction ratios achieved for each PBUT. We are aware that some authors advise against the use of reduction ratio for estimation of the total PBUT plasma levels decrease.[99] Nevertheless, we opted for this approach as a measure of the change in dialysis associated PBUT concentration in the patients' plasma due to its prevalence in the literature, straight-forwardness and, most importantly, high clinical relevance. Altogether, a comprehensive overview of the efficacy of kidney replacement therapies and relevant supplements for the reduction of protein-bound uremic solutes in plasma reported in the literature is presented.

PBUTs showed the highest reduction ratios with a variety of treatments; at present there is no predominant technique providing optimal lowering of all types of protein-bound retention solutes. The explanation for this might be the lack of comprehensive clinical studies that analyze reduction of all known protein-bound solutes by a given techniques, as well as the heterogeneous nature of the solutes and differences in their protein-binding forces. Promising technologies that demonstrated significant RRs in the acute studies, such as the FPAD

system,[19] increased mass transfer area coefficient and dialysate flow in nocturnal HD[92] and 8-hour HDF,[70] are still not yet being investigated for long-term effects. Canonical HD, on the other hand, failed to reach any significant reduction in most long-term studies while demonstrating a great variety of reduction ratios, up to 72.0% for hippuric acid[110], in single session analyses. Interestingly, reduction ratios reported in the acute studies were, for most PBTUs, much higher than those in longitudinal trials. These findings indicate that the results from single session measurements are of limited value and might not always reflect long-term treatment efficacy.

The highest reduction ratios were achieved in long-term trials for PBUTs, like indoxyl sulfate, *p*-cresyl sulfate and homocysteine, with oral or intravenous supplements as an addition to hemodialysis treatment. In particular, AST-120 (Kremezin®), an oral charcoal adsorbent, significantly reduced serum levels of IS and *p*-cresyl sulfate by adsorbing their bacteria-derived precursors in the intestine.[18,40,74,77] Multiple prospective and retrospective clinical trials showed beneficial effects of long-term AST-120 treatment towards slowing down the progression of CKD.[123] It was also shown that there are no significant changes in adsorption of the fat-soluble vitamins, such as vitamin D and K, in patients after AST-120 administration for 12 weeks.[40] On the other hand, AST-120 treatment did not result in any significant positive effects for pre-dialysis patients in the prospective randomized controlled trials EPPIC1-2 and K-STAR.[138,139] Even though a *post-hoc* re-evaluation of these studies revealed that AST-120 might delay kidney function decline and decrease the risk for cardiovascular events,[140,141] these results should be interpreted with cautious due to statistical limitations of the *post-hoc* analysis.[142] Additionally, it is not known whether AST-120 has an effect on plasma levels of other PBUTs with a different origin in CKD patients.

We acknowledge certain limitations of our study. First, we did not implement any statistical tools for comparison of the efficacy of different kidney replacement therapies. Comparisons were solely based on the reported reduction ratios or plasma concentrations of the PBUTs, without correction for sample size, strength, or duration of the study. With this approach, we achieved the maximum inclusiveness, which is particularly important given the low number of homogeneous studies on this topic. However, only half of the longitudinal studies,[22,25,28,40,61,77] and few acute studies[31,41,43,95] reporting high RR values, were based on sample sizes of more than 40 patients. Thus, the power of the included studies is different. Second, some of the highest RRs for different toxins were achieved within the same studies,[19,23,31,41,43,70,74,77,92] which bears a risk of bias for the analysis. Nevertheless, these preliminary results are encouraging and do demonstrate promising approaches for higher PBUT reduction rates, as well as highlight the need for further investigation into the pertinent methods in larger cohorts, for longer periods of time, and, ideally, for clinically relevant endpoints.

The ineffective lowering of PBUT levels by conventional dialysis techniques (e.g. low-flux hemodialysis, hemodiafiltration, or peritoneal dialysis) may be related to the fact that only the water-soluble fraction of these toxins is able to pass through the dialysis membranes by convection and/or diffusion.[143] Substances, such as indoxyl sulfate and *p*-cresyl sulfate bind

with high affinity to plasma albumin, for instance, through two binding sites[144] forming a high molecular weight complex that exceeds the cut-off limit of the dialyzer membranes. Thus, one possible approach could be increasing the protein-leaking property of the dialyzers. Of note, several studies on protein-leaking dialyzers conducted in the early 2000s demonstrated only a moderate reduction ratio of 32.0% for homocysteine (in acute study) and 15.0-34.5% (acute and 6-month-trial, respectively) for the advanced glycation end product pentosidine.[51,65,71] However, the use of protein-leaking dialyzers for HD patients raised concerns regarding the possible risks of malnutrition and disturbances in protein and amino acid metabolism.[145] In addition, most PBUTs are hydrophobic molecules,[19] which might partly explain their hampered removal by aqueous solutions used in dialysis.

*How can we achieve more effective elimination of PBUTs in the future?* From the early development of dialysis until the present, PBUT elimination was solely based on passive diffusion and filtration, using various types of membranes. The other physical elimination measure, namely adsorption, has not attracted major interest of nephrologists and dialysis companies so far. For instance, vitamin E-coated membranes[146] which combine filtration and adsorption, have only scarcely been used. Albumin dialysis is also based on the principle of adsorption, however this technique is mainly used in liver failure and is not practiced in CKD.[147] Nevertheless, several promising novel adsorption technologies such as zirconium-based metal−organic frameworks,[148] mixed-matrix membrane,[149] and nanoporous adsorbent monolith[150] have emerged showing almost complete removal of IS and *p*-cresyl sulfate from human plasma in *ex vivo* experiments.

Another promising recent strategy, which demonstrated the highest acute RR in our investigation, aims to increase the free fraction of hydrophobic uremic toxins in relation to the protein-bound fraction. In this context, it has already been shown that separation rates of hydrophobic toxins can be improved by increasing the ionic strength of an adsorber (FPAD system).[19] This approach was proven in a limited clinical study demonstrating principle technical and clinical feasibility. The application of high-frequency fields for the increased release of hydrophobic uremic toxins has also been tested and patented (patent numbers: WO2013004604A1, EP2729198A1, US20140246367A1), although a clinical study on this approach is pending. Hopefully, these new approaches, as well as further developments in elimination techniques may allow a better control of cardiovascular complications in end-stage renal disease, the most prominent cause of death in chronic kidney disease patients.[151]

In conclusion, to date, although undoubtedly needed, there is no highly effective method for effective reduction of all types of PBUTs for routine clinical use. Taking the limitations of our study into account, only a very preliminary ranking of the available techniques is possible at present. With this in mind, it can be summarized that supplements, such as AST-120 and intravenous folates, significantly improve the performance of canonical dialysis techniques for the lowering of certain PBUTs plasma levels in the long term. Therefore, there is an urgent need for comprehensive research in this area, considering that protein-bound uremic toxins

are significant contributors to the burden of chronic kidney failure. Long-term, multicenter, cross-over designed studies are essential in this context.

## Authors' contribution

V.S.K. and J.J. designed the study; V.S.K., J.J. and S.O.A. performed analysis of the publications against inclusion/ exclusion criteria; V.S.K., J.J. and W.Z. drafted the manuscript; D.F., V.J. and E.B. revised the paper; all authors approved the final version of the manuscript.

## Acknowledgements

This work was supported by the German Research Foundation (DFG) SFB/TRR219 (S-03, C-04, C-08) and the Interreg V-A EMR program (EURLIPIDS, EMR23). Also, this project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 764474 (CaReSyAn).

## Disclosures

J.J. has research cooperation with Fresenius Medical Care and B Braun.

# References


1. Hill, N. R. *et al.* Global prevalence of chronic kidney disease - A systematic review and meta-analysis. *PLoS One* **11**, (2016).
2. Webster, A. C., Nagler, E. V, Morton, R. L. & Masson, P. Seminar Chronic kidney disease. *www.thelancet.com* **389**, (2017).
3. Gansevoort, R. T. *et al.* Chronic kidney disease and cardiovascular risk: Epidemiology, mechanisms, and prevention. *The Lancet* **382**, 339–352 (2013).
4. Duranton, F. *et al.* Normal and Pathologic Concentrations of Uremic Toxins. **23**, 1258–1270 (2012).
5. Vanholder, R. *et al.* Review on uremic toxins: Classification, concentration, and interindividual variability. *Kidney Int* **63**, 1934–1943 (2003).
6. Jourde-Chiche, N. *et al.* Protein-bound toxins--update 2009. *Semin Dial* **22**, 334–339 (2009).
7. Massry, S. G. Is Parathyroid Hormone a Uremic Toxin? *Nephron* **19**, 125–130 (1977).
8. Glassock, R. J. Uremic Toxins: What Are They? An Integrated Overview of Pathobiology and Classification. *Journal of Renal Nutrition* **18**, 2–6 (2008).
9. Vanholder, R., Pletinck, A., Schepers, E. & Glorieux, G. Biochemical and clinical impact of organic uremic retention solutes: A comprehensive update. *Toxins (Basel)* **10**, (2018).
10. Lekawanvijit, S., Kompa, A. R. & Krum, H. Protein-bound uremic toxins: A long overlooked culprit in cardiorenal syndrome. *Am J Physiol Renal Physiol* **311**, F52–F62 (2016).
11. Dou, L. *et al.* The uremic solutes p-cresol and indoxyl sulfate inhibit endothelial proliferation and wound repair. *Kidney Int* **65**, 442–451 (2004).
12. Sun, C. Y., Chang, S. C. & Wu, M. S. Uremic toxins induce kidney fibrosis by activating intrarenal renin-angiotensin-aldosterone system associated epithelial-to-mesenchymal transition. *PLoS One* **7**, e34026 (2012).
13. Yu, M., Kim, Y. J. & Kang, D. H. Indoxyl sulfate-induced endothelial dysfunction in patients with chronic kidney disease via an induction of oxidative stress. *Clinical Journal of the American Society of Nephrology* **6**, 30–39 (2011).
14. Bammens, B., Evenepoel, P., Keuleers, H., Verbeke, K. & Vanrenterghem, Y. Free serum concentrations of the protein-bound retention solute p-cresol predict mortality in hemodialysis patients. *Kidney Int* **69**, 1081–1087 (2006).
15. Wu, I. W. *et al.* Serum free p-cresyl sulfate levels predict cardiovascular and all-cause mortality in elderly hemodialysis patients-A prospective cohort study. *Nephrology Dialysis Transplantation* **27**, 1169–1175 (2012).
16. Liabeuf, S. *et al.* Free p-cresylsulphate is a predictor of mortality in patients at different stages of chronic kidney disease. *Nephrology Dialysis Transplantation* **25**, 1183–1191 (2010).
17. Itoh, Y., Ezawa, A., Kikuchi, K., Tsuruta, Y. & Niwa, T. Protein-bound uremic toxins in hemodialysis patients measured by liquid chromatography/tandem mass spectrometry and their effects on endothelial ROS production. *Anal Bioanal Chem* **403**, 1841–1850 (2012).
18. Niwa, T. *et al.* Oral sorbent suppresses accumulation of albumin-bound indoxyl sulphate in serum of haemodialysis patients. *Nephrology, dialysis, transplantation* **6**, 105–109 (1991).
19. Brettschneider, F. *et al.* Removal of Protein-Bound, Hydrophobic Uremic Toxins by a Combined Fractionated Plasma Separation and Adsorption Technique. *Artif Organs* **37**, 409–416 (2013).
20. Meijers, B. K. *et al.* Removal of the uremic retention solute p-cresol using fractionated plasma separation and adsorption. *Artif Organs* **32**, 214–219 (2008).
21. Yamamoto, S. *et al.* Adsorption of Protein-Bound Uremic Toxins Through Direct Hemoperfusion With Hexadecyl-Immobilized Cellulose Beads in Patients Undergoing Hemodialysis. *Artif Organs* **42**, 88–93 (2018).
22. Chen, S.-J. *et al.* Combination of maintenance hemodialysis with hemoperfusion: a safe and effective model of artificial kidney. *Int J Artif Organs* **34**, 339–347 (2011).
23. Rocchetti, M. T. *et al.* Efficacy of divinylbenzenic resin in removing indoxyl sulfate and p-cresol sulfate in hemodialysis patients: Results from an in vitro study and an in vivo pilot Trial. *Toxins (Basel)* **12**, 170 (2020).
24. Madero, M. *et al.* Removal of Protein-Bound Uremic Toxins during Hemodialysis Using a Binding Competitor. *Clin J Am Soc Nephrol* **14**, 394–402 (2019).

25. Perna, A. F. *et al.* Therapy ofHyperhomocysteinemia in Hemodialysis Patients: Effects of Folates and N-Acetylcysteine. *J Ren Nutr* **22**, 507-514.e1 (2012).
26. Urquhart, B. L., Freeman, D. J., Spence, J. D. & House, A. A. The effect of mesna on plasma total homocysteine concentration in hemodialysis patients. *American Journal of Kidney Diseases* **49**, 109–117 (2007).
27. Scholze, A. *et al.* Acetylcysteine reduces plasma homocysteine concentration and improves pulse pressure and endothelial function in patients with end-stage renal failure. *Circulation* **109**, 369–374 (2004).
28. Ducloux, D. *et al.* Hyperhomocysteinaemia therapy in haemodialysis patients: Folinic versus folic acid in combination with vitamin B6 and B12. *Nephrology Dialysis Transplantation* **17**, 865–870 (2002).
29. Nevalainen, P. I., Lahtela, J. T., Mustonen, J. & Pasternack, A. Intraperitoneal insulin reduces plasma leptin concentration in diabetic patients on CAPD. *Peritoneal Dialysis International* **20**, 27–32 (2000).
30. Moher, D. *et al.* Preferred reporting items for systematic reviews and meta-analyses: The PRISMA statement. *PLoS Medicine* vol. 6 e1000097 Preprint at https://doi.org/10.1371/journal.pmed.1000097 (2009).
31. van Gelder, M. K. *et al.* Protein-bound uremic toxins in hemodialysis patients relate to residual kidney function, are not influenced by convective transport, and do not relate to outcome. *Toxins (Basel)* **12**, 234 (2020).
32. Ouseph, R., Hutchison, C. A., Ward, R. A., R. H. Ouseph, C. A. & Ward, R. A. Differences in solute removal by two high-flux membranes of nominally similar synthetic polymers. *Nephrol Dial Transplant* **23**, 1704–1712 (2008).
33. De Smet, R. *et al.* Effect of the super-flux cellulose triacetate dialyser membrane on the removal of non-protein-bound and protein-bound uraemic solutes. *Nephrol Dial Transplant* **22**, 2006–2012 (2007).
34. Paats, J. *et al.* Serum levels and removal by haemodialysis and haemodiafiltration of tryptophan-derived uremic toxins in ESKD patients. *Int J Mol Sci* **21**, 1522 (2020).
35. Gugliucci, A. *et al.* Acrolein inactivates paraoxonase 1: changes in free acrolein levels after hemodialysis correlate with increases in paraoxonase 1 activity in chronic renal failure patients. *Clinica Chimica Acta* **384**, 105–112 (2007).
36. Krieter, D. H., Kerwagen, S., Rüth, M., Lemke, H.-D. & Wanner, C. Differences in dialysis efficacy have limited effects on protein-bound uremic toxins plasma levels over time. *Toxins (Basel)* **11**, 47 (2019).
37. Mandolfo, S., Borlandelli, S. & Imbasciati, E. Leptin and beta2-microglobulin kinetics with three different dialysis modalities. *Int J Artif Organs* **29**, 949–955 (2006).
38. Panichi, V. *et al.* Effects on inflammatory and nutritional markers of haemodiafiltration with online regeneration of ultrafiltrate (HFR) vs online haemodiafiltration: a cross-over randomized multicentre trial. *Nephrol Dial Transplant* **21**, 756–762 (2006).
39. Belmouaz, M. *et al.* Comparison of the removal of uraemic toxins with medium cut-off and high-flux dialysers: A randomized clinical trial. *Nephrology Dialysis Transplantation* **35**, 328–335 (2019).
40. Schulman, G. *et al.* A multicenter, randomized, double-blind, placebo-controlled, dose-ranging study of AST-120 (Kremezin) in patients with moderate to severe CKD. *Am J Kidney Dis* **47**, 565–577 (2006).
41. Snauwaert, E. *et al.* Haemodiafiltration does not lower protein-bound uraemic toxin levels compared with haemodialysis in a paediatric population. *Nephrol Dial Transplant* **35**, 648–656 (2019).
42. Testa, A., Gentilhomme, H., Le Carrer, D. & Orsonneau, J. L. In vivo removal of high- and low-molecular-weight compounds in hemodiafiltration with on-line regeneration of ultrafiltrate. *Nephron Clin Pract* **104**, 55–60 (2006).
43. Lenglet, A. *et al.* Does the administration of sevelamer or nicotinamide modify uremic toxins or endotoxemia in chronic hemodialysis patients? *Drugs* **79**, 855–862 (2019).
44. Gomolka, M. *et al.* Protein-Bound Solute Clearance During Hemodialysis. *Adv Exp Med Biol* **1153**, 69–77 (2019).
45. Agalou, S. *et al.* Advanced glycation end product free adducts are cleared by dialysis. *Ann N Y Acad Sci* **1043**, 734–739 (2005).
46. Chen, X. *et al.* Acute effect of one session of hemodiafiltration with endogenous reinfusion on uremic toxins and inflammatory mediators. *International Journal of Artificial Organs* **43**, 437–443 (2020).
47. Beerenhout, C. H. *et al.* Pre-dilution on-line haemofiltration vs low-flux haemodialysis: A randomized prospective study. *Nephrology Dialysis Transplantation* **20**, 1155–1163 (2005).
48. Etinger, A. *et al.* The effect of isohydric hemodialysis on the binding and removal of uremic retention solutes. *PLoS One* **13**, e0192770 (2018).

49. Czupryniak, A., Nowicki, M., Chwatko, G., Jander, A. & Bald, E. Peritoneal clearance of homocysteine with icodextrin or standard glucose solution exchange. *Nephrology* **10**, 571–575 (2005).
50. Kalim, S. *et al.* Extended duration nocturnal hemodialysis and changes in plasma metabolite profiles. *Clinical Journal of the American Society of Nephrology* **13**, 436–444 (2018).
51. Galli, F. *et al.* Glycoxidation and inflammatory markers in patients on treatment with PMMA-based protein-leaking dialyzers. *Kidney Int* **67**, 750–759 (2005).
52. Albalate Ramón, M. *et al.* Asymmetric cellulose triacetate is a safe and effective alternative for online haemodiafiltration. *Nefrologia* **38**, 315–320 (2018).
53. Krieter, D. H. *et al.* Clinical cross-over comparison of mid-dilution hemodiafiltration using a novel dialyzer concept and post-dilution hemodiafiltration. *Kidney Int* **67**, 349–356 (2005).
54. Martinez, A. W., Recht, N. S., Hostetter, T. H. & Meyer, T. W. Removal of P-cresol sulfate by hemodialysis. *Journal of the American Society of Nephrology* **16**, 3430–3436 (2005).
55. Esquivias-Motta, E. *et al.* Hemodiafiltration With Endogenous Reinfusion Improved Microinflammation and Endothelial Damage Compared With Online-Hemodiafiltration: A Hypothesis Generating Study. *Artif Organs* **41**, 88–98 (2017).
56. Mudge, D. W. *et al.* Randomized trial of FX high flux vs standard high flux dialysis for homocysteine clearance. *Nephrology Dialysis Transplantation* **20**, 2178–2185 (2005).
57. Krieter, D. H. *et al.* Haemodiafiltration at increased plasma ionic strength for improved protein-bound toxin removal. *Acta Physiologica* **219**, 510–520 (2017).
58. Santoro, A. *et al.* Mid-Dilution : The Perfect Balance between Convection and Diffusion. *Contrib Nephrol* **149**, 107–114 (2005).
59. Panichi, V. *et al.* Long term variation of serum levels of uremic toxins in patients treated by post-dilution high volume on-line hemodiafiltration in comparison to standard low-flux bicarbonate dialysis: results from the REDERT study. *J Nephrol* **30**, 583–591 (2017).
60. Bammens, B. *et al.* Removal of the protein-bound solute p-cresol by convective transport: a randomized crossover study. *Am J Kidney Dis* **44**, 278–285 (2004).
61. Sirich, T. L. *et al.* Limited reduction in uremic solute concentrations with increased dialysis frequency and time in the Frequent Hemodialysis Network Daily Trial. *Kidney Int* **91**, 1186–1192 (2017).
62. Hohmann, C. *et al.* Detection of free advanced glycation end products in vivo during hemodialysis. *J Agric Food Chem* **65**, 930–937 (2017).
63. Splendiani, G. *et al.* Selective adsorption of homocysteine using an HFR-ON LINE technique. *Artif Organs* **28**, 592–595 (2004).
64. Abad, S. *et al.* Protein-bound toxins: added value in their removal with high convective volumes. *Nefrologia* **36**, 637–642 (2016).
65. Tessitore, N. *et al.* Effect of protein leaking BK-F PMMA-based hemodialysis on plasma pentosidine levels. *J Nephrol* **17**, 707–714 (2004).
66. Camacho, O. *et al.* Effect of a sustained difference in hemodialytic clearance on the plasma levels of p-cresol sulfate and indoxyl sulfate. *Nephrol Dial Transplant* **31**, 1335–1341 (2016).
67. van Tellingen, A. *et al.* Enhanced long-term reduction of plasma leptin concentrations by super-flux polysulfone dialysers. *Nephrology Dialysis Transplantation* **19**, 1198–1203 (2004).
68. Chazot, C. *et al.* Effect of membrane permeability on cardiovascular risk factors and β2m plasma levels in patients on long-term haemodialysis: a randomised crossover trial. *Nephron* **129**, 269–275 (2015).
69. De Vriese, A. S. *et al.* Effect of dialyser membrane pore size on plasma homocysteine levels in haemodialysis patients. *Nephrology Dialysis Transplantation* **18**, 2596–2600 (2003).
70. Cornelis, T. *et al.* Protein-bound uraemic toxins, dicarbonyl stress and advanced glycation end products in conventional and extended haemodialysis and haemodiafiltration. *Nephrol Dial Transplant* **30**, 1395–1402 (2015).
71. Galli, F. *et al.* The effect of PMMA-based protein-leaking dialyzers on plasma homocysteine levels. *Kidney Int* **64**, 748–755 (2003).
72. Eloot, S. *et al.* Protein-bound solute removal during extended multipass versus standard hemodialysis. *BMC Nephrol* **16**, 57 (2015).
73. Opatrná, S., Opatrný, K., Racek, J., Sefrna, F. & Cox, T. Effect of Icodextrin-Based Dialysis Solution on Peritoneal Leptin Clearance. *Educational Research* **24**, 223–227 (2003).
74. Yamamoto, S. *et al.* Continuous reduction of protein-bound uraemic toxins with improved oxidative stress by using the oral charcoal adsorbent AST-120 in haemodialysis patients. *Sci Rep* **5**, 14381 (2015).
75. Arnadottir, M., Wingren, K., Hultberg, B. & Hegbrant, J. The postdialytic rise in the plasma total homocysteine concentration is delayed. *Blood Purif* **20**, 334–337 (2002).

76. Galli, F. *et al.* Blood thiol status and erythrocyte glutathione-S-transferase in chronic kidney disease patients on treatment with frequent (daily) hemodialysis. *Free Radic Res* **48**, 273–281 (2014).
77. Lee, C.-T. *et al.* Effects of AST-120 on blood concentrations of protein-bound uremic toxins and biomarkers of cardiovascular risk in chronic dialysis patients. *Blood Purif* **37**, 76–86 (2014).
78. Fagugli, R. M., De Smet, R., Buoncristiani, U., Lameire, N. & Vanholder, R. Behavior of non-protein-bound and protein-bound uremic solutes during daily hemodialysis. *Am J Kidney Dis* **40**, 339–347 (2002).
79. Pedrini, L. A., Krisp, C., Gmerek, A. & Wolters, D. A. Patterns of proteins removed with high-flux membranes on high-volume hemodiafiltration detected with a multidimensional LC-MS/MS strategy. *Blood Purif* **38**, 115–126 (2014).
80. Lüdemann, P., Zwernemann, S. & Lerchl, A. Clearance of melatonin and 6-sulfatoxymelatonin by hemodialysis in patients with end-stage renal disease. *J Pineal Res* **31**, 222–227 (2001).
81. Riccio, E. *et al.* Evidence that p-cresol and IL-6 are adsorbed by the HFR cartridge: towards a new strategy to decrease systemic inflammation in dialyzed patients? *PLoS One* **9**, e95811 (2014).
82. Van Tellingen, A. *et al.* Long-term reduction of plasma homocysteine levels by super-flux dialyzers in hemodialysis patients. *Kidney Int* **59**, 342–347 (2001).
83. Sirich, T. L., Funk, B. A., Plummer, N. S., Hostetter, T. H. & Meyer, T. W. Prominent accumulation in hemodialysis patients of solutes normally cleared by tubular secretion. *J Am Soc Nephrol* **25**, 615–622 (2014).
84. House, A. A., Wells, G. A., Donnelly, J. G., Nadler, S. P. & Hébert, P. C. Randomized trial of high-flux vs low-flux haemodialysis: Effects on homocysteine and lipids. *Nephrology Dialysis Transplantation* **15**, 1029–1034 (2000).
85. Lesaffer, G. *et al.* Intradialytic removal of protein-bound uraemic toxins: role of solute characteristics and of dialyser membrane. *Nephrol Dial Transplant* **15**, 50–57 (2000).
86. C. B. Kneis, W. *et al.* Elimination of middle-sized uremic solutes with high-flux and high-cut-off membranes: A randomized in vivo study. *Blood Purif* **36**, 287–294 (2013).
87. Sequera, P. De *et al.* A comparison of the effectiveness of two online haemodiafiltration modalities: mixed versus post-dilution. *Nefrologia* **33**, 779–787 (2013).
88. Ducloux, D. *et al.* Dialysis adequacy and homocyst(e)ine concentrations in peritoneal dialysis patients. *Nephrology Dialysis Transplantation* **14**, 728–731 (1999).
89. Eloot, S., Dhondt, A., Van Landschoot, M., Waterloos, M. A. & Vanholder, R. Removal of water-soluble and protein-bound solutes with reversed mid-dilution versus post-dilution haemodiafiltration. *Nephrol Dial Transplant* **27**, 3278–3283 (2012).
90. Jadoul, M. *et al.* Influence of hemodialysis membrane type on pentosidine plasma level, a marker of 'carbonyl stress'. *Kidney Int* **55**, 2487–2492 (1999).
91. Kim, D. J. *et al.* The effect of continuous ambulatory peritoneal dialysis on change in serum leptin. in *Peritoneal Dialysis International* vol. 19 (1999).
92. Sirich, T. L., Luo, F. J. G., Plummer, N. S., Hostetter, T. H. & Meyer, T. W. Selectively increasing the clearance of protein-bound uremic solutes. *Nephrology Dialysis Transplantation* **27**, 1574–1579 (2012).
93. Biasioli S, Schiavon R, Petrosino L, Cavallini L, Cavalcanti G, D. F. E. Dialysis kinetics of homocysteine and reactive oxygen species. *ASAIO Journal* **44**, M423-32 (1998).
94. Badiou, S. *et al.* Does hemodiafiltration improve the removal of homocysteine? *Hemodial Int* **15**, 515–521 (2011).
95. Wiesholzer, M., Harm, F., Hauser, A. C., Pribasnig, A. & Balcke, P. Inappropriately high plasma leptin levels in obese haemodialysis patients can be reduced by high flux haemodialysis and haemodiafiltration. *Clin Sci* **94**, 431–435 (1998).
96. Nakazono, H., Nagake, Y., Ichikawa, H. & Makino, H. Serum leptin concentrations in patients on hemodialysis. *Nephron* **80**, 35–40 (1998).
97. Meert, N. *et al.* Comparison of removal capacity of two consecutive generations of high-flux dialysers during different treatment modalities. *Nephrology Dialysis Transplantation* **26**, 2624–2630 (2011).
98. Miyata, T. *et al.* Clearance of pentosidine, an advanced glycation end product, by different modalities of renal replacement therapy. *Kidney Int* **51**, 880–887 (1997).
99. Meijers, B. *et al.* Reduction in protein-bound solutes unacceptable as marker of dialysis efficacy during alternate-night nocturnal hemodialysis. *Am J Nephrol* **34**, 226–232 (2011).
100. Friedlander, M. A., Wu, Y. C., Elgawish, A. & Monnier, V. M. Early and advanced glycosylation end products: Kinetics of formation and clearance in peritoneal dialysis. *Journal of Clinical Investigation* **97**, 728–735 (1996).

101. Pedrini, L. A. *et al.* Long-term effects of high-efficiency on-line haemodiafiltration on uraemic toxicity. A multicentre prospective randomized study. *Nephrology Dialysis Transplantation* **26**, 2617–2624 (2011).
102. Tamura, T., Johnston, K. E., Bergman, S. M. S. S. M., Johnston, K. E. & Bergman, S. M. S. S. M. Homocysteine and folate concentrations in blood from patients treated with hemodialysis. *Journal of the American Society of Nephrology* **7**, 2414–2418 (1996).
103. Krieter, D. H. *et al.* Protein-bound uraemic toxin removal in haemodialysis and post-dilution haemodiafiltration. *Nephrology Dialysis Transplantation* **25**, 212–218 (2010).
104. Friedlander, M. A., Wu, Y. C., Schulak, J. A., Monnier, V. M. & Hricik, D. E. Influence of dialysis modality on plasma and tissue concentrations of pentosidine in patients with end-stage renal disease. *Am J Kidney Dis* **25**, 445–51 (1995).
105. Meert, N. *et al.* Prospective evaluation of the change of predialysis protein-bound uremic solute concentration with postdilution online hemodiafiltration. *Artif Organs* **34**, 580–585 (2010).
106. Odetti, P. *et al.* Plasma advanced glycosylation end-products in maintenance haemodialysis patients. *Nephrol Dial Transplant* **10**, 2110–2113 (1995).
107. Righetti, M. *et al.* Internal hemodiafiltration versus low-flux bicarbonate dialysis: Results from a long-term prospective study. *International Journal of Artificial Organs* **33**, 796–802 (2010).
108. Vaziri, N. D. *et al.* Serum melatonin and 6-sulfatoxymelatonin in end-stage renal disease: Effect of hemodialysis. *Artif Organs* **17**, 764–769 (1993).
109. Luo, F. J.-G. *et al.* Effect of increasing dialyzer mass transfer area coefficient and dialysate flow on clearance of protein-bound solutes: a pilot crossover trial. *Am J Kidney Dis* **53**, 1042–1049 (2009).
110. Vanholder, R. C., De Smet, R. V & Ringoir, S. M. Assessment of urea and other uremic markers for quantification of dialysis efficacy. *Clin Chem* **38**, 1429 (1992).
111. Maduell, F. *et al.* Mid-dilution hemodiafiltration: A comparison with pre- and postdilution modes using the same polyphenylene membrane. *Blood Purif* **28**, 268–274 (2009).
112. Meert, N. *et al.* Effective removal of protein-bound uraemic solutes by different convective strategies: a prospective trial. *Nephrol Dial Transplant* **24**, 562–570 (2009).
113. Vanholder, R., Krause, A., De Smet, R. & Ringoir, S. In vivo solute extraction by a new polysulphone membrane with low ultrafiltration capacity. *ASAIO Trans* **34**, 598–601 (1988).
114. Susantitaphong, P. *et al.* A simple efficient technique of 'mid-dilution' on-line hemodiafiltration. *Blood Purif* **28**, 93–101 (2009).
115. Wichman, Z., Pajdak, W., Smoleski, O. & Kope, J. Detection of plasma proteins during sequential ultrafiltration/dialysis. *Kidney Int* **28**, 558–565 (1985).
116. Krieter, D. H. *et al.* Matching efficacy of online hemodiafiltration in simple hemodialysis mode. *Artif Organs* **32**, 903–909 (2008).
117. M. V. V. Pahl, N. D., Pahl, M. V. & Vaziri, N. D. In-vivo and in-vitro hemodialysis studies of thiocyanate. *Clin Toxicol* **19**, 965–974 (1982).
118. Mandolfo, S. *et al.* Pilot study to assess increased dialysis efficiency in patients with limited blood flow rates due to vascular access problems. *Hemodialysis International* **12**, 55–61 (2008).
119. Röckel, A., Gilge, U., Liewald, A. & Heidland, A. Elimination of Low Molecular Weight Proteins During Hemofiltration. *Artif Organs* **6**, 307–311 (1982).
120. Mair, R. D., Sirich, T. L. & Meyer, T. W. Uremic toxin clearance and cardiovascular toxicities. *Toxins (Basel)* **10**, 226 (2018).
121. Leong, S. C. & Sirich, T. L. Indoxyl sulfate-review of toxicity and therapeutic strategies. *Toxins (Basel)* **8**, 358 (2016).
122. Vanholder, R., Schepers, E., Pletinck, A., Nagler, E. V. & Glorieux, G. The uremic toxicity of indoxyl sulfate and p-cresyl sulfate: A systematic review. *Journal of the American Society of Nephrology* **25**, 1897–1907 (2014).
123. Liu, W. C., Tomino, Y. & Lu, K. C. Impacts of indoxyl sulfate and p-Cresol sulfate on chronic kidney disease and mitigating effects of AST-120. *Toxins (Basel)* **10**, 367 (2018).
124. Sato, B. *et al.* Relation of plasma indoxyl sulfate levels and estimated glomerular filtration rate to left ventricular diastolic dysfunction. *American Journal of Cardiology* **111**, 712–716 (2013).
125. Hsu, C. C. *et al.* Levels of indoxyl sulfate are associated with severity of coronary atherosclerosis. *Clinical and Investigative Medicine* **36**, E42–E49 (2013).
126. Cao, X. Sen *et al.* Association of indoxyl sulfate with heart failure among patients on hemodialysis. *Clinical Journal of the American Society of Nephrology* **10**, 111–119 (2015).
127. Lin, C. J. *et al.* Serum protein-bound uraemic toxins and clinical outcomes in haemodialysis patients. *Nephrology Dialysis Transplantation* **25**, 3693–3700 (2010).

128. Melamed, M. L. *et al.* Retained organic solutes, patient characteristics and all-cause and cardiovascular mortality in hemodialysis: Results from the retained organic solutes and clinical outcomes (ROSCO) investigators. *BMC Nephrol* **14**, 134 (2013).
129. Meijers, B. K. I. *et al.* p-Cresol and cardiovascular risk in mild-to-moderate kidney disease. *Clinical Journal of the American Society of Nephrology* **5**, 1182–1189 (2010).
130. Veeranna, V. *et al.* Homocysteine and reclassification of cardiovascular disease risk. *J Am Coll Cardiol* **58**, 1025–1033 (2011).
131. Au-Yeung, K. K. W. *et al.* Hyperhomocysteinemia activates nuclear factor-κB in endothelial cells via oxidative stress. *Circ Res* **94**, 28–36 (2004).
132. Weigand, K. M. *et al.* Uremic solutes modulate hepatic bile acid handling and induce mitochondrial toxicity. *Toxicology in Vitro* **56**, 52–61 (2019).
133. Yu, T. H. *et al.* Association between hippuric acid and left ventricular hypertrophy in maintenance hemodialysis patients. *Clinica Chimica Acta* **484**, 47–51 (2018).
134. Dou, L. *et al.* The cardiovascular effect of the uremic solute indole-3 acetic acid. *Journal of the American Society of Nephrology* **26**, 876–887 (2015).
135. Brocca, A., Virzì, G. M., De Cal, M., Cantaluppi, V. & Ronco, C. Cytotoxic effects of p-cresol in renal epithelial tubular cells. *Blood Purif* **36**, 219–225 (2014).
136. Poesen, R. *et al.* Cardiovascular disease relates to intestinal uptake of p-cresol in patients with chronic kidney disease. *BMC Nephrol* **15**, 1–8 (2014).
137. Chrysant, S. G. & Chrysant, G. S. The current status of homocysteine as a risk factor for cardiovascular disease: a mini review. *Expert Rev Cardiovasc Ther* **16**, 559–565 (2018).
138. Cha, R. H. *et al.* A randomized, controlled trial of oral intestinal sorbent AST-120 on renal Function deterioration in patients with advanced renal dysfunction. *Clinical Journal of the American Society of Nephrology* **11**, 559–567 (2016).
139. Schulman, G. *et al.* Randomized placebo-controlled EPPIC trials of AST-120 in CKD. *Journal of the American Society of Nephrology* **26**, 1732–1746 (2015).
140. Schulman, G. *et al.* The effects of AST-120 on chronic kidney disease progression in the United States of America: a post hoc subgroup analysis of randomized controlled trials. *BMC Nephrol* **17**, 1–8 (2016).
141. Cha, R. H. *et al.* Sustained uremic toxin control improves renal and cardiovascular outcomes in patients with advanced renal dysfunction: Post-hoc analysis of the kremezin study against renal disease progression in Korea. *Kidney Res Clin Pract* **36**, 68–78 (2017).
142. Srinivas, T. R., Ho, B., Kang, J. & Kaplan, B. Post hoc analyses: After the facts. *Transplantation* **99**, 17–20 (2015).
143. Meyer, T. W. *et al.* Increasing dialysate flow and dialyzer mass transfer area coefficient to increase the clearance of protein-bound solutes. *Journal of the American Society of Nephrology* **15**, 1927–1935 (2004).
144. Viaene, L. *et al.* Albumin is the main plasma binding protein for indoxyl sulfate and p-cresyl sulfate. *Biopharm Drug Dispos* **34**, 165–175 (2013).
145. Ward, R. A. Protein-leaking membranes for hemodialysis: A new class of membranes in search of an application? *Journal of the American Society of Nephrology* **16**, 2421–2430 (2005).
146. Piroddi, M., Pilolli, F., Aritomi, M. & Galli, F. Vitamin e as a functional and biocompatibility modifier of synthetic hemodialyzer membranes: An overview of the literature on vitamin E-modified hemodialyzer membranes. *Am J Nephrol* **35**, 559–572 (2012).
147. Tsipotis, E., Shuja, A. & Jaber, B. L. Albumin dialysis for liver failure: A systematic review. *Advances in Chronic Kidney Disease* vol. 22 382–390 Preprint at https://doi.org/10.1053/j.ackd.2015.05.004 (2015).
148. Kato, S. *et al.* Zirconium-Based Metal-Organic Frameworks for the Removal of Protein-Bound Uremic Toxin from Human Serum Albumin. *J Am Chem Soc* **141**, 2568–2576 (2019).
149. Pavlenko, D. *et al.* New low-flux mixed matrix membranes that offer superior removal of protein-bound toxins from human plasma. *Sci Rep* **6**, 34429 (2016).
150. Sandeman, S. R. *et al.* A haemocompatible and scalable nanoporous adsorbent monolith synthesised using a novel lignin binder route to augment the adsorption of poorly removed uraemic toxins in haemodialysis. *Biomed Mater* **12**, 35001 (2017).
151. Thompson, S. *et al.* Cause of death in patients with reduced kidney function. *Journal of the American Society of Nephrology* **26**, 2504–2511 (2015).

# Supplementary material

## I. Full Search Query

MEDLINE:

((((((((((Renal replacement therapy) OR "Hemodialysis") OR Hemofiltration) OR Hemodiafiltration) OR Peritoneal dialysis) OR Renal Dialysis) OR "Adsorption dialysis") OR "Extracorporeal Dialysis"))
AND
((((((((((((((((((((((((((((((("Protein-bound"[All Fields] OR "hydrophobic uremic toxins"[All Fields]) OR "Protein bound uremic toxins"[All Fields]) OR "methoxyresorcinol"[All Fields]) OR ("3-deoxyglucosone"[Supplementary Concept] OR "3-deoxyglucosone"[All Fields] OR "3 deoxyglucosone"[All Fields])) OR ("3-carboxy-4-methyl-5-propyl-2-furanpropionic acid"[Supplementary Concept] OR "3-carboxy-4-methyl-5-propyl-2-furanpropionic acid"[All Fields] OR "3 carboxy 4 methyl 5 propyl 2 furanpropionate"[All Fields])) OR ("fructosyl-lysine"[Supplementary Concept] OR "fructosyl-lysine"[All Fields] OR "fructose lysine"[All Fields])) OR "Fructoselysine"[All Fields]) OR ("glyoxal"[MeSH Terms] OR "glyoxal"[All Fields])) OR ("hippuric acid"[Supplementary Concept] OR "hippuric acid"[All Fields])) OR ("homocysteine"[MeSH Terms] OR "homocysteine"[All Fields])) OR ("hydroquinone"[Supplementary Concept] OR "hydroquinone"[All Fields])) OR ("indoleacetic acid"[Supplementary Concept] OR "indoleacetic acid"[All Fields] OR "indole 3 acetic acid"[All Fields])) OR "Indoxyl sulfate"[All Fields]) OR ("indoxyl glucuronide"[Supplementary Concept] OR "indoxyl glucuronide"[All Fields] OR "indoxyl beta d glucuronide"[All Fields])) OR ("kynurenine"[MeSH Terms] OR "kynurenine"[All Fields])) OR ("kynurenic acid"[MeSH Terms] OR "kynurenic acid"[All Fields])) OR ("leptin"[MeSH Terms] OR "leptin"[All Fields])) OR ("melatonin"[MeSH Terms] OR "melatonin"[All Fields])) OR ("pyruvaldehyde"[MeSH Terms] OR "pyruvaldehyde"[All Fields] OR "methylglyoxal"[All Fields])) OR "N(6)-carboxymethyllysine"[Supplementary Concept]) OR "N(6)-carboxymethyllysine"[All Fields]) OR ("4-cresol"[Supplementary Concept] OR "4-cresol"[All Fields] OR "p cresol"[All Fields] OR "para cresol"[All Fields] OR "para-cresol"[All Fields])) OR ("p-Cresyl sulfate"[All Fields] OR "para-cresyl sulfate"[All Fields])) OR ("pentosidine"[Supplementary Concept] OR "pentosidine"[All Fields])) OR ("phenols"[MeSH Terms] OR "phenols"[All Fields] OR "phenol"[All Fields] OR "phenol"[MeSH Terms])) OR ("4-hydroxyhippuric acid"[Supplementary Concept] OR "4-hydroxyhippuric acid"[All Fields] OR "4 hydroxyhippuric acid"[All Fields])) OR ("putrescine"[MeSH Terms] OR "putrescine"[All Fields])) OR ("quinolinic acid"[MeSH Terms] OR "quinolinic acid"[All Fields])) OR ("retinol-binding proteins"[MeSH Terms] OR "retinol-binding proteins"[All Fields] OR "retinol binding protein"[All Fields])) OR ("spermidine"[MeSH Terms] OR "spermidine"[All Fields])) OR ("spermine"[MeSH Terms] OR "spermine"[All Fields])) OR ("dihydroxyphenylalanine"[MeSH Terms] OR "dihydroxyphenylalanine"[All Fields])) OR ("indican"[MeSH Terms] OR "indican"[All Fields])) OR ("acrolein"[MeSH Terms] OR "acrolein"[All Fields])) OR ("thiocyanate"[Supplementary Concept] OR "thiocyanate"[All Fields] OR "thiocyanates"[MeSH Terms] OR "thiocyanates"[All Fields] OR ("phenylacetic acid"[Supplementary Concept] OR "phenylacetic acid"[All Fields])))))
AND
((((("filtration"[MeSH Terms] OR ("adsorption"[MeSH Terms] OR "adsorption"[All Fields])) OR "Clearance"[All Fields]) OR "Removal"[All Fields]) OR "Separation"[All Fields]))
AND

English[Language]) AND ("1980/01/01"[Date - Publication] : "2020/02/01"[Date - Publication]) AND "humans"[MeSH Terms]

Embase:

'renal replacement therapy'/exp OR 'renal replacement therapy' OR 'hemodialysis'/exp OR 'hemodialysis' OR 'hemofiltration'/exp OR 'hemofiltration' OR 'hemodiafiltration'/exp OR 'hemodiafiltration' OR 'peritoneal dialysis'/exp OR 'peritoneal dialysis' OR 'adsorption dialysis' OR 'renal dialysis'/exp OR 'renal dialysis' OR 'extracorporeal dialysis'/exp OR 'extracorporeal dialysis' OR 'haemofiltration' OR 'haemodiafiltration' OR ('peritoneal' AND 'dialysis') OR ('renal' AND 'dialysis')
AND
'protein-bound' OR 'hydrophobic uremic toxins' OR 'protein bound uremic toxins' OR '(2-) methoxyresorcinol' OR 'methoxyresorcinol' OR '3 deoxyglucosone'/exp OR '3 deoxyglucosone' OR '3-carboxy-4-methyl-5-propyl-2-furanpropionate' OR '3 carboxy 4 methyl 5 propyl 2 furanpropionate' OR 'fructose-lysine' OR 'fructoselysine' OR 'glyoxal'/exp OR 'glyoxal' OR 'hippuric acid'/exp OR 'hippuric acid' OR 'homocysteine'/exp OR 'homocysteine' OR 'hydroquinone'/exp OR 'hydroquinone' OR 'indoleacetic acid'/exp OR 'indoleacetic acid' OR 'indole 3 acetic acid' OR 'indoxyl sulfate'/exp OR 'indoxyl sulfate' OR 'indoxyl glucuronide' OR 'indoxyl beta d glucuronide' OR 'kynurenine'/exp OR 'kynurenine' OR 'kynurenic acid'/exp OR 'kynurenic acid' OR 'leptin'/exp OR 'leptin' OR 'melatonin'/exp OR 'melatonin' OR 'methylglyoxal'/exp OR 'methylglyoxal' OR 'pyruvaldehyde' OR 'n 6 carboxymethyllysine'/exp OR 'n 6 carboxymethyllysine' OR 'para cresol'/exp OR 'para cresol' OR '4 cresol' OR 'p cresyl sulfate'/exp OR 'p cresyl sulfate' OR 'pentosidine'/exp OR 'pentosidine' OR 'phenol'/exp OR 'phenol' OR '4 hydroxyhippuric acid'/exp OR '4 hydroxyhippuric acid' OR 'putrescine'/exp OR 'putrescine' OR 'quinolinic acid'/exp OR 'quinolinic acid' OR 'retinol binding protein'/exp OR 'retinol binding protein' OR 'spermidine'/exp OR 'spermidine' OR 'spermine'/exp OR 'spermine' OR 'dopa'/exp OR 'dopa' OR 'dihydroxyphenylalanine' OR 'indican'/exp OR 'indican' OR 'acrolein'/exp OR acrolein OR 'thiocyanate'/exp OR thiocyanate
AND
'filtration'/exp OR 'adsorption'/exp OR 'adsorption' OR 'clearance'/exp OR 'clearance' OR 'separation'/exp OR 'separation' OR 'removal' AND [1980-2020]/py AND [english]/lim AND [humans]/lim AND ([article]/lim OR [article in press]/lim)

## II. Supplementary Tables

**Table 11. Reduction ratios (RR) of retinol binding protein.**

**Longitudinal studies**

| First author, year | Technique | Analysis period | Results | RR |
|---|---|---|---|---|
| Röckel, 1982[1] | high-flux HF | 6 months | before 21.8 ± 1.1 after 18.3 ± 2.1 mg/dl, ns | 16.1% |

**Acute studies**

| First author, year | Technique | Results | RR |
|---|---|---|---|
| Sequera, 2013[2] | FXCorDiax1000 dialyser in mixed-dilution OLHDF | RR: 43.2% | 43.2% |
| Sequera, 2013[2] | FXCorDiax1000 dialyser in post-dilution OLHDF | RR: 39% | 39.0% |
| Maduell, 2009[3] | Olpur MD 220 2.2 m2 Polyphenylene dilayzer in mid-dilution OLHDF | RR: 30.5% | 30.5% |

| | | | |
|---|---|---|---|
| Meert, 2011[4] | Polynephron dialyser in pre-dilution HDF | approx. RR: 30% | 30.0% |
| Meert, 2011[4] | Polynephron dialyser in post-dilution HDF | approx. RR: 30% | 30.0% |
| Maduell, 2009[3] | Olpur MD 190 1.9 m2 Polyphenylene dilyser mid-dilution OLHDF | RR: 29.2% | 29.2% |
| Maduell, 2009[3] | Philther HF 170 1.7 m2 Polyphenylene dialyser post-dilution in OLHDF | RR: 23.5% | 23.5% |
| Maduell, 2009[3] | Philther HF 170 1.7 m2 Polyphenylene dialyser pre-dilution OLHDF | RR: 22.5% | 22.5% |
| Krieter, 2005[5] | Nephros OLpur MD 190 hemodiafilter in mid-dilution HDF | RR: 22% | 22.0% |
| Krieter, 2008[6] | PES membrane in HD | RR: 22% | 22.0% |
| Meert, 2011[4] | Polynephron dialyser in HD | approx. RR: 20% | 20.0% |
| Ramon, 2018[7] | asymmetric CTA membrane in OLHDF | RR: 18.6 ± 7.6% | 18.6% |
| Krieter, 2008[6] | PS HF 80 S dialyser in post-dilution OLHDF | approx. RR: 16% | 16.0% |
| Meert, 2011[4] | DIAPES-HF800 dilayser in post-dilution HDF | approx. RR: 15% | 15.0% |
| Krieter, 2008[6] | Polyflux 170 H dialyser in post-dilution OLHDF | approx. RR: 14% | 14.0% |
| Krieter, 2005[5] | high-flux 80 S dialyser in post-dilution OLHDF | approx. RR: 12% | 12.0% |
| Meert, 2011[4] | DIAPES-HF800 dilayser in HD | approx. RR: 10% | 10.0% |
| Meert, 2011[4] | DIAPES-HF800 dilayser in pre-dilution HDF | approx. RR: 10% | 10.0% |
| Wichman, 1985[8] | sequential ultrafiltration/dialysis | before 301.7 ± 104.8 after 276.5 ± 85.6 mg/l, ns | 8.4% |
| Testa, 2006[9] | OLHDF | before 131.2 ± 24 after 145.6 ± 46.5 mg/l | 0.0% |

*Abbreviations: CTA – cellulose triacetate; HD – hemodialysis; HDF – hemodiafiltration; OLHDF – on-line hemodiafiltration; HF – hemofiltration; ns – not significant; PES – polyethersulfone; and PS – polysulphone.*

**Table 12. Reduction ratios (RR) of pentosidine (total, protein-bound and free fractions).**

**Longitudinal studies**

| First author, year | Technique | Intervention period | Results | RR |
|---|---|---|---|---|
| Galli, 2005[10] | protein-leaking dialyser (BF-K) in HD | 6 months | total, before 23.5±9.3 after 15.4±5.2 pmol/mg protein, p<0.01 | 34.5% |
| Galli, 2005[10] | non-protein-leaking dialyser in HD | 6 months | before 26.5±8.7 after 25.0±11.1 pmol/mg protein, ns | 5.7% |

**Acute studies**

| | First author, year | Technique | Results | RR |
|---|---|---|---|---|
| Total pentosidine | Odetti, 1995[11] | HF | before 23.94±5.31 after 21.36±3.74 pmol/mg protein, ns | 10.8% |
| | De Smet, 2007[12] | super-flux CTA (Sureflux-150FH) membrane in HD | RR: 3.3±25.7% | 3.3% |
| | De Smet, 2007[12] | Low-flux CTA (Sureflux-150L) membrane in HD | LF RR: -1.24±21.5% | 0.0% |
| | Odetti, 1995[11] | chronical HD | before 23.72±2.01 after 26.46±2.15 pmol/mg protein, ns | 0.0% |
| | Odetti, 1995[11] | bicarbonate HD | before 23.83±3.20 after 30.02±3.90 pmol/mg protein, ns | 0.0% |
| | Odetti, 1995[11] | acetate-free biofiltration | before 23.44 ±3.11 after 24.32± 1.88 pmol/mg protein, ns | 0.0% |
| Protein-bound pentosidine | Tessitore, 2004[13] | BK-F dialyser in HD | approx. RR= 15% | 15.0% |
| | Friedlander, 1995[14] | canonical HD | before 23.7±12.3 after 21.4±9.4 pmol/ mg prot, ns | 9.7% |
| | Jadoul, 1999[15] | AN69 membrane in HD | before 25.4±8.4 after 23.4±5.6 pmol/mg prot | 7.9% |
| | Jadoul, 1999[15] | PMMA membrane in HD | before 24.3±8.5 after 22.8±8.3 pmol/mg prot | 6.2% |

| | | | | |
|---|---|---|---|---|
| | Jadoul, 1999[15] | PS membrane in HD | before 14.6±6.2 after 13.8±6.6 pmol/mg prot | 5.5% |
| | Jadoul, 1999[15] | Cellulosic membrane in HD | before 21.8±6.4 after 22.1±5.8 pmol/mg prot | 0.0% |
| | Cornelis, 2015[16] | 8-hour HDF | no reduction | 0.0% |
| | Cornelis, 2015[16] | 8-hour HD | no reduction | 0.0% |
| | Cornelis, 2015[16] | 4-hour HDF | no reduction | 0.0% |
| | Cornelis, 2015[16] | canonical HD | no reduction | 0.0% |
| | Miyata, 1997[17] | canonical HD | before 26.64±9.15 after 27.16±9.86 pmol/ mg albumin | 0.0% |
| Free pentosidine | Hohmann, 2017[18] | canonical HD | before 4±2 after non-detectable, RR: 100% | 100.0% |
| | Agalou, 2005[19] | canonical HD | HD RR: 86 ± 7% | 86.0% |
| | Friedlander, 1996[20] | canonical HD | from 105.2±37.9 to 53.6±30.7 pmol/ml, p< 0.05 | 49.0% |
| | Friedlander, 1996[20] | CAPD | from 63.9±31 to 60.3±29.3 pmol/ml, ns | 5.6% |

*Abbreviations: CAPD – continuous ambulatory peritoneal dialysis; CTA – cellulose triacetate; HD – hemodialysis; HDF – hemodiafiltration; HF – hemofiltration; ns – not significant; and PS – polysulphone.*

**Table 13. Reduction ratios (RR) of 3-carboxy-4-methyl-5-propyl-2-furanpropionate (CMPF).**

**Longitudinal studies**

| First author, year | Technique | Analysis period | Results | RR |
|---|---|---|---|---|
| Meert, 2010[21] | post-dilution HDF | 9 weeks | before 0.72±0.52 after 0.64±0.46 mg/dl, -11%, p < 0.01 | 11.1% |
| Snauwaert, 2019[22] | high-flux HD | 12 months | after 0.12 (0.03; 0.34), change -0.01 (-0.07; 0.06) mg/dl | 7.7% |
| Lenglet, 2019[23] | nicotinamide in HD | 24 weeks | before 6992 (2341 - 8856) after 6465 ± 6599 ng/ml, ns | 7.5% |
| Snauwaert, 2019[22] | low-flux HD | 12 months | after 0.14 (0.04; 0.39), change -0.01 (-0.08; 0.01) mg/dl | 6.7% |
| Snauwaert, 2019[22] | post-dilution HDF | 12 months | after 0.16 (0.07; 0.57), change -0.00 (-0.03; 0.03) mg/dl | 0.0% |
| Lenglet, 2019[23] | sevelamer-hydrochloride in HD | 24 weeks | before 6581 ± 6135 after 6746 ± 6135 ng/ml, ns | 0.0% |

**Acute studies**

| First author, year | Technique | Results | RR |
|---|---|---|---|
| Cornelis, 2015[16] | 8-hour HD | approx. RR: 10%, ns | 10.0% |
| Meert, 2009[24] | post-dilution HDF | RR: 7.1 ± 5.7% | 7.1% |
| Meert, 2009[24] | pre-dilution HDF | RR: 5.9 ± 6.7% | 5.9% |
| Eloot, 2012[25] | post-dilution HDF | RR: 5.5 ± 11.1% | 5.5% |
| Eloot, 2012[25] | mid-dilution HDF | RR: 4.4 ± 9.1% | 4.4% |
| Meert, 2009[24] | pre-dilution HF | RR: 4.0 ± 7.8% | 4.0% |
| Cornelis, 2015[16] | 4-hour HDF | approx. RR: 3%, ns | 3.0% |
| Cornelis, 2015[16] | 8-hour HDF | approx. RR: 1%, ns | 1.0% |
| Cornelis, 2015[16] | canonical HD | no reduction | 0.0% |
| De Smet, 2007[12] | low-flux CTA membrane (Sureflux-150L) in HD | RR: -17.7±25.8%, increase | 0.0% |
| De Smet, 2007[12] | Super-flu CTA membrane (Sureflux-150FH) in HD | RR: -10.2±13.3%, increase | 0.0% |
| Fagugli, 2002[26] | daily short hemodialysis | before 0.36±0.20 after 0.41±0.26 mg/dl | 0.0% |

| | | | |
|---|---|---|---|
| Fagugli, 2002[26] | canonical HD | before 0.37±0.25 after 0.49±0.33 mg/dl | 0.0% |
| Lesaffer, 2000[27] | high-flux PS (F60) membrane in HD | before 1.97±1.03 after 2.25±1.30 mg/100ml, p< 0.01 | 0.0% |
| Lesaffer, 2000[27] | high-flux CTA (Nissho Nipro) membrane in HD | before 1.76±0.77 after 2.00±1.11 mg/100ml, ns | 0.0% |
| Lesaffer, 2000[27] | low-flux PS (F8) membrane in HD | LF PS: before 1.71±0.89 after 1.83±0.93 mg/100ml, ns | 0.0% |

*Abbreviations: CTA – cellulose triacetate; HD – hemodialysis; HDF – hemodiafiltration; HF – hemofiltration; ns – not significant and PS – polysulphone.*

**Table 14. Reduction ratios (RR) of p-cresol in the acute studies.**

| First author, year | Technique | Results | RR |
|---|---|---|---|
| Susantitaphong, 2009[28] | mid-dilution OLHDF | RR: 52.2±20.5% | 52.2% |
| Riccio, 2014[29] | hemofiltrate-reinfusion system in HDF | RR: 52% | 52.0% |
| Susantitaphong, 2009[28] | pre-dilution OLHDF | RR: 48.0±20.5% | 48.0% |
| Susantitaphong, 2009[28] | post-dilution OLHDF | RR: 41.9±15.7% | 41.9% |
| Lesaffer, 2000[27] | high-flux PS (F60) membrane in HD | before 0.72±0.30 after 0.42±0.20 mg/100ml, p< 0.01 | 41.7% |
| Bammens, 2004[30] | pre-dilution HDF with 20L FX80 dialyser | before 23.6±1.8 after 14.5±1.1 mg/l | 38.6% |
| Riccio, 2014[29] | canonical HD | RR: 37% | 37.0% |
| Bammens, 2004[30] | post-dilution HDF with FX80 dialyser | before 21.7±2.6 after 13.9±1.7 mg/l | 35.9% |
| Lesaffer, 2000[27] | low-flux PS (F8) membrane in HD | before 0.76±0.41 after 0.49±0.25 mg/100ml, p< 0.01 | 35.5% |
| Lesaffer, 2000[27] | high-flux CTA (Nissho Nipro) membrane in HD | before 0.72±0.31 after 0.47±0.17 mg/100ml, p< 0.01 | 34.7% |
| Bammens, 2004[30] | FX80 dialyser in HD | before 23.1±2.7 after 15.4±1.7 mg/l | 33.3% |
| Fagugli, 2002[26] | canonical HD | before 0.96±0.59 after 0.66±0.38mg/dl | 31.3% |
| Bammens, 2004[30] | pre-dilution HDF with 60L FX80 dialyser | before 19.5±2.0 after 13.5±1.5 mg/l | 30.8% |
| Bammens, 2004[30] | HF80(S) dialyser in HD | before 23.5±2.2 mg/l after 16.3±1.7 mg/l | 30.6% |
| Fagugli, 2002[26] | daily short hemodialysis | before: 0.78±0.33 after 0.64±0.32 mg/dl | 17.9% |

*Abbreviations: CTA – cellulose triacetate; HD – hemodialysis; HDF – hemodiafiltration; HF – hemofiltration; OLHDF – on-line hemodiafiltration; and PS – polysulphone.*

**Table 15. Reduction ratios (RR) of *p*-cresylglucuronide (PCG).**

**Longitudinal studies**

| First author, year | Technique | Analysis period | Results | RR |
|---|---|---|---|---|
| van Gelder, 2020[31] | low-flux HD | 6 months | RR: 7.0 (38.1 to -69.8)%, ns | 7.0% |
| Lenglet, 2019[23] | sevelamer-hydrochloride in HD | 24 weeks | before 5458±6528 after 5874±5395 ng/ml. ns | 0.0% |
| Lenglet, 2019[23] | nicotinamide in HD | 24 weeks | before 6070±6766 after 6565±6973 ng/ml. ns | 0.0% |
| van Gelder, 2020[31] | post-dilution high-flux OLHDF | 6 months | increase -7.4 (37.3 to -65.3)%, ns | 0.0% |
| Snauwaert, 2019[22] | post-dilution HDF | 12 months | 0.09 (0.04; 0.30). change -0.00 (-0.10; 0.16) mg/dl | 0.0% |
| Snauwaert, 2019[22] | high-flux HD | 12 months | after: 0.17 (0.05; 0.36). change 0.04 (-0.03; 0.10) mg/dl | 0.0% |
| Snauwaert, 2019[22] | low-flux HD | 12 months | after 0.25 (0.18; 0.53). change 0.02 (-0.04; 0.11) mg/dl | 0.0% |

**Acute studies**

| First author, year | Technique | Results | RR |
|---|---|---|---|
| Cornelis, 2015[16] | 8-hour HD | approx. RR: 85% | 85.0% |
| Cornelis, 2015[16] | 8-hour HDF | approx. RR: 84% | 84.0% |
| Cornelis, 2015[16] | 4-hour HDF | approx. RR: 83% | 83.0% |
| Cornelis, 2015[16] | canonical HD | approx. RR: 81% | 81.0% |
| Eloot, 2015[32] | canonical HD | RR: 81 ± 7% | 81.0% |
| Etinger, 2018[33] | isohydric dialysis (25 mM buffer bicarbonate) | before 20.83 ± 17.77 after 5.28 ± 3.26 µmol/l | 74.7% |
| Etinger, 2018[33] | canonical (37 mM buffer bicarbonate) | before 21.86 ± 17.74 after 7.54 ± 6.34 µmol/l | 65.5% |
| Eloot, 2015[32] | multipass HD | RR: 55±6% | 55.0% |

*Abbreviations: HD – hemodialysis; HDF – hemodiafiltration; and OLHDF – on-line hemodiafiltration.*

**Table 16. Reduction ratios (RR) of kynurenic acid (KA) and kynurenine (KY).**

**Longitudinal studies**

| First author, year | Techniques | Analysis period | Results | RR |
|---|---|---|---|---|
| van Gelder, 2020[31] | low-flux HD | 6 months | KY RR: 7.7 (22.6 to -14.5)%; KA RR: increase -5.6 (8.6 to -69.1)%, ns | 7.7% |
| van Gelder, 2020[31] | post-dilution high-flux OLHDF | 6 months | KY RR: 5.9 (20.9 to -29.3)%; KA RR: increase -3.2 (−22.1 to 39.5)%, ns | 5.9% |
| Lenglet, 2019[23] | sevelamer-hydrochloride in HD | 24 weeks | KY: before 1065 ± 526 after 1190 ± 584 ng/ml, ns; KA before 488 ± 315 after 483 ± 293 ng/ml, ns | 1.0% |
| Lenglet, 2019[23] | nicotinamide in HD | 24 weeks | KY: before 1225 ± 555 after 1244 ± 559 ng/ml, ns; KA: before 363 ± 183 after 445 ± 261 ng/ml, p= 0.056 | 0.0% |

**Acute studies**

| First author, year | Technique | Results | RR |
|---|---|---|---|
| Luo, 2009[34] | increased KoA and Qd | RR: 58±7% | 58.0% |
| Etinger, 2018[33] | canonical (37 mM buffer bicarbonate) | before 4.26 ± 1.22 after 2.72 ± 0.77 µmol/l | 36.2% |
| Etinger, 2018[33] | isohydric dialysis (25 mM buffer bicarbonate) | before 4.03 ± 1.30 after 2.63 ± 0.63 µmol/l | 34.7% |
| Luo, 2009[34] | canonical HD | RR: 34±10% | 34.0% |

*Abbreviations: HD – hemodialysisHF – hemofiltration; KoA - mass transfer area coefficient; OLHDF – on-line hemodiafiltration; and Qd - dialysate flow.*

**Table 17. Reduction ratios (RR) of Ne-(carboxymethyl)lysine (CML), glyoxal, methylglyoxal, 3-deoxyglucosone (3-DG), phenylacetic acid (PAA), melatonin, indican, thiocyanate and acrolein.**

| PBUT | First author, year | Technique | Results | RR |
|---|---|---|---|---|
| CML, free | Agalou, 2005[19] | canonical HD | RR: 84 ± 5% | 84.0% |
| CML, free | Hohmann, 2017[18] | canonical HD | RR: 76% | 76.0% |
| CML, protein-bound | Cornelis, 2015[16] | 8-hour HD | no reduction | 0.0% |
| CML, protein-bound | Cornelis, 2015[16] | 8-hour HDF | no reduction | 0.0% |
| CML, protein-bound | Cornelis, 2015[16] | 4-hour HDF | no reduction | 0.0% |
| CML, protein-bound | Cornelis, 2015[16] | canonical HD | no reduction | 0.0% |

| | | | | |
|---|---|---|---|---|
| Glyoxal | Cornelis, 2015[16] | 8-hour HDF | approx. RR: 65% | 65.0% |
| Glyoxal | Cornelis, 2015[16] | 8-hour HD | approx. RR: 62% | 62.0% |
| Glyoxal | Cornelis, 2015[16] | 4-hour HDF | approx. RR: 58% | 58.0% |
| Glyoxal | Cornelis, 2015[16] | canonical HD | approx. RR: 55% | 55.0% |
| Glyoxal | Agalou, 2005[19] | canonical HD | RR: 52 ± 9% for free fractions | 52.0% |
| Methylglyoxal | Cornelis, 2015[16] | 8-hour HD | approx. RR: 72% | 72.0% |
| Methylglyoxal | Cornelis, 2015[16] | 8-hour HDF | approx. RR: 70% | 70.0% |
| Methylglyoxal | Cornelis, 2015[16] | 4-hour HDF | approx. RR: 62% | 62.0% |
| Methylglyoxal | Cornelis, 2015[16] | canonical HD | approx. RR: 60% | 60.0% |
| Methylglyoxal | Agalou, 2005[19] | canonical HD | RR: 74 ± 8% for free fractions | 74.0% |
| 3-DG, free | Agalou, 2005[19] | canonical HD | RR: 70 ± 14% for free fraction | 70.0% |
| 3-DG | Cornelis, 2015[16] | 8-hour HD | approx. RR: 10% | 10.0% |
| 3-DG | Cornelis, 2015[16] | 8-hour HDF | approx. RR: 10% | 10.0% |
| 3-DG | Cornelis, 2015[16] | 4-hour HDF | approx. RR: 10% | 10.0% |
| 3-DG | Cornelis, 2015[16] | canonical HD | approx. RR: 10% | 10.0% |
| PAA | Brettschneider, 2013[35] | FPAD system | before 1377±227 after 223±75 µmol/l | 83.8% |
| PAA | Brettschneider, 2013[35] | high-flux HD | before 1201±506 after 443±122 µmol/l | 63.1% |
| Melatonin | Ludemann, 2001[36] | canonical HD | before 40.6 after 20.3 pg/ml, p< 0.001 | 50.0% |
| Melatonin | Vaziri, 1993[37] | single-pass, volumetric controlled dialysate delivery systems in HD | before 16.7±7.0 after 10.8±3.2 pg/ml, ns | same as healthy |
| Indican | Martinez, 2005[38] | membrane (Fresenius F70NR kidneys) in HD | RR: 30±7% | 30.0% |
| Thiocyanate | Pahl, 1982[39] | 2-hour HD | before 28.5 ug/ml after 17.8 ug/ml; n= 1 | 37.5% |
| Acrolein | Gugliucci, 2007[40] | canonical HD | Average decrease 32% | 32.0% |

*Abbreviations: FPAD – fractionated plasma separation, adsorption, and dialysis; HD – hemodialysis; HDF – hemodiafiltration; and OLHDF – on-line hemodiafiltration.*

# References


1. Röckel, A., Gilge, U., Liewald, A. & Heidland, A. Elimination of Low Molecular Weight Proteins During Hemofiltration. *Artif Organs* **6**, 307–311 (1982).
2. Sequera, P. De *et al.* A comparison of the effectiveness of two online haemodiafiltration modalities: mixed versus post-dilution. *Nefrologia* **33**, 779–787 (2013).
3. Maduell, F. *et al.* Mid-dilution hemodiafiltration: A comparison with pre- and postdilution modes using the same polyphenylene membrane. *Blood Purif* **28**, 268–274 (2009).
4. Meert, N. *et al.* Comparison of removal capacity of two consecutive generations of high-flux dialysers during different treatment modalities. *Nephrology Dialysis Transplantation* **26**, 2624–2630 (2011).
5. Krieter, D. H. *et al.* Clinical cross-over comparison of mid-dilution hemodiafiltration using a novel dialyzer concept and post-dilution hemodiafiltration. *Kidney Int* **67**, 349–356 (2005).
6. Krieter, D. H. *et al.* Matching efficacy of online hemodiafiltration in simple hemodialysis mode. *Artif Organs* **32**, 903–909 (2008).
7. Albalate Ramón, M. *et al.* Asymmetric cellulose triacetate is a safe and effective alternative for online haemodiafiltration. *Nefrologia* **38**, 315–320 (2018).
8. Wichman, Z., Pajdak, W., Smoleski, O. & Kope, J. Detection of plasma proteins during sequential ultrafiltration/dialysis. *Kidney Int* **28**, 558–565 (1985).
9. Testa, A., Gentilhomme, H., Le Carrer, D. & Orsonneau, J. L. In vivo removal of high- and low-molecular-weight compounds in hemodiafiltration with on-line regeneration of ultrafiltrate. *Nephron Clin Pract* **104**, 55–60 (2006).
10. Galli, F. *et al.* Glycoxidation and inflammatory markers in patients on treatment with PMMA-based protein-leaking dialyzers. *Kidney Int* **67**, 750–759 (2005).
11. Odetti, P. *et al.* Plasma advanced glycosylation end-products in maintenance haemodialysis patients. *Nephrol Dial Transplant* **10**, 2110–2113 (1995).
12. De Smet, R. *et al.* Effect of the super-flux cellulose triacetate dialyser membrane on the removal of non-protein-bound and protein-bound uraemic solutes. *Nephrol Dial Transplant* **22**, 2006–2012 (2007).
13. Tessitore, N. *et al.* Effect of protein leaking BK-F PMMA-based hemodialysis on plasma pentosidine levels. *J Nephrol* **17**, 707–714 (2004).
14. Friedlander, M. A., Wu, Y. C., Schulak, J. A., Monnier, V. M. & Hricik, D. E. Influence of dialysis modality on plasma and tissue concentrations of pentosidine in patients with end-stage renal disease. *Am J Kidney Dis* **25**, 445–51 (1995).
15. Jadoul, M. *et al.* Influence of hemodialysis membrane type on pentosidine plasma level, a marker of 'carbonyl stress'. *Kidney Int* **55**, 2487–2492 (1999).
16. Cornelis, T. *et al.* Protein-bound uraemic toxins, dicarbonyl stress and advanced glycation end products in conventional and extended haemodialysis and haemodiafiltration. *Nephrol Dial Transplant* **30**, 1395–1402 (2015).
17. Miyata, T. *et al.* Clearance of pentosidine, an advanced glycation end product, by different modalities of renal replacement therapy. *Kidney Int* **51**, 880–887 (1997).
18. Hohmann, C. *et al.* Detection of free advanced glycation end products in vivo during hemodialysis. *J Agric Food Chem* **65**, 930–937 (2017).
19. Agalou, S. *et al.* Advanced glycation end product free adducts are cleared by dialysis. *Ann N Y Acad Sci* **1043**, 734–739 (2005).
20. Friedlander, M. A., Wu, Y. C., Elgawish, A. & Monnier, V. M. Early and advanced glycosylation end products: Kinetics of formation and clearance in peritoneal dialysis. *Journal of Clinical Investigation* **97**, 728–735 (1996).
21. Meert, N. *et al.* Prospective evaluation of the change of predialysis protein-bound uremic solute concentration with postdilution online hemodiafiltration. *Artif Organs* **34**, 580–585 (2010).
22. Snauwaert, E. *et al.* Haemodiafiltration does not lower protein-bound uraemic toxin levels compared with haemodialysis in a paediatric population. *Nephrol Dial Transplant* **35**, 648–656 (2019).
23. Lenglet, A. *et al.* Does the administration of sevelamer or nicotinamide modify uremic toxins or endotoxemia in chronic hemodialysis patients? *Drugs* **79**, 855–862 (2019).
24. Meert, N. *et al.* Effective removal of protein-bound uraemic solutes by different convective strategies: a prospective trial. *Nephrol Dial Transplant* **24**, 562–570 (2009).

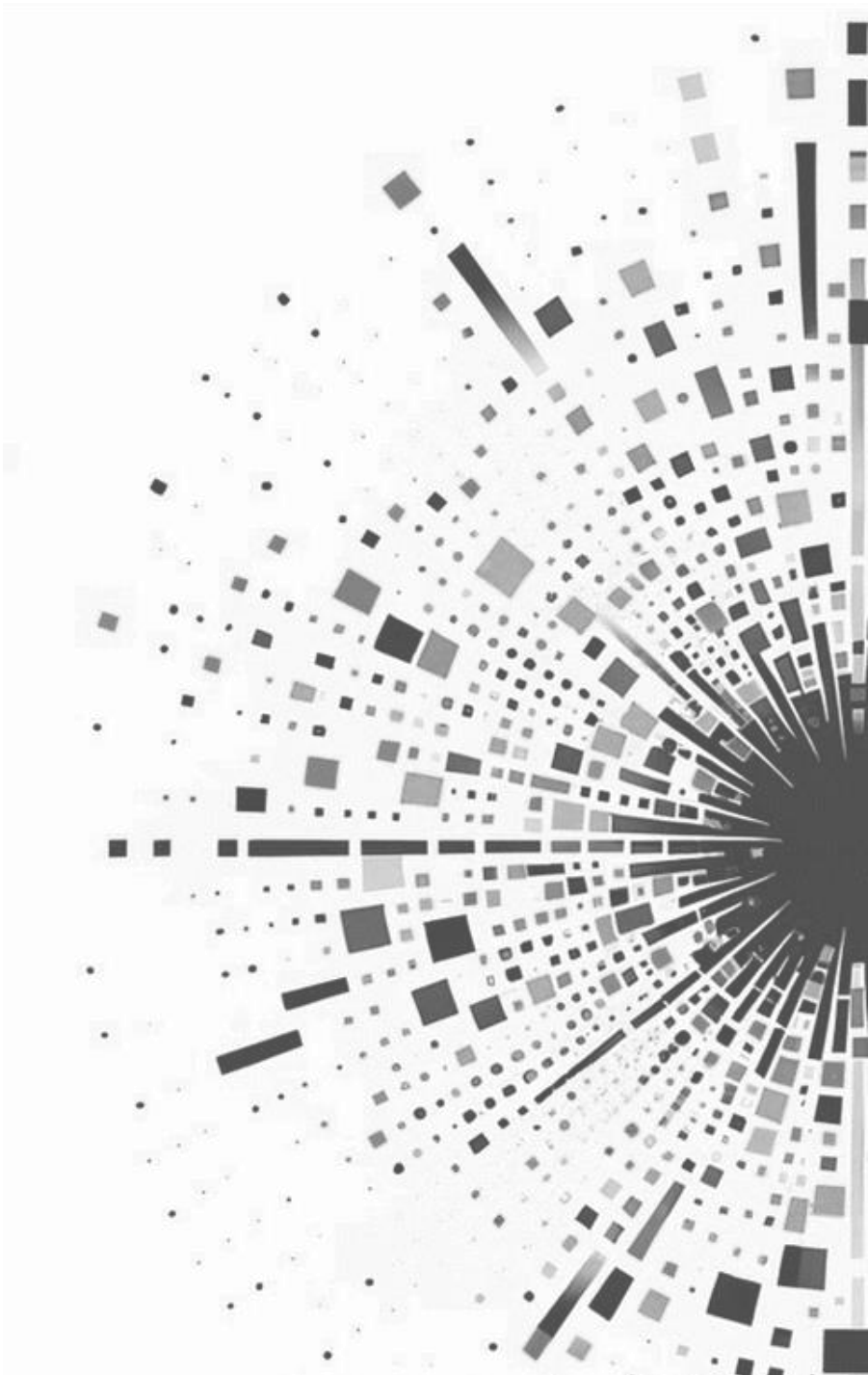
25. Eloot, S., Dhondt, A., Van Landschoot, M., Waterloos, M. A. & Vanholder, R. Removal of water-soluble and protein-bound solutes with reversed mid-dilution versus post-dilution haemodiafiltration. *Nephrol Dial Transplant* **27**, 3278–3283 (2012).
26. Fagugli, R. M., De Smet, R., Buoncristiani, U., Lameire, N. & Vanholder, R. Behavior of non-protein-bound and protein-bound uremic solutes during daily hemodialysis. *Am J Kidney Dis* **40**, 339–347 (2002).
27. Lesaffer, G. *et al.* Intradialytic removal of protein-bound uraemic toxins: role of solute characteristics and of dialyser membrane. *Nephrol Dial Transplant* **15**, 50–57 (2000).
28. Susantitaphong, P. *et al.* A simple efficient technique of 'mid-dilution' on-line hemodiafiltration. *Blood Purif* **28**, 93–101 (2009).
29. Riccio, E. *et al.* Evidence that p-cresol and IL-6 are adsorbed by the HFR cartridge: towards a new strategy to decrease systemic inflammation in dialyzed patients? *PLoS One* **9**, e95811 (2014).
30. Bammens, B. *et al.* Removal of the protein-bound solute p-cresol by convective transport: a randomized crossover study. *Am J Kidney Dis* **44**, 278–285 (2004).
31. van Gelder, M. K. *et al.* Protein-bound uremic toxins in hemodialysis patients relate to residual kidney function, are not influenced by convective transport, and do not relate to outcome. *Toxins (Basel)* **12**, 234 (2020).
32. Eloot, S. *et al.* Protein-bound solute removal during extended multipass versus standard hemodialysis. *BMC Nephrol* **16**, 57 (2015).
33. Etinger, A. *et al.* The effect of isohydric hemodialysis on the binding and removal of uremic retention solutes. *PLoS One* **13**, e0192770 (2018).
34. Luo, F. J.-G. *et al.* Effect of increasing dialyzer mass transfer area coefficient and dialysate flow on clearance of protein-bound solutes: a pilot crossover trial. *Am J Kidney Dis* **53**, 1042–1049 (2009).
35. Brettschneider, F. *et al.* Removal of Protein-Bound, Hydrophobic Uremic Toxins by a Combined Fractionated Plasma Separation and Adsorption Technique. *Artif Organs* **37**, 409–416 (2013).
36. Lüdemann, P., Zwernemann, S. & Lerchl, A. Clearance of melatonin and 6-sulfatoxymelatonin by hemodialysis in patients with end-stage renal disease. *J Pineal Res* **31**, 222–227 (2001).
37. Vaziri, N. D. *et al.* Serum melatonin and 6-sulfatoxymelatonin in end-stage renal disease: Effect of hemodialysis. *Artif Organs* **17**, 764–769 (1993).
38. Martinez, A. W., Recht, N. S., Hostetter, T. H. & Meyer, T. W. Removal of P-cresol sulfate by hemodialysis. *Journal of the American Society of Nephrology* **16**, 3430–3436 (2005).
39. M. V. V. Pahl, N. D., Pahl, M. V. & Vaziri, N. D. In-vivo and in-vitro hemodialysis studies of thiocyanate. *Clin Toxicol* **19**, 965–974 (1982).
40. Gugliucci, A. *et al.* Acrolein inactivates paraoxonase 1: changes in free acrolein levels after hemodialysis correlate with increases in paraoxonase 1 activity in chronic renal failure patients. *Clinica Chimica Acta* **384**, 105–112 (2007).

# Chapter 3

## Plaque Protein Carbamylation Is Associated with Atherosclerosis Progression and Uremia and Accumulates in Foam Cells

V Saar-Kovrov, A Pawlowska, A Guillot, MJJ Gijbels, GE Fazzi, JC Sluimer, BME Mees, F Tacke, J Jankowski, MMPC Donners, and EAL Biessen

*In preparation*

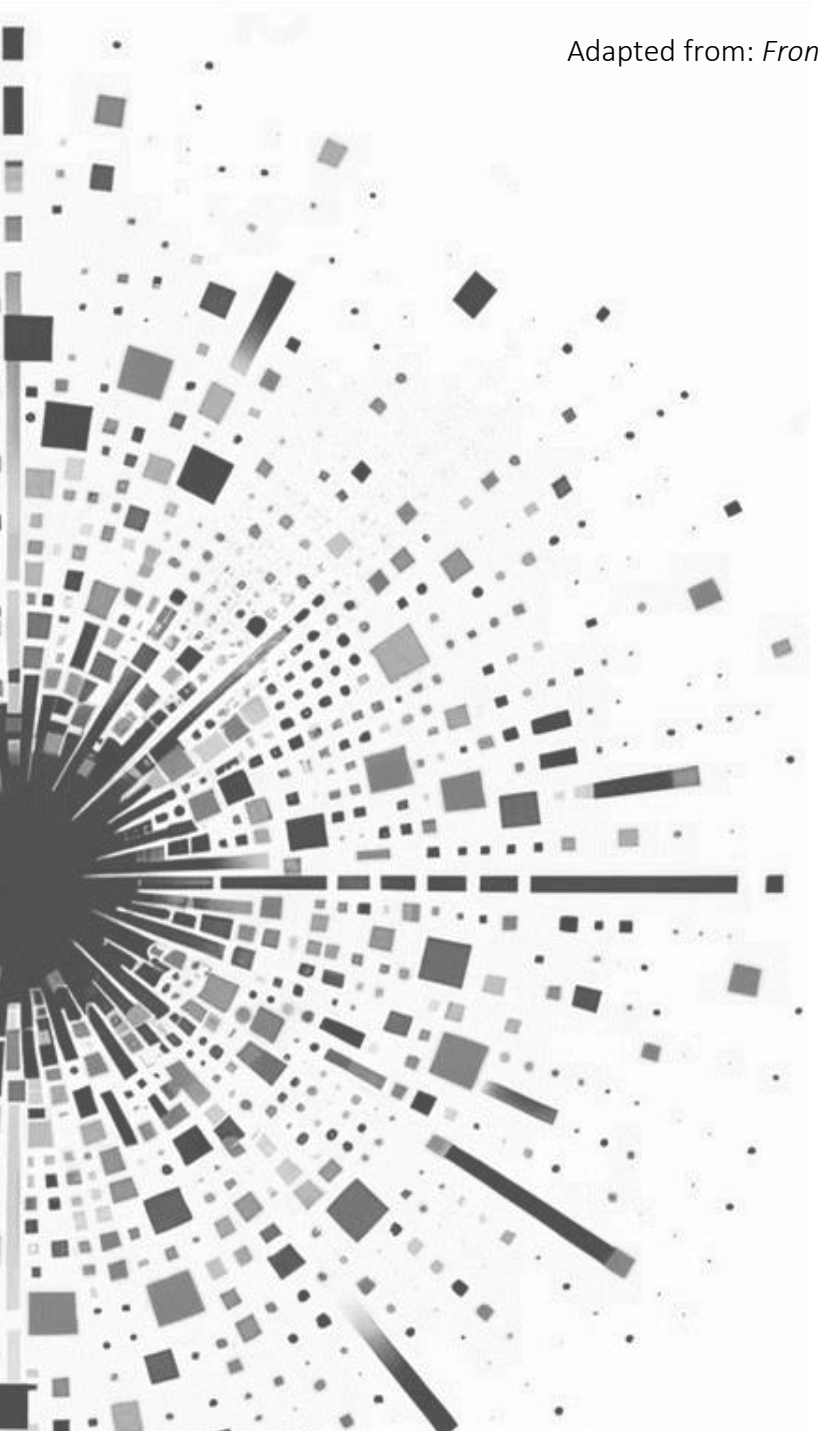


# Chapter 4

## Shedding of Klotho: Functional Implications in Chronic Kidney Disease and Associated Vascular Disease

V Saar-Kovrov, MMPC Donners, and EPC van der Vorst

Adapted from: *Front in Cardiovascular Medicine, 2021, 7:617842*



## Abstract

α-Klotho (Klotho) exists in two different forms, a membrane-bound and soluble form, which are highly expressed in the kidney. Both forms play an important role in various physiological and pathophysiological processes. Recently, it has been identified that soluble Klotho arises exclusively from shedding or proteolytic cleavage. In this review, we will highlight the mechanisms underlying the shedding of Klotho and the functional effects of soluble Klotho, especially in CKD and the associated cardiovascular complications.

Klotho can be cleaved by a process called shedding, releasing the ectodomain of the transmembrane protein. A disintegrin and metalloproteases ADAM10 and ADAM17 have been demonstrated to be mainly responsible for this shedding, resulting in either full-length fragments or sub-fragments called KL1 and KL2.

Reduced levels of soluble Klotho have been associated with kidney disease, especially chronic kidney disease (CKD). In line with a protective effect of soluble Klotho in vascular function and calcification, CKD and the reduced levels of soluble Klotho herein are associated with cardiovascular complications. Interestingly, although it has been demonstrated that soluble Klotho has a multitude of effects its direct impact on vascular cells and the exact underlying mechanisms remain largely unknown and should therefore be a major focus of further research. Moreover, functional implications of the cleavage process resulting in KL1 and KL2 fragments remain to be elucidated.

## Introduction

α-Klotho (Klotho) is a type I transmembrane protein which is highly conserved among human, mouse and rat (up to 94% homological sequence)[1] and primarily expressed in the kidney, in both proximal and distal tubuli, though some expression has been shown in choroid plexus, parathyroid gland and sinoatrial node[2]. Membrane-bound Klotho plays an important role in a wide range of physiological and pathophysiological processes, as recently reviewed in[3]. For example, Klotho has not only been demonstrated to play an important role in renal function [4] and controls the brain-immune system interface[5]. The membrane-bound Klotho protein forms a complex with the fibroblast growth factor receptors (FGFR), which is crucial for the binding of FGF23[6]. FGF23 exerts several endocrine functions, like regulating phosphate, calcium and vitamin D homeostasis[7]. Dysregulation of this FGF23-Klotho axis is not only associated with chronic kidney disease[8], but also with vascular and skeletal anomalies which are mainly caused by an altered phosphate turnover, as reviewed in[9]. This abnormal phosphate regulation is the mechanism by which deficiency of FGF23 and Klotho is associated with accelerated aging[6,10], which can be rescued by low phosphate diet feeding to restore the phosphate balance[11].

Besides this membrane-bound form, Klotho is also released in soluble form. This soluble Klotho is detectable in cerebrospinal fluid, after being shed from the choroid plexus, and in urine and blood, after being mainly shed from the kidney[12,13]. Soluble Klotho has not only a local impact on renal function but also systemic effects on the cardiovascular system (see **Chapter 3**). Initially this soluble form was believed to arise from both proteolytic cleavage, a process called shedding, as well as secretion of an alternatively spliced Klotho variant. This spliced variant has a 50bp insertion containing an in-frame stop codon, resulting in a truncated Klotho protein[14]. Interestingly, this spliced, truncated version of Klotho has not been detected *in vivo* thus far[3,13,15]. Recently, Mencke *et al.* described that this alternatively spliced variant is subjected to nonsense-mediated mRNA decay and therefore not secreted[15]. Soluble Klotho thus solely arises from the shedding process, which will be further elaborated on in the next section.

## Klotho Shedding

Various type I transmembrane protein, like Klotho, can be cleaved by a process called shedding, which releases the ectodomain of the transmembrane protein[16]. In this manner, shedding is a post-translational modification that controls the levels and function of hundreds of membrane proteins. Alpha secretases (e.g. “a disintegrin and metalloprotease” (ADAM)) as well as beta-secretases ( e.g. “beta-site APP cleaving enzyme” (BACE)) have been described as the main sheddases, although in recent years a broader range of proteases has been identified to play a role in protein shedding[16].

Regarding Klotho, both ADAM10 and ADAM17 were shown to be responsible for its shedding. Chen *et al.* demonstrated that overexpression of either ADAM10 or ADAM17 in Klotho-

transfected COS-7 cells increased release of soluble Klotho, while this shedding could be abolished by using the metalloprotease inhibitor TAPI-1[17]. Similarly, the metalloprotease inhibitor TNF484 or the ADAM10-selective inhibitor GI254023X inhibited endogenous Klotho shedding in HEK cells[18]. Interestingly, besides the 130-kDa full-length ectodomain Klotho product, another smaller Klotho product of approximately 70-kDa could also be detected in the Klotho-expressing COS-7 cells[17]. Based on the predicted primary structure it is known that the extracellular domain of Klotho consists of two tandem internal repeats, KL1 and KL2 (**Figure 1**), which only share 21% amino acid identity[19]. As the antibodies used for Klotho detection specifically recognize the KL1 domain[20], the smaller product should correspond to the cleaved KL1 domain, which was also confirmed using mass spectrometry by other groups[17,18]. The full-length shed extracellular Klotho domain was shown to be much more abundant in the cell media than the cleaved KL1, and the cleavages that produce these forms have been termed α- and β-cut, respectively[17]. For the β-cut, it could be demonstrated that membrane anchoring is essential as transfection of COS-7 cells with a truncated version of Klotho, lacking the transmembrane domain, did not generate detectable KL1 products in the medium or cell lysate[17]. On the other hand, it seems that this anchoring is not necessary for the α-cut, releasing full-length Klotho in the cell media[17]. Interestingly, treatment of the Klotho-expressing cells with a broad metalloprotease inhibitor Timp-3 significantly reduced the amount of KL1 not only in the medium but also in the cell lysate samples, indicating that the β-cut also takes place intracellularly[17].

To determine the exact Klotho cleavage sites of the proteinases ADAM10 and ADAM17, highly conserved regions of 34 known substrates for ADAM10 and ADAM17[21] were analyzed and two potential recognition sites in the proximity of the Klotho transmembrane domain could be identified[22]. Transfection of COS-7 cells with Klotho constructs in which these sites were mutated demonstrated that deletion of amino acids at positions 958 and 959 at the juxtamembrane site decreased soluble Klotho levels by 50-60%[22]. Furthermore, deletion of the region between amino acids 954−962 almost completely abolished the presence of the 130-kDa product in the medium and the 70-kDa product in both medium and cell lysate, suggesting that not only membrane anchoring[17] but also intact α-cut sequence is required for the β-cut to occur. Overexpression of ADAM10 or ADAM17 did not result in increased shedding of the mutated Klotho, while it did in case of the intact protein, primarily when co-transfected with ADAM17[22]. In contrast to previous observations[17], however, co-transfection of the cells with ADAM10 did not increase Klotho shedding[22]. Hence, it seemed that shedding by ADAM17 is prevailing over that by ADAM10 in COS-7 cells. Nonetheless, overexpression of ADAM10 in Klotho-expressing HEK293 cells did increase the amount of soluble Klotho in the medium, as shown by Bloch *et al* [23]. Therefore, the exact involvement of ADAM10 in Klotho shedding compared to ADAM17 remains to be further evaluated. The fact that ADAM10 is predominantly responsible for the constitutive shedding of many of its substrates, while ADAM17 is implicated in induced shedding events[24], might explain the discrepancies regarding Klotho shedding in the different studies and cell-types.

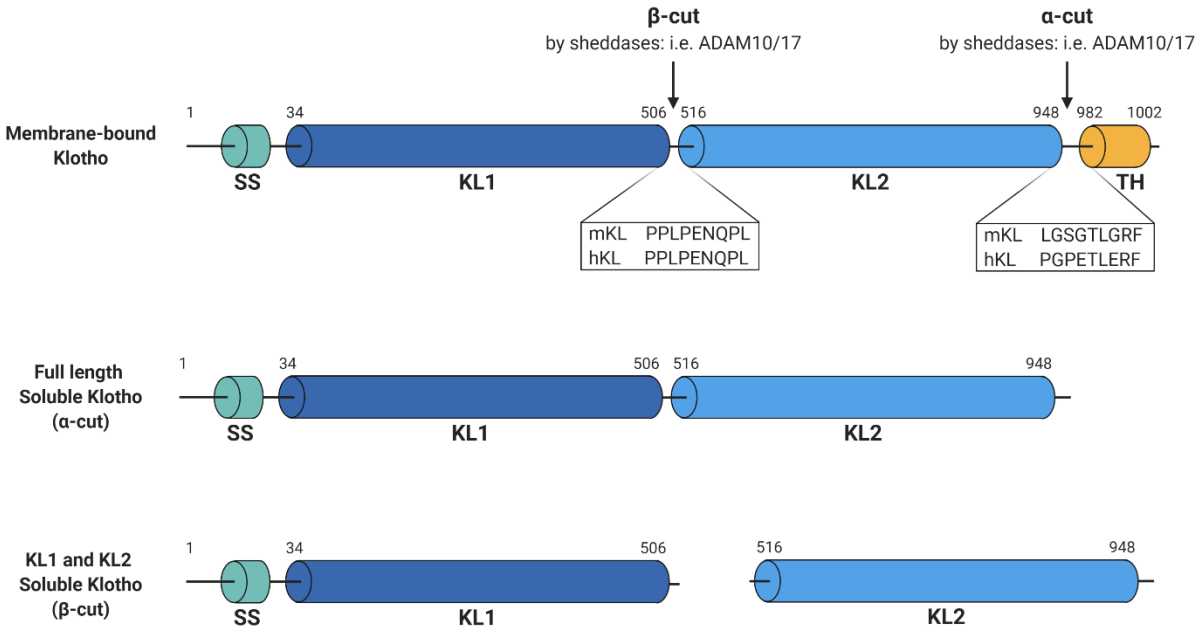



**Figure 1. Schematic overview of Klotho structure and cleavage.** Membrane-bound Klotho consists of four main domains, being the signal sequence (SS), KL1, KL2, and the transmembrane domain (TM). Several sheddases can cut this membrane-bound Klotho forming full-length soluble Klotho (a-cut) or KL1 and KL2 soluble Klotho (both a- and b-cut). Illustrated sequences reflect the suggested cleavage sites at which these cuts occur. Figure is created with BioRender.com.

Using a similar approach, by analyzing the ADAMs' substrate compilation from Caescu *et al.*[21], the precise region of the β-cut could be identified as well[25]. This was confirmed in COS-7 cells by transfecting the cells with Klotho mutated at the predicted β-cut site, which completely abolished KL1 fragments in the cell lysates and media[25]. Moreover, the full-length Klotho product was also significantly decreased indicating that successful Klotho cleavage at either of the cuts is dependent on intact sequences at both sites, and that both α- and β-cut can occur simultaneously[25]. However, it is difficult to determine whether the reduced cleavage of Klotho is caused by a mutation of the recognition sequence itself or due to potential conformational changes induced by the mutation that would render the cleavage site inaccessible for the proteinases. In either case, it also remains to be determined whether both ADAM10 and ADAM17 are responsible for both cleavages equally or if one of them is dominating in certain conditions.

Besides ADAM10 and ADAM17, Klotho was also shown to be shed by a β-secretase β-APP cleaving enzyme 1 (BACE1), as BACE1-specific siRNAs treatment of Klotho-expressing HEK293 cells resulted in a significantly decreased soluble full-length Klotho protein in the media[23]. In line with this observation, overexpression of BACE1 in these cells increased the amount of shed Klotho[23]. In addition, the remaining transmembrane Klotho domain is further processed by γ-secretase, since the small 5-kDa product corresponding to the Klotho stub was only visible when the cells were treated with γ-secretase inhibitors[23]. Unfortunately, the exact cleavage

site of BACE1 has not yet been elucidated and remains an interesting focus of future research. Nevertheless, these results are in line with previous findings that several type-I transmembrane proteins can be processed by α- and β-, as well as γ-secretases[16]. Shedding by different secretases results in distinct fragments with specific properties. Such divergent effects could, for example, already be shown for amyloid precursor protein (APP; reviewed in[26]). Shedding of APP by an α-secretase, mainly by ADAM10, generates a soluble APP fragment that has neuroprotective properties[27]. In sharp contrast, shedding of APP by the β-secretase BACE1 is amyloidogenic and results in the formation of amyloid β which is a major component of amyloid plaques in Alzheimer's disease[28]. Due to the similarities in Klotho and APP structure and processing, it would be highly interesting to investigate the individual roles of ADAMs and BACE1 in soluble Klotho formation and determine the presence of any functional differences between the products of the different cleavages.

## Functional role of soluble Klotho in disease

### *3.1. Soluble Klotho as a biomarker for renal disease*

As the kidney is the main source of soluble Klotho, it is not surprising that soluble levels of Klotho drastically drop in patients with CKD. Soluble Klotho levels have been observed to decrease in both blood and urine upon CKD progression[29,30]. Strikingly, this drop already occurs very early in disease development, in CKD stage 2 and often even already in CKD stage 1[31]. Associated with the reduced Klotho levels, FGF23 and mineral parameters increase during CKD development[32,33]. Although the decreased soluble levels have been attributed to decreased expression of membrane Klotho[32], it may also arise from reduced shedding capacity, however this fact remains to be determined. In line with an important local role for soluble Klotho in the kidney, higher soluble Klotho levels are also independently associated with a lower risk of decline in renal function[34]. Several studies have investigated whether soluble Klotho could be a potential biomarker for CKD or at least for impaired kidney function[8]. However, so far, the outcomes are rather contradictory and therefore not conclusive, especially since mostly small cohorts were investigated. Another limitation for this research field is the fact that soluble Klotho is difficult to reliably measure in patient material[8] as, for example, Klotho levels seem to be correlated with age[35]. Furthermore, it is not possible to distinguish between full length soluble Klotho and the KL1 or KL2 fragments, although it remains to be elucidated whether these different products exert distinct functions. Therefore, further studies and larger cohorts need to be screened before drawing conclusions about the potential use of soluble Klotho as biomarker.

### *3.2. Clinical relevance of Klotho for renal disease from mice models*

When originally discover by Kuro-o and colleagues, Klotho mutation in mice caused severe manifestations of premature aging and significantly shortened life expectancy[19]. Full Klotho knockout mice (Klotho -/-) do not display any abnormalities until the week 3-4 of age, however

further development is arrested, and mice generally die at the age of 8-9 weeks. Klotho -/- mice also exhibit cardiac dysfunction, sterility, skin atrophy, Monckeberg type arteriosclerosis, ectopic calcifications as well as decline in renal function[36,37]. Full Klotho knockout mice are too fragile and rarely survive surgery[38], which makes them a difficult model to work with. Notwithstanding, heterozygous Klotho-deficient mice (Klotho +/-) display less striking phenotype and their life expectancy is comparable to the wild type mice. At the later age, however, Klotho +/- mice develop impaired kidney function with glomerulosclerosis, interstitial fibrosis and increased albuminuria[39,40]. These mice were shown to be more prone to develop pathological response to injury, such as unilateral ureteral obstruction (UUO) or bilateral ischemia-reperfusion injury (IRI), which significantly exacerbated kidney fibrosis in the Klotho +/- mice comparing to the wild type[41,42]. On the other hand, mice ubiquitously overexpressing Klotho seemed to be protected against renal function deterioration in case of the acute kidney injury as well as in glomerulonephritis model[42–44]. Moreover, using adeno-associated virus (AAV)-mediated gene transfer of Klotho it could be observed that delivery of Klotho has beneficial effects in not only acute kidney injury, but also in CKD models[45,46]. Additionally, administration of recombinant soluble Klotho showed comparable effects as it reduced renal fibrosis in AKI and UUO, suggesting these effects are primarily driven by soluble Klotho[29,47–49]. Besides local renal functions, soluble Klotho has also been shown to have systemic impact on the cardiovascular system like for example on vascular calcification as described below. A short overview of mice models used for studying effects of Klotho on renal and cardiovascular system is given in **Table 1**.

| | *Mouse model* | **Reported outcome** | **Reference** |
|---|---|---|---|
| **Klotho in renal disease** | Aged Klotho +/- mice | Impaired kidney function with glomerulosclerosis, interstitial fibrosis and increased albuminuria | Zhou, 2015[39]<br>Zhou, 2016[40] |
| | UUO in Klotho +/- mice | Exacerbated kidney fibrosis | Sugiura, 2012[41] |
| | UUO in Klotho transgenic mice<br><br>UUO in Klotho +/- mice | Reduced tubulointerstitial fibrosis<br><br>Enhanced tubulointerstitial fibrosis | Satoh, 2012[44] |
| | Recombinant Klotho treatment in UUO | Alleviation of UUO-induced EndoMT, reduced fibrosis and improved kidney function | Li, 2019[48] |
| | Bilateral IRI in Klotho +/- mice<br><br>IRI in Klotho transgenic mice<br><br>Recombinant Klotho treatment after AKI in mice or rats | Faster progression to CKD<br><br>Improved preservation of kidney function after AKI<br><br>Accelerated recovery and reduced renal fibrosis | Shi 2016[42]<br>Hu, 2010[29] |
| | Adenoviral delivery of Klotho in rats with IRI | Reduced renal damage | Sugiura, 2005[46] |
| | ICR-derived glomerulonephritis in Klotho transgenic mice | Improved renal function and survival | Haruna, 2007[43] |
| | Klotho +/- mice or transgenic mice with uni-Nx with IRI in contralateral kidney | Reduced or improved renal function and vascular calcification | Hu, 2011[37] |
| **Klotho in cardiovascular complications** | Klotho -/- mice with diabetic nephropathy<br><br>AAV-mediated delivery of soluble Klotho | Hyperphosphatemia and enhanced vascular calcification<br><br>Rescued phosphate levels and prevention of calcification | Hum, 2017[45] |
| | Aortic ring culture and hind limb ischemia in Klotho -/- and -/+ mice | Impaired angiogenesis and vasculogenesis | Shimada, 2004[50] |
| | Klotho -/- and +/- mice | Impaired vasodilation/vasorelaxation, rescued by parabiosis with wt mice | Saito, 1998[51] |
| | Klotho +/- mice | Cardiac dysfunction, hypertrophy, and fibrosis | Hu, 2015[52] |
| | Klotho administration in mice with uni-Nx with IRI in contralateral kidney | Attenuated CKD-associated cardiac remodeling | Hu, 2017[49] |

*Abbreviations: UUO - unilateral ureteral obstruction; EndoMT - endothelial-to-mesenchymal transition; IRI - ischemia-reperfusion injury; CKD - chronic kidney disease; AKI - acute kidney injury; Nx - nephrectomy; and wt - wild type*.

### *3.3. Klotho in cardiovascular complications of renal disease*

Vascular calcification appears early in the course of CKD and becomes more prevalent as kidney function decreases and thereby causes a high risk of cardiovascular mortality in patients with CKD[4,53]. Obviously, considering its major role in regulating mineral (Ca/phosphate) homeostasis, Klotho deficiency causes high circulating phosphate levels and thereby strongly enhances vascular calcification in mice with CKD[37]. It could be also shown that delivery of AAV expressing soluble Klotho into Klotho deficient mice reduces phosphate levels and, in line with this, vascular calcification[45]. Moreover, Klotho deficiency in CKD enhances renal tubule and vascular cell senescence which impairs angiogenesis and vasculogenesis[50]. Together, these results clearly demonstrate that Klotho plays a protective role in vascular calcification and CKD, although cell-specific effects remain rather elusive. These protective effects of Klotho are probably mostly indirect in nature as they are related to its ability to regulate the effects of several growth factors such as FGF23, and ion-channels, as discussed before. However, soluble Klotho also suppresses the activity of the WNT/β-catenin pathway in stem and progenitor cells in a direct manner, which has been shown to be important not only for vascular calcification, but also aging[54]. Interestingly, WNT/β-catenin, in turn, inhibits renal Klotho expression. Via this loop Klotho and WNT signaling interact and play an important role in CKD and associated complications[55].

Furthermore, elevated levels of soluble Klotho in plasma are independently associated with a lower risk of cardiovascular disease[56]. This can at least partly be explained by the observed vasculoprotective effects of soluble Klotho on the endothelium, as production of nitric oxide and vasodilation are impaired in heterozygous Klotho deficient rodent[51]. Additionally, soluble Klotho has been identified as an anti-inflammatory modulator, since a bidirectional negative relationship between Klotho and NF-κB could be identified in which Klotho impairs translocation and hence activation of NF-κB in cultured endothelial cells[57]. Thereby Klotho also suppresses expression of the adhesion molecules intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in endothelial cells[57]. Klotho also reduces the expression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), a major receptor for oxidized LDL, in cultured endothelial cells[58]. However, the direct impact on vascular cells and the exact mechanism of action remains poorly understood, especially as receptors that mediate the effects remain largely unknown and the effects of soluble Klotho seem to be at least partially FGFR23 independent[59]. Not surprisingly, as all of the described mechanisms play a crucial role in atherosclerosis development, it could be shown that lower levels of serum soluble Klotho were associated with increased carotid artery intima-media thickness and could thereby be considered an early predictor of atherosclerosis[56,60]. Although the ectopic expression of Klotho is still under debate, recent studies demonstrate its expression in

cardiomyocytes and highlight the impact of Klotho on cardiac diseases, like myocardial infarction and left ventricular hypertrophy, as reviewed in[61,62]. In line with this, subjects at high risk for atherosclerotic/cardiovascular events have a reduced expression of Klotho in cardiomyocytes[63], associated with increased oxidative stress, inflammation and fibrosis, although the direct impact of Klotho on cardiomyocytes has not been examined in this study.

## Discussion and future directions

Over the course of more than two decades after the serendipitous identification of the Klotho protein[19], research has focused on elucidating the exact function of this protein in health and disease. It has already been described that Klotho plays a role in a multitude of processes and this list will only grow further over time. At the moment, the role of Klotho in aging, kidney disease, more particularly CKD, and the vasculature is quite well described. Yet, the direct vs indirect functions of soluble Klotho on vascular cells, receptor(s) involved, and the exact underlying mechanisms of action remain largely unknown or contradictory and should therefore be a focus of future research.

Recently, it was demonstrated that all of the soluble Klotho arises from shedding of membrane-bound Klotho as the alternatively spliced variant is subject to nonsense-mediated mRNA decay and degraded[15]. While ADAM10, ADAM17 as well as BACE have been implicated in Klotho shedding, other proteases may be involved as well. Shedding of Klotho can result in different fragments, being either a full-length fragment or smaller sub-fragments called KL1 and KL2. However, until now most studies did not clearly distinguish between these different fragments or mainly used full-length soluble Klotho. Therefore, further studies are needed to elucidate which fragments are produced by the different shedding enzymes and determine the specific functional implication of the cleavage process resulting in KL1 and KL2 fragments.

In order to fully comprehend the function of soluble Klotho and to enable potential therapeutic targeting it is highly important that future research focuses on the elucidation of the exact underlying mechanisms. Only recently the crystal structure of Klotho has been described elucidating the exact structure of Klotho protein[64]. This might give an important impulse to the research field. Recently, a potential mechanism of action of soluble Klotho has been suggested, as it could be identified that soluble Klotho binds to membrane lipid rafts which alters the lipid organization in the cell membrane[65,66]. However, further functional studies are needed to elucidate the importance of this interaction.

In conclusion, soluble Klotho plays an important role in health and disease and is thereby a promising therapeutic target. However, further research is first needed to improve our understanding of the exact effects and especially the regulation of Klotho shedding.

## Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author Contributions

V.S.K. wrote the manuscript
M.M.P.C.D. made critical revisions to the manuscript
E.P.C.v.d.V. wrote the manuscript

## Funding

The authors' research is supported by a grant from the Interdisciplinary Center for Clinical Research within the faculty of Medicine at the RWTH Aachen University, the DZHK (German Centre for Cardiovascular Research) and the BMBF (German Ministry of Education and Research), and NWO-ZonMw Veni (91619053) to E.P.C.v.d.V. This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 764474 (CaReSyAn).

## Acknowledgments

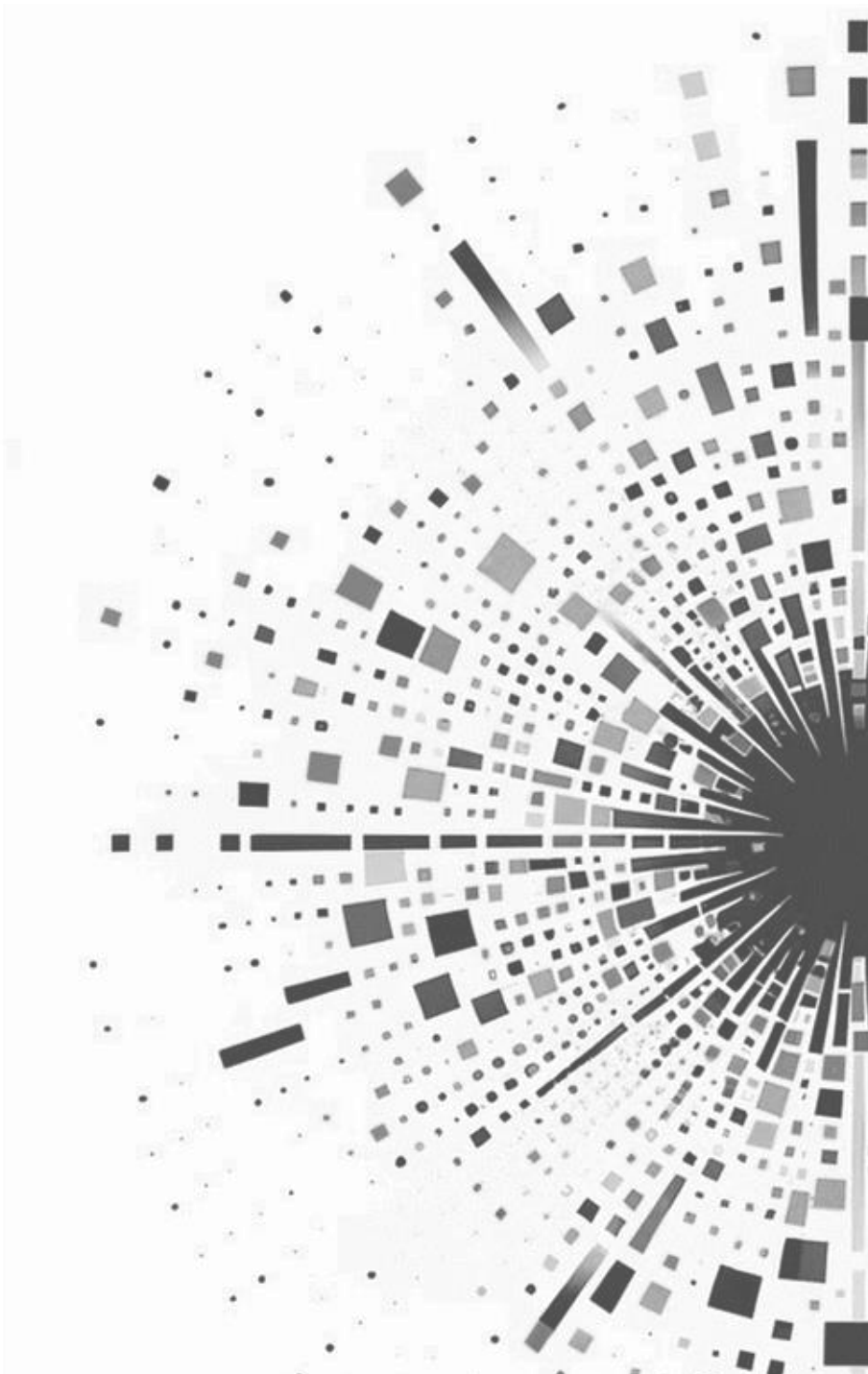
Figure has been prepared using BioRender.com.

# References


1. Wang, Y., Kuro-o, M. & Sun, Z. Current understanding of klotho. *Ageing Res Rev* **8**, 43–51 (2009).
2. Dalton, G. D., Xie, J., An, S. W. & Huang, C. L. New insights into the mechanism of action of soluble klotho. *Front Endocrinol (Lausanne)* **8**, (2017).
3. Kuro-o, M. The Klotho proteins in health and disease. *Nat Rev Nephrol* **15**, 27–44 (2019).
4. Buchanan, S., Combet, E., Stenvinkel, P. & Shiels, P. G. Klotho, Aging, and the Failing Kidney. *Front Endocrinol (Lausanne)* **11**, (2020).
5. Zhu, L. *et al.* Klotho controls the brain–immune system interface in the choroid plexus. *Proc Natl Acad Sci U S A* **115**, E11388–E11396 (2018).
6. Hu, M. C., Shiizaki, K., Kuro-O, M. & Moe, O. W. Fibroblast growth factor 23 and klotho: Physiology and pathophysiology of an endocrine network of mineral metabolism. *Annu Rev Physiol* **75**, 503–533 (2013).
7. Bär, L., Stournaras, C., Lang, F. & Föller, M. Regulation of fibroblast growth factor 23 (FGF23) in health and disease. *FEBS Lett* **593**, 1879–1900 (2019).
8. Zou, D., Wu, W., He, Y., Ma, S. & Gao, J. The role of klotho in chronic kidney disease. *BMC Nephrol* **19**, (2018).
9. Razzaque, M. S. The FGF23-Klotho axis: Endocrine regulation of phosphate homeostasis. *Nat Rev Endocrinol* **5**, 611–619 (2009).
10. Shimada, T. *et al.* Targeted ablation of Fgf23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. *Journal of Clinical Investigation* **113**, 561–568 (2004).
11. Stubbs, J. R. *et al.* Role of hyperphosphatemia and 1,25-dihydroxyvitamin D in vascular calcification and mortality in fibroblastic growth factor 23 null mice. *Journal of the American Society of Nephrology* **18**, 2116–2124 (2007).
12. Hu, M. C. *et al.* Renal Production, Uptake, and Handling of Circulating αklotho. *Journal of the American Society of Nephrology* **27**, 79–90 (2016).
13. Imura, A. *et al.* Secreted Klotho protein in sera and CSF: Implication for post-translational cleavage in release of Klotho protein from cell membrane. *FEBS Lett* **565**, 143–147 (2004).
14. Matsumura, Y. *et al.* Identification of the human klotho gene and its two transcripts encoding membrane and secreted klotho protein. *Biochem Biophys Res Commun* **242**, 626–630 (1998).
15. Mencke, R. *et al.* Human alternative Klotho mRNA is a nonsense-mediated mRNA decay target inefficiently spliced in renal disease. *JCI Insight* **2**, (2017).
16. Lichtenthaler, S. F., Lemberg, M. K. & Fluhrer, R. Proteolytic ectodomain shedding of membrane proteins in mammals—hardware, concepts, and recent developments. *EMBO J* **37**, 99456 (2018).
17. Chen, C. Di, Podvin, S., Gillespie, E., Leeman, S. E. & Abraham, C. R. Insulin stimulates the cleavage and release of the extracellular domain of Klotho by ADAM10 and ADAM17. *Proc Natl Acad Sci U S A* **104**, 19796–19801 (2007).
18. van Loon, E. P. M. M. *et al.* Shedding of klotho by ADAMs in the kidney. *Am J Physiol Renal Physiol* **309**, F359–F368 (2015).
19. Kuro-o, M. *et al.* Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* **390**, 45–51 (1997).
20. Kato, Y. *et al.* Establishment of the anti-klotho monoclonal antibodies and detection of klotho protein in kidneys. *Biochem Biophys Res Commun* **267**, 597–602 (2000).
21. Caescu, C. I., Jeschke, G. R. & Turk, B. E. Active-site determinants of substrate recognition by the metalloproteinases TACE and ADAM10. *Biochemical Journal* **424**, 79–88 (2009).
22. Chen, C. Di *et al.* Identification of cleavage sites leading to the shed form of the anti-aging protein klotho. *Biochemistry* **53**, 5579–5587 (2014).
23. Bloch, L. *et al.* Klotho is a substrate for α-, β- and γ-secretase. *FEBS Lett* **583**, 3221–3224 (2009).
24. van der Vorst, E., Weber, C. & Donners, M. A Disintegrin and Metalloproteases (ADAMs) in Cardiovascular, Metabolic and Inflammatory Diseases: Aspects for Theranostic Approaches. *Thromb Haemost* **118**, 1167–1175 (2018).
25. Chen, C. Di *et al.* Identification of the cleavage sites leading to the shed forms of human and mouse antiaging and cognition-enhancing protein Klotho. *PLoS One* **15**, (2020).
26. Zheng, H. & Koo, E. H. The amyloid precursor protein: beyond amyloid. *Mol Neurodegener* **1**, 5 (2006).
27. Chasseigneaux, S. & Allinquant, B. Functions of Aβ, sAPPα and sAPPβ : similarities and differences. *J Neurochem* **120**, 99–108 (2012).

28. Cole, S. L. & Vassar, R. The Role of Amyloid Precursor Protein Processing by BACE1, the β-Secretase, in Alzheimer Disease Pathophysiology. *Journal of Biological Chemistry* **283**, 29621–29625 (2008).
29. Hu, M. C. *et al.* Klotho deficiency is an early biomarker of renal ischemia-reperfusion injury and its replacement is protective. *Kidney Int* **78**, 1240–1251 (2010).
30. Izquierdo, M. C. *et al.* Klotho, phosphate and inflammation/ageing in chronic kidney disease. *Nephrology Dialysis Transplantation* **27**, iv6–iv10 (2012).
31. Pavik, I. *et al.* Secreted Klotho and FGF23 in chronic kidney disease Stage 1 to 5: A sequence suggested from a cross-sectional study. *Nephrology Dialysis Transplantation* **28**, 352–359 (2013).
32. Barker, S. L. *et al.* The demonstration of αKlotho deficiency in human chronic kidney disease with a novel synthetic antibody. *Nephrology Dialysis Transplantation* **30**, 223–233 (2015).
33. Dhayat, N. A. *et al.* Fibroblast growth factor 23 and markers of mineral metabolism in individuals with preserved renal function. *Kidney Int* **90**, 648–657 (2016).
34. Drew, D. A. *et al.* Association between soluble klotho and change in kidney function: The health aging and body composition study. *Journal of the American Society of Nephrology* **28**, 1859–1866 (2017).
35. Yamazaki, Y. *et al.* Establishment of sandwich ELISA for soluble alpha-Klotho measurement: Age-dependent change of soluble alpha-Klotho levels in healthy subjects. *Biochem Biophys Res Commun* **398**, 513–518 (2010).
36. Nabeshima, Y. Klotho deficient mouse: an in vivo model for human aging. *Drug Discov Today Dis Models* **1**, 223–227 (2004).
37. Hu, M. C. *et al.* Klotho deficiency causes vascular calcification in chronic kidney disease. *Journal of the American Society of Nephrology* **22**, 124–136 (2011).
38. Mencke, R., Olauson, H. & Hillebrands, J. L. Effects of Klotho on fibrosis and cancer: A renal focus on mechanisms and therapeutic strategies. *Advanced Drug Delivery Reviews* vol. 121 85–100 Preprint at https://doi.org/10.1016/j.addr.2017.07.009 (2017).
39. Zhou, X., Chen, K., Lei, H. & Sun, Z. Klotho gene deficiency causes salt-sensitive hypertension via monocyte chemotactic protein-1/CC chemokine receptor 2-mediated inflammation. *Journal of the American Society of Nephrology* **26**, 121–132 (2015).
40. Zhou, X. *et al.* Antiaging gene klotho regulates adrenal CYP11B2 expression and aldosterone synthesis. *Journal of the American Society of Nephrology* **27**, 1765–1776 (2016).
41. Sugiura, H. *et al.* Reduced klotho expression level in kidney aggravates renal interstitial fibrosis. *Am J Physiol Renal Physiol* **302**, 1252–1264 (2012).
42. Shi, M. *et al.* Aklotho Mitigates Progression of Aki To Ckd Through Activation of Autophagy. *Journal of the American Society of Nephrology* **27**, 2331–2345 (2016).
43. Haruna, Y. *et al.* Amelioration of progressive renal injury by genetic manipulation of Klotho gene. *Proc Natl Acad Sci U S A* **104**, 2331–2336 (2007).
44. Satoh, M. *et al.* Klotho protects against mouse renal fibrosis by inhibiting Wnt signaling. *Am J Physiol Renal Physiol* **303**, 1641–1651 (2012).
45. Hum, J. M. *et al.* Chronic hyperphosphatemia and vascular calcification are reduced by stable delivery of soluble klotho. *Journal of the American Society of Nephrology* **28**, 1162–1174 (2017).
46. Sugiura, H. *et al.* Klotho reduces apoptosis in experimental ischaemic acute renal failure. *Nephrology Dialysis Transplantation* **20**, 2636–2645 (2005).
47. Doi, S. *et al.* Klotho inhibits transforming growth factor-β1 (TGF-β1) signaling and suppresses renal fibrosis and cancer metastasis in mice. *Journal of Biological Chemistry* **286**, 8655–8665 (2011).
48. Li, S., Yu, L., He, A. & Liu, Q. Klotho Inhibits Unilateral Ureteral Obstruction-Induced Endothelial-to-Mesenchymal Transition via TGF-β1/Smad2/Snail1 Signaling in Mice. *Front Pharmacol* **10**, 348 (2019).
49. Hu, M. C. *et al.* Recombinant α-Klotho may be prophylactic and therapeutic for acute to chronic kidney disease progression and uremic cardiomyopathy. *Kidney Int* **91**, 1104–1114 (2017).
50. Shimada, T. *et al.* Angiogenesis and vasculogenesis are impaired in the precocious-aging klotho mouse. *Circulation* **110**, 1148–1155 (2004).
51. Saito, Y. *et al.* Klotho protein protects against endothelial dysfunction. *Biochem Biophys Res Commun* **248**, 324–329 (1998).
52. Hu, M. C. *et al.* Klotho and phosphate are modulators of pathologic uremic cardiac remodeling. *Journal of the American Society of Nephrology* **26**, 1290–1302 (2015).
53. Vervloet, M. G., Adema, A. Y., Larsson, T. E. & Massy, Z. A. *The Role of Klotho on Vascular Calcification and Endothelial Function in Chronic Kidney Disease*. *Seminars in Nephrology* vol. 34 578–585 (W.B. Saunders, 2014).

54. Liu, H. *et al.* Augmented Wnt signaling in a mammalian model of accelerated aging. *Science (1979)* **317**, 803–806 (2007).
55. Muñoz-Castañeda, J. R. *et al.* Klotho/FGF23 and Wnt Signaling as Important Players in the Comorbidities Associated with Chronic Kidney Disease. *Toxins (Basel)* **12**, (2020).
56. Semba, R. D. *et al.* Plasma klotho and cardiovascular disease in adults. *J Am Geriatr Soc* **59**, 1596–1601 (2011).
57. Maekawa, Y. *et al.* Klotho suppresses TNF-α-induced expression of adhesion molecules in the endothelium and attenuates NF-κB activation. *Endocrine* **35**, 341–346 (2009).
58. Yao, Y., Wang, Y., Zhang, Y. & Liu, C. Klotho ameliorates oxidized low density lipoprotein (ox-LDL)-induced oxidative stress via regulating LOX-1 and PI3K/Akt/eNOS pathways. *Lipids Health Dis* **16**, (2017).
59. Quarles, L. D. Fibroblast growth factor 23 and α-Klotho co-dependent and independent functions. *Curr Opin Nephrol Hypertens* **28**, 16–25 (2019).
60. Keles, N. *et al.* Low serum level of Klotho is an early predictor of atherosclerosis. *Tohoku Journal of Experimental Medicine* **237**, 17–23 (2015).
61. Olejnik, A., Franczak, A., Krzywonos-Zawadzka, A., Kałużna-Oleksy, M. & Bil-Lula, I. The Biological Role of Klotho Protein in the Development of Cardiovascular Diseases. *Biomed Res Int* **2018**, 17 (2018).
62. Navarro-García, J. A. *et al.* PTH, vitamin D, and the FGF-23–klotho axis and heart: Going beyond the confines of nephrology. *European Journal of Clinical Investigation* vol. 48 Preprint at https://doi.org/10.1111/eci.12902 (2018).
63. Corsetti, G. *et al.* Decreased expression of Klotho in cardiac atria biopsy samples from patients at higher risk of atherosclerotic cardiovascular disease. *Journal of Geriatric Cardiology* **13**, 701–711 (2016).
64. Chen, G. *et al.* α-Klotho is a non-enzymatic molecular scaffold for FGF23 hormone signalling. *Nature* **553**, 461–466 (2018).
65. Dalton, G. *et al.* Soluble klotho binds monosialoganglioside to regulate membrane microdomains and growth factor signaling. *Proc Natl Acad Sci U S A* **114**, 752–757 (2017).
66. Wright, J. D. *et al.* Modeled structural basis for the recognition of a2-3-sialyllactose by soluble Klotho. *FASEB Journal* **31**, 3574–3586 (2017).

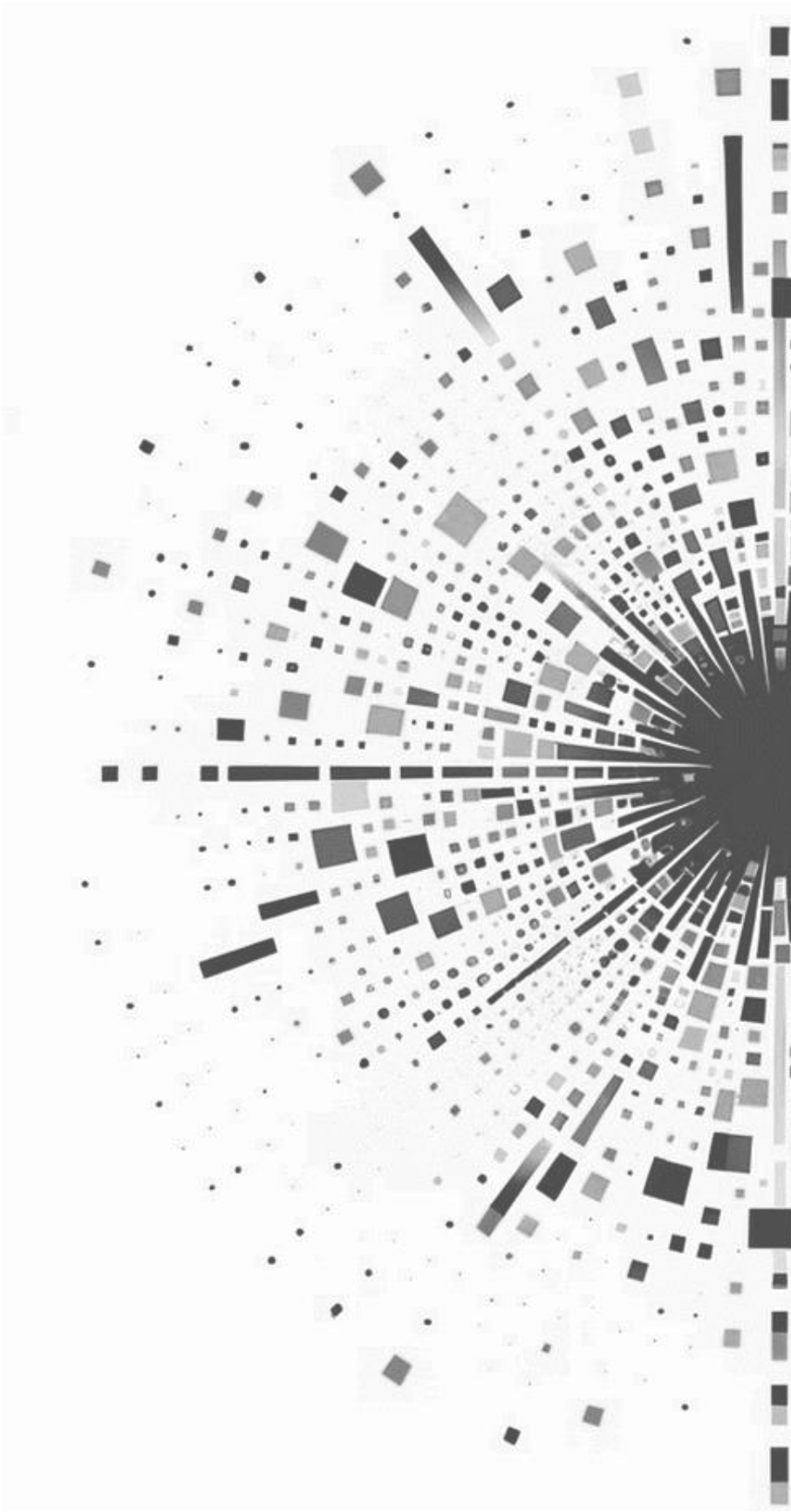


# Chapter 5

## Myeloid-Specific CDK5 Knockdown Reduces Atherosclerotic Plaque Calcification

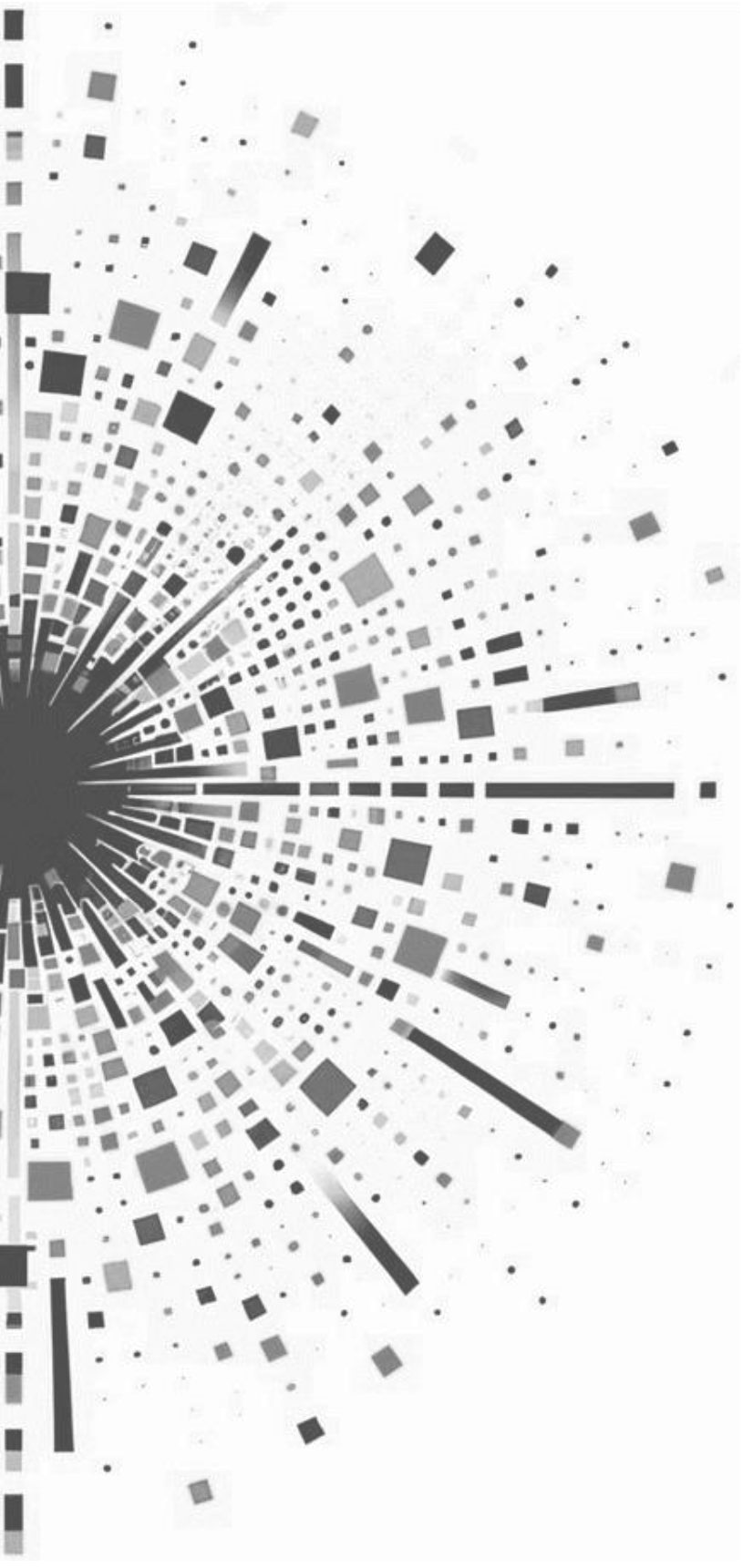
OJ Waring, V Saar-Kovrov, H Jin, C Lu, A Ruder, MJJ Gijbels, J Nagenborg, T Timmerman, M Giacca, S Vettorazzi, CG Schalkwijk, BME Mees, AMG Jamasoot, LJ Schurgers, M Stoll, P Goossens, L Matic, MMPC Lutgens, and EAL Biessen

*In preparation*



# Chapter 6

General Discussion



## Main Findings of the Thesis

Cardiorenal syndrome is a debilitating condition characterized by a complex interplay between decline in renal function and consequential development and aggravation of cardiovascular disease (CVD). In healthy individuals, kidneys fulfil numerous functions for sustaining homeostasis, e.g. regulation of blood pressure, maintenance of fluid and mineral balance, and removal of waste products. Pathological changes in kidney, such as loss of podocytes, tubular cell injury and subsequent tubulointerstitial fibrosis[1], will lead to kidney function decline, which is strongly associated with cardiovascular morbidity and mortality[2–4]. Increase in uremic toxins, protein modifications (e.g. carbamylation), inflammation, as well as vascular calcification, phenomena commonly seen in patient with chronic kidney disease (CKD)[4,5], are known to worsen cardiovascular condition and contribute to the development of atherosclerosis, a major manifestation of CVD[6]. However, the exact molecular mechanisms behind the cardiorenal syndrome are not properly understood thus far. Such knowledge would be of a high importance, since the limited clinically available approaches do not provide sufficient treatment for patients with CKD, and related cardiovascular disease, ranking as the 12$^{th}$ leading cause of death worldwide in 2017[7].

The aim of this thesis was to a) determine the best available clinical technique for reduction of protein-bound uremic toxins in the circulation of dialysis patients; b) investigate the role of a prominent uraemia-associated protein modification (carbamylation) in atherosclerosis; and c) study calcification-associated factors affected by kidney disorder.

In this chapter I will discuss the following main findings of my thesis:

1. Systematic review of 101 publication on kidney replacement therapies revealed orally administered charcoal adsorbent AST-120 (Kremezin®) and fractionated plasma separation and adsorption dialysis as the most effective approaches for reduction of blood levels of well-studied protein-bound uremic toxins (PBUTs) indoxyl sulfate and *p*-cresyl sulfate. However, there is currently no universal method for effective reduction of all PBUTs (**Chapter 2**).

2. In **Chapter 3**, we demonstrated that plaque carbamylation levels increase with plaque progression in patients with kidney insufficiency. Plaque protein carbamylation, at least partly, appears to reflect uptake of carbamylated low density lipoproteins (LDL) by macrophages that leads to foam cell formation.

3. Soluble Klotho is an important calcification-inhibiting protein that originates from truncation of a membrane-bound form and is significantly reduced in patients with CKD. However, as is highlighted by the review in **Chapter 4**, the differential effects of various soluble Klotho fragments on the organism remain to be fully elucidated.

4. Our study identifies macrophage CDK5 as a hub gene associated with plaque calcification and an important regulator of several inflammatory pathways, promoting smooth muscle cell-dependent calcification *in vitro* and *in vivo* (**Chapter 5**), which makes it a promising target for drug development.

# 1. CVD-promoting effects of CKD: uremic toxins and protein modifications

## *1.1. Protein-bound uremic toxins*

The discovery of urea in urine can be traced back to the 17th century with more detailed description of its chemical and physiological properties in the 18th and 19th century[8]. However, the first protein-bound solutes associated with kidney function, such as indican (commonly known as indoxyl sulfate), and their physiological role in CKD were reported much later facilitated by the advancement in biochemical methods[9–11]. Since then, indoxyl sulfate and another major sulphated solute, *p*-cresyl sulfate, are the most studied PBUTs in respect to their effects on cardiovascular system and methods of removal from plasma, as is evident from the systematic review presented in **Chapter 2**. This high scientific interest could be explained by the fact that both indoxyl sulfate and *p*-cresyl sulfate exhibit large protein-binding fraction of 80-90% and are shown to be the most toxic PBUTs to the cardiovascular system[12].

Albumin is the prime binding target for most of the PBUTs due to its high abundance in the plasma and strong affinity for amphiphilic compounds. Uremic toxins can bind to plasma albumin through electrostatic, dipolar, and van der Waals forces, or steric factors such as amino acids arrangement in the protein[13] depending on their chemical structure. Indoxyl sulfate, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), *p*-cresyl sulfate, hippuric acid and indoleacetic acid (IAA) demonstrate exceptionally strong affinity to albumin with CMPF showing more than 99% of its plasma pool to be protein-bound. This makes them particularly difficult to remove during haemodialysis procedure[14–16]. The reason for the variable protein-binding affinity between the compounds lay in their highly different physico-chemical properties. Additionally, albumin has specific binding sites for toxins indoxyl sulfate and *p*-cresyl sulfate called Sudlow's sites I and II[17]. In fact, the binding of a variety of endogenous and exogenous compounds (e.g. vitamins and drugs), but also uremic toxins, is one of the main physiological functions of plasma albumin[17]. Albumin binding often lowers compounds' toxicity and hence protects the organism from their deleterious effects. Impaired kidney function causes increased levels of uremic toxins in the circulation (up to 116 times of the normal concentration in case of indoxyl sulfate[18]), leading to saturation of albumin binding sites and increased levels of unbound toxic compounds. Moreover, saturation of albumin binding capacity also prevents other drugs from binding increasing their toxicity and interfering with delivery to the targets, which can be a serious limitation in heavily medicated people with chronic conditions [19,20].

Due to their high protein affinity, PBUTs behave more like middle or high than small molecular weight toxins. This fact makes them difficult to remove with the conventional dialysis techniques optimized for removal of small free solutes in the plasma. Thus, different approaches are required for adequate removal of these molecules from the circulation.

Prevention of production or elimination of their precursors could be an effective approach to reduce PBUT levels. Most PBUTs, or their precursors, originate from the gut[21]. This is true, for example, for indoxyl sulphate precursor, indole, which is a product of (often protein derived) tryptophan metabolism by bacteria *Escherichia coli*[22]. Gut derived indole is taken up into circulation and is transported to the liver where it is converted into indoxyl sulfate[11,23]. Hence, high dietary protein intake is known to increase blood indoxyl sulfate levels. Toxins indoleacetic acid and indoxyl-β-d-glucuronide are generated in a similar fashion. Phenolic compounds, such as *p*-cresyl sulphate, are also produced by gut bacteria[23,24].

Thus, it is not surprising that several uremic toxin eliminating methods interfere with their formation in the intestine. For instance, AST-120 (Kremezin®) is an oral spherical activated carbon that is prescribed to patients with CKD to delay the need for dialysis and improve the symptoms of uremia[25–27]. It acts by adsorbing and improving removal of low molecular weight substances from the intestinal lumen due to its high porous surface area. In **Chapter 2**, we demonstrate that oral supplementation of patient on haemodialysis with AST-120 showed the highest reduction ratios of both indoxyl sulphate and *p*-cresyl sulphate in longitudinal trials. AST-120 was also shown in a recent systematic review and meta-analysis, that involved integrative analysis of data from 3,763 patients from 15 randomized controlled trials, to have significant beneficial effects towards delaying the progression of the kidney disease in pre-dialysis patients[28]. Hence, this therapy proves to be an effective technique for reduction of the gut-originating PBUTs such as indoxyl sulphate and *p*-cresyl sulphate.

Other approaches for the PBUTs removal described in **Chapter 2** include the use of adsorbents or binding competitors, increasing the frequency or duration of dialysis or the size of the dialyzer, or applying hemofiltration or hemodiafiltration. One particularly effective technique to reduce PBUT levels in our study was fractionated plasma separation and adsorption dialysis by an extracorporeal liver support adsorption-dialysis system. This system affects separation of plasma albumin and clearance of the bound toxins by adsorbing materials in a separate circuit[29,30]. It is an effective procedure for removal of both water-soluble and hydrophobic toxins commonly used in liver failure. This technique could be a promising approach for reduction of the PBUTs in CKD parties, however, to the best of our knowledge, it has not been implemented for this purpose on a regular basis so far.

Removal of uremic toxins in CKD is relevant for preserving cardiovascular system in the patients since PBUTs have been linked to worsened outcomes in this population. Barreto and colleagues conducted a study on 139 patients with CKD and found that high levels of serum indoxyl sulfate were associated with mortality, including cardiovascular-related death, and was predictive for mortality independently of other conventional markers [31]. Moreover, free (non-protein-bound) concentrations of *p*-cresyl sulfate, but not total concentrations, have also been directly associated with CVD and overall mortality in pre-dialysis as well as dialysis patients[32–35]. In addition, free *p*-cresyl glucuronide, a metabolite formed by glucuronidation of *p*-cresol, was linked to all-cause and cardiovascular mortality in patients with CKD and showed predictive power for cardiovascular events beyond traditional markers[36,37]. Taken

together, these findings and numerous *in vitro* studies of cytotoxic effects of indoxyl and *p*-cresyl sulphates (reviewed[12]) strongly suggest that uremic toxins have a causal relationship to CVD.

By now, 33 PBUTs have been identified, including indoles, phenols, advanced glycation end products, and others[16]. In view of their chemical heterogeneity, it is unlikely that a single solution exists for effective PBTUs removal. Our findings show that, at least at the current moment, there is no universal technique suitable for reduction of all PBUTs. To bypass this limitation and achieve the best possible outcome for the patients, kidney therapies could be tuned to the needs of a particular patient, combining several kidney replacement techniques as well as supplements based on a patient's PBTU profile, individual metabolic status, co-morbidities, gut microbiota, and other factors. Such personalized approach requires further research before it can be implemented in the clinic.

## *1.2. Protein carbamylation and cardiorenal disease*

As mentioned above, kidney function decline is associated with a significant, up to 10-fold, increase in plasma urea concentrations[38]. Urea is known for its cellular toxicity as well as modifying properties resulting in protein carbamylation. Protein carbamylation is demonstrated to be significantly increased with kidney function decline and, similarly to some PBUTs, associated with decreased survival rates in CKD population showing strong adverse effects on the cardiovascular system[39–42]. Amongst others, this modification affects protein charge resulting in alterations of its folding, function, and interactions with other molecules[43]. However, unlike PBUTs, binding of the carbamoyl group to proteins is covalent which renders it difficult, if not impossible, to remove.

**Chapter 3** demonstrates that protein carbamylation is abundant in advanced atherosclerotic plaque, which is in line with published data[44,45]. We showed that total carb-lys positive area percentage increases with the plaque progression, particularly in patients with reduced kidney function. This could be caused by increased intracellular accumulation of carbamylated LDL particles taken up by plaque macrophages which causes foam cell formation and inflammatory pathway activation, albeit differently than that by the oxLDL uptake.

What could this accumulation mean functionally for the macrophages and, consequently, for the plaque phenotype? The fact that carbLDL uptakes caused no stimulatory effect on PLIN2, MPO or CD36 expression could indicate that carbLDL elements are not recognized by the peroxisome proliferator-activated receptor gamma (PPARγ) since CD36 is a known reporter of PPARγ [46] while both MPO and PLIN2 expression is induced by this nuclear receptor and transcription factor[47–49]. PPARγ recognizes such molecules as oxidized free fatty acids and phospholipid LPA which are common components of oxidized LDL[46,50]. Since carbLDL was not subjected to oxidation, such elements are likely lacking in these particles. CarbLDL uptake, however, seem to trigger another nuclear receptor, liver x receptor alpha (LXRα) which, in turn, activates expression of the cholesterol transporters ABCA1 and ABCG1[51]. It is known as a "cholesterol sensor" recognizing oxysterols molecules in the cell[52] indicating that these

molecules are preserved in the carbamylated LDL. Upregulation of the cholesterol transporters by carbLDL uptake in the absent of PLIN2 induction could indicate faster cholesterol turnover since PLIN2 was shown to protect lipid droplets from disintegration and processing by lipases[53,54]. Additionally, carbLDL uptake did not cause ROS production or apoptosis in the macrophages unlike oxLDL treatment. Taken together, it seems that carbLDL particles are in general less harmful to macrophages, resulting in lower inflammatory response, however still promoting foam cell phenotype. Nevertheless, considering the diverse role of PPARγ in macrophage functioning, its involvement in both lipid uptake and cholesterol efflux[55], the exact functional consequences of carbLDL uptake, and its effects on other PPARs, need to be elucidated further. RNA sequencing and electron microscopy could help solve these questions.

The physiological relevance of these findings for the atherosclerosis development are, however, yet to be established. A complicating factor is that carbLDL and oxLDL will be generated simultaneously and thus taken up in parallel, as also suggested by the correlation between PLIN2 and carb-lys signals in the absence of the effects on PLIN2 expression levels by the carbLDL *in vitro*. What is more, double modified carb-oxLDL levels were shown by Apostolov *et al.* to exceed those of single modified LDL in CKD mouse model[56]. Thus, it is unclear if reduction of carbLDL levels or prevention of its uptake would have any physiologically significant effects on the cells and atherosclerosis progression. As certain differences were observed between carbLDL and oxLDL mode of action, further investigation is required to fully elucidate the mechanistic consequences of the carbLDL and how this could be intervened.

Besides LDL, also its functional counterpart HDL, known to mediate reverse cholesterol transport from periphery to the liver[57], was recently reported to be affected by carbamylation. Carbamylation of ApoA-1, the major protein constituent of HDL, was shown to impair its cholesterol efflux properties and induce lipid droplet formation in the macrophages by disrupting the interaction between the HDL particles and scavenger receptor class B, type I[58,59]. Additionally, Hazen and coworkers have demonstrated abundant presence of carbamylated ApoA-1 in plaque and discovered its impaired cholesterol efflux-promoting ability as well as pro-inflammatory properties on endothelial cells[45,60]. Besides ApoA-1, also paraoxonase-1, another HDL associated protein, was shown to be affected by carbamylation in patients with uremia, disrupting the antioxidant activity of HDL[61]. A different group also observed that *in vitro* carbamylated HDL particles significantly inhibited migration, angiogenesis, and proliferation in endothelial cells *in vitro*[62]. However, it is important to remember that the cyanate concentrations used for the *in vitro* modification are often much higher than those found in patients with CKD to compensate for the shorter exposure time e.g., 1-100 mM vs 141 nM[63], respectively. Hence, *in vitro* experimental results should be interpreted with caution.

In general, the effects of carbamylation seem to be protein specific. For instance, there is ample literature showing that carbamylation of such long turnover proteins as collagen, fibrin,

and albumin causes their structural alterations, loss of cell binding ability, and resistance to degradation [64–68]. We have also observed more diffused extracellular carb-lys signal in human atherosclerotic plaque (**Chapter 3**) indicating potential modification of the extracellular matrix proteins. The significance of these findings would need to be elucidated in further studies. Carbamylation was also shown to cause gain of function of some proteins. For example, carbamylation of haemoglobin is known to increase its oxygen binding abilities, a finding that is used in sickle-cell disease therapy[69]. Additionally, carbamylation of histone 1 was shown to play a role in gene expression regulation. As Joshi *et al*. demonstrated, this is mediated by carbamoyl phosphate synthase 1 which is recruited to DNA enhancer sites in the genome. This, in turn, results in enzyme-independent carbamylation of specific lysines of the linker histone H1 protein, leading to conformational changes of the chromatin and promoting gene expression[70].

Several papers highlight the positive correlation between carbamylated plasma protein levels and increased all-cause and cardiovascular mortality in CKD patients[39–41,71,72]. This could be caused by the deleterious effects of carbamylated proteins on the vasculature as reviewed by Delanghe *et al*. and Gorisse *et al*.[43,73]. In addition to the above mentioned pro-inflammatory and pro-atherogenic effects of modified lipoproteins, carbamylated collagen type I, for instance, showed significantly higher monocyte adhesion and activation ability than non-modified collagen[67], while carbamylated but not non-modified sortilin was shown to promote smooth muscle cell calcification[74]. End-stage kidney disease patients that showed greater reduction of carbamylated albumin blood levels after initiation of maintenance haemodialysis demonstrated higher survival rates compared to the less responsive patient group[39]. Thus, reduction or possibly prevention of carbamylation seems to be beneficial for the CKD patients. In addition to reduction of blood urea levels with dialysis, attenuating inflammation and MPO activity in general with healthy lifestyle and anti-inflammatory agents, e.g. poly-unsaturated free fatty acids[75,76], could potentially be another way of managing protein carbamylation levels in the organism. However, data on direct anti-carbamylation therapies is scarce. One study describes a successful *in vitro* experiment using glycylglycine that allowed to reduce albumin carbamylation by 63%[41]. However, the therapy was not implemented in humans thus far. Another approach, supplementation of dialysis patients with free amino acids, showed promising results in carbamylation levels reduction in a pilot clinical trial[77] and is undergoing further investigation at the moment (clinical trial number NCT02472834).

Carbamylated proteins have also been shown to play a significant role in rheumatoid arthritis with certain patient population developing anti-carbamylated protein (anti-CarP) autoantibodies that are detectable already at the early onset of the disease[78]. The anti-CarP antibodies were shown to be predictive for more severe disease course and joint damage and proposed as biomarkers by multiple studies[78–82]. Presence of anti-CarP in patients with kidney disease remains to be described. This would be especially interesting in the context of cardiorenal syndrome since anti-CarP were associated with endothelial disfunction and atherosclerosis in rheumatoid arthritis patients without evident cardiovascular disease or

traditional risk factors[83]. Of note, autoantibodies against other factors, e.g. oxLDL, ApoA1 and phospholipids, were previously proposed as biomarkers for increased risks of cardiovascular disease (reviwed[84]).

Carbamylation was proposed in several studies as a biomarker for kidney disease progression as well as mortality risks. Homocitrulline, formed as a result of lysine carbamylation, is a promising biomarker that allows to distinguish between acute and chronic renal patients[85]. It was also shown that carbamylated albumin levels are associated with elevated serum markers of cardiac stress as well as cardiovascular mortality. Moreover, carbamylated HDL was proposed as biomarker for kidney disease progression[86]. However, carbamylation measurements are not implemented in a standard clinical practise thus far.

### *1.3. Methods and hurdles of PTM analysis*

Most powerful and reliable method for PTM detection to this day remains to be mass spectrometry (MS), which provides highly specific, large-scale, quantitative profiling of proteins and their PTMs[87]. Conventionally, after initial degradation by proteolytic enzymes, peptides can be subjected to an enrichment step by e.g. cationic exchange, ion affinity or immunoaffinity chromatography. After that, the peptides are analyzed using high-performance liquid chromatography (HPLC)-coupled tandem MS/MS technique and their generated spectra are compared to existing databases with the adjustment for the mass of a specific modification[88]. Quantification of the modification levels is also possible if a reference molecule with known concentration is available. Due to substoichiometric, transient, and fragile nature of PTMs, additional experimental step, such as enrichment, is frequently necessary for preservation and identification of the modifications. This step, however, depends on the availability of a good technique for a particular modification, e.g. antibodies, affinity chromatography or metabolic tagging, and might suffer from low specificity[89].

MS-based techniques are usually accompanied by high costs, requiring special equipment and extensive training of the personnel and are thus not implemented broadly in the clinics. An alternative strategy for PTMs analysis involves antibody-based approaches such as ELISA, Western blot, and immunohistochemistry (IHC). These approaches, however, are strongly dependent on the antibody specificity and might suffer from weak signal due to low abundancy of modification, low affinity of the antibody or its cross-reaction with other substances giving a false-positive result. Attempts have been made to improve affinity and decrease non-specific binding of the antibodies based on the crystal structure of the antibody-PTM binding sites[90]. Additionally, many PTMs have no antibodies developed for their detection so far (e.g. guanidinylation).

Nowadays, modern techniques allow retrieval of spatial information of the PTM localization in a tissue sample by applying MS imaging and spatial multiomics. For instance, Krahmer and colleagues developed a mass spectrometric workflow for protein and phosphopeptide correlation profiling. This method revealed subcellular rearrangements and organelle-specific phosphorylation accompanying the development of hepatic steatosis[91]. Additionally, spatial

multiomics approaches can be used for in-depth characterization of the epigenetic landscape by e.g. combining histone modification data with transcriptomics which provides spatially resolved information on epigenetic priming, differentiation, and gene regulation processes on a cellular level[92]. Progress in spatial multiomics approaches increases our understanding of the convoluted cellular processes and sheds light on underlying mechanisms of the disease development facilitating discovery of new biomarkers, development of novel therapeutic approaches, and guiding precision medicine[93–97].

To summarize, kidney function decline is accompanied by augmented cardiovascular toxicity that manifests, amongst others, through increase in toxic uremic milieu and undesirable protein modifications. PTMs, in particular, can serve as an example of how minute alterations in molecular composition can result in dramatic changes in protein function leading to pathological consequences for the organism. Thus, deepening our understanding of these intricate mechanisms is crucial for finding of an effective preventive and therapeutic solutions for cardiorenal syndrome.

# 2. CVD-promoting effects of CKD: vascular calcification

Vascular calcification is one of the major processes connecting CKD to cardiovascular diseases. In CKD condition, distortion of mineral balance, elevated parathyroid hormone levels, changes in vitamin D metabolism, as well as increased inflammation and oxidative stress[4,98] result in increased calcium and phosphate deposition in the vascular wall, osteochondrogenic dedifferentiation of smooth muscle cells and apoptosis that leads to release of apoptotic bodies and formation of calcium crystal nucleation sites[99]. These processes give rise to vascular stiffening, arterial stenosis, hypertension, plaque vulnerability, and are associated with increased cardiovascular mortality in CKD patients[4,100,101]. CKD was also seen to affect activity and levels of important anti-calcification factors, such as matrix gla protein and Klotho, an effect that is detectable already at an early stage of the disease[102–104].

## *2.1. Anti-calcification agents: soluble Klotho*

Low levels of Klotho are associated with increased vascular calcification, fibrosis, and inflammation[105–108]. As discussed in detail in **Chapter 4**, Klotho is an important cofactor, produced by kidney tubule cells, that forms a complex with the fibroblast growth factor receptors (FGFR), thus allowing binding of FGF23[109]. FGF23, in turn, serves as a mediator for several processes, e.g. regulation of phosphate-calcium balance, and vitamin D homeostasis[110]. Hence, it is not surprising that dysregulation in membrane-bound Klotho promotes calcification[111]. In addition, soluble Klotho, generated through proteolytic cleavage of the membrane-bound form[105,112], was demonstrated to have anti-inflammatory and vascular-protective properties[113,114] and was independently associated with a lower likelihood of CVD in a population study[115]. However, the exact mechanisms of soluble Klotho regulation and its vasoactive properties are not fully understood.

Due to its apparent beneficial properties, Klotho represents a promising therapeutic target for various indications including vascular disease, kidney disease, diabetes, and cancer[116]. However, no therapies based on Klotho protein reached clinical trials stage so far[117]. This could be partially attributed to the difficulties associated with Klotho delivery such as a relatively large size of the protein (~130 kDa for the full-length)[105] and its instability[118]. Nevertheless, extensive studies continue to address various possibilities of recombinant Klotho delivery, as well as target mechanisms of its upregulation (reviewed[116,117]).

## *2.2. Novel calcification regulators: CDK5*

Advanced computational analysis, data mining, as well as single cell sequencing approaches allow to identify novel players in calcification and other conditions that could have been otherwise left unnoticed. Thus, in this work, using weighted gene co-expression network analysis, we have discovered macrophage cyclin-dependent kinase 5 (CDK5) to exert pro-calcifying activity (**Chapter 5**). CDK5 was demonstrated earlier to regulate inflammation, as inhibition of CDK5 resulted in reduced inducible nitric oxide synthase and cytokine expression and NO generation in inflammatory macrophages[119,120] highlighting its pro-inflammatory properties. Our study confirms these findings showing significantly supressed cytokine secretion by the CDK5-deficient macrophages after inflammatory stimulation. A link between vascular calcification and inflammation is well established with inflammation preceding and exacerbating calcification, and both processes being major factors contributing to atherosclerosis development[121,122]. However, the connection between CDK5 and calcification has not been studied so far. Our results demonstrate that conditional deletion of CDK5 in mouse myeloid cells resulted in a less severe plaque phenotype and reduced calcification propensity. Bai and colleagues have shown earlier that pharmacological long term CDK5 inhibition with semi-specific drug roscovitine, that also shows inhibitory properties towards other CDKs[123], attenuated cellular senescence and aorta atherosclerosis in ApoE-deficient mice[124], making CDK5 an interesting drug target for CVD therapy. It is important to remember, however, that CDK5 is ubiquitously expressed and plays an important role in various physiological cell processes, e.g. adhesion, cytoskeleton regulation, and cell cycle, as well as in neuronal development[125]. Whole-body CKD5 knockout mice die shortly after birth[126] underpinning its critical function in organism's development. Thus, to avoid possible undesirable side effects, a more targeted cell-specific approach would be preferable in tackling atherosclerosis through CDK5. Solution may lay, for instance, in novel techniques using antibody-drug conjugates that have been actively implemented in cancer therapy allowing cell-specific delivery of cytotoxic compounds[127,128]. If similar techniques could be applied to deliver protein inhibitors to hamper atherosclerosis development remains to be investigated.

Besides atherosclerosis, CDK5 inhibition could potentially be particularly relevant for patients with diabetic nephropathy and acute kidney injury, where CDK5 upregulation promotes tubular fibrosis and aggravates kidney damage[129,130]. Further studies are required to investigate CDK5 regulation and activity in kidney disease-associated cardiovascular

complications, which might not be strictly mediated through the myeloid cell types. Additionally, inhibitors of CDK5 and other CDK isoforms, are currently being actively studied for treatment of various types of cancer. For instance, R-roscovitine has recently completed Phase I clinical trials for treatment of advanced solid tumours (study NCT00999401), while small molecule AT7519 is in Phase I trial at the moment for advanced or metastatic solid tumours (study NCT02503709)[131].

## Concluding remarks and future perspectives

Cardiorenal syndrome, in particular type 4 discussed in this thesis, is a complex medical disorder arising from bidirectional relationship between chronic kidney disease and cardiovascular complications. Taking into account the heterogeneity and multifactorial character of the pathological mechanisms underlying cardiorenal syndrome, it is important to consider this condition from various perspectives. Thus, in this work we addressed the role of uremic toxins, protein modification induced by uremia, as well as the role of macrophage gene programs in plaque calcification regulation.

It can be concluded that:

1) Protein-bound uremic toxins exhibit high physico-chemical heterogeneity rendering it difficult to find a universal technique for their effective reduction in the CKD patients. Hence, more personalized approaches customized to individual patients' metabolic status, co-morbidities, gut microbiota, and other factors combining various kidney replacement therapies and supplements might be needed to achieve better outcomes for the patients.

2) Pathological protein modifications, such as carbamylation, have only limitedly been elucidated so far. They play a crucial role in mediating proteins' biology and interaction with the surrounding environment, thus more research should be attempted to comprehend the full spectrum of different protein modifications and their impact on cellular behaviour and disease outcomes before this knowledge can be translated into effective therapies.

3) Modern omics and computational techniques allow identification of novel players in known pathological processes. Firstly, this enhances our understanding of the complex pathophysiological mechanisms of e.g. plaque calcification, revealing macrophage inflammation as important regulatory cue in this process. Secondly, its implications go further also allowing discovery of promising drug development targets. Indeed, we provide *in vitro* and *in vivo* evidence for the validity of this approach and the potential of macrophage CDK5 as a target for intervention.

To advance our understanding of the mechanisms behind cardiorenal syndrome and reveal novel therapeutic opportunities, further research is required. For instance, long-term,

multicentre, cross-over clinical trials combining several promising techniques, e.g. haemodiafiltration or high-flux haemodialysis and oral supplement AST-120, are necessary to confirm the effectiveness of this approach. Additionally, latest advances in multiomics techniques including epigenomics, transcriptomics, metabolomics, in combination with global analyses of PTMs, will allow to map protein modifications in healthy and diseased tissue, shedding light on their role in cellular processes and opening opportunities for novel therapies. This stands true as well for the discovery of novel protein biomarkers and drug targets, such as CDK5, that previously escaped our attention.

Taken together, this work highlights the importance of a holistic approach in tackling such complex health disorders as cardiorenal syndrome that needs to be guided by the individual differences of the involved players. Thus, continuous improvement of the research techniques could make it possible to develop highly precise and personalized combinational therapies to provide better solutions for treatment of cardiovascular disease in the future.

# References

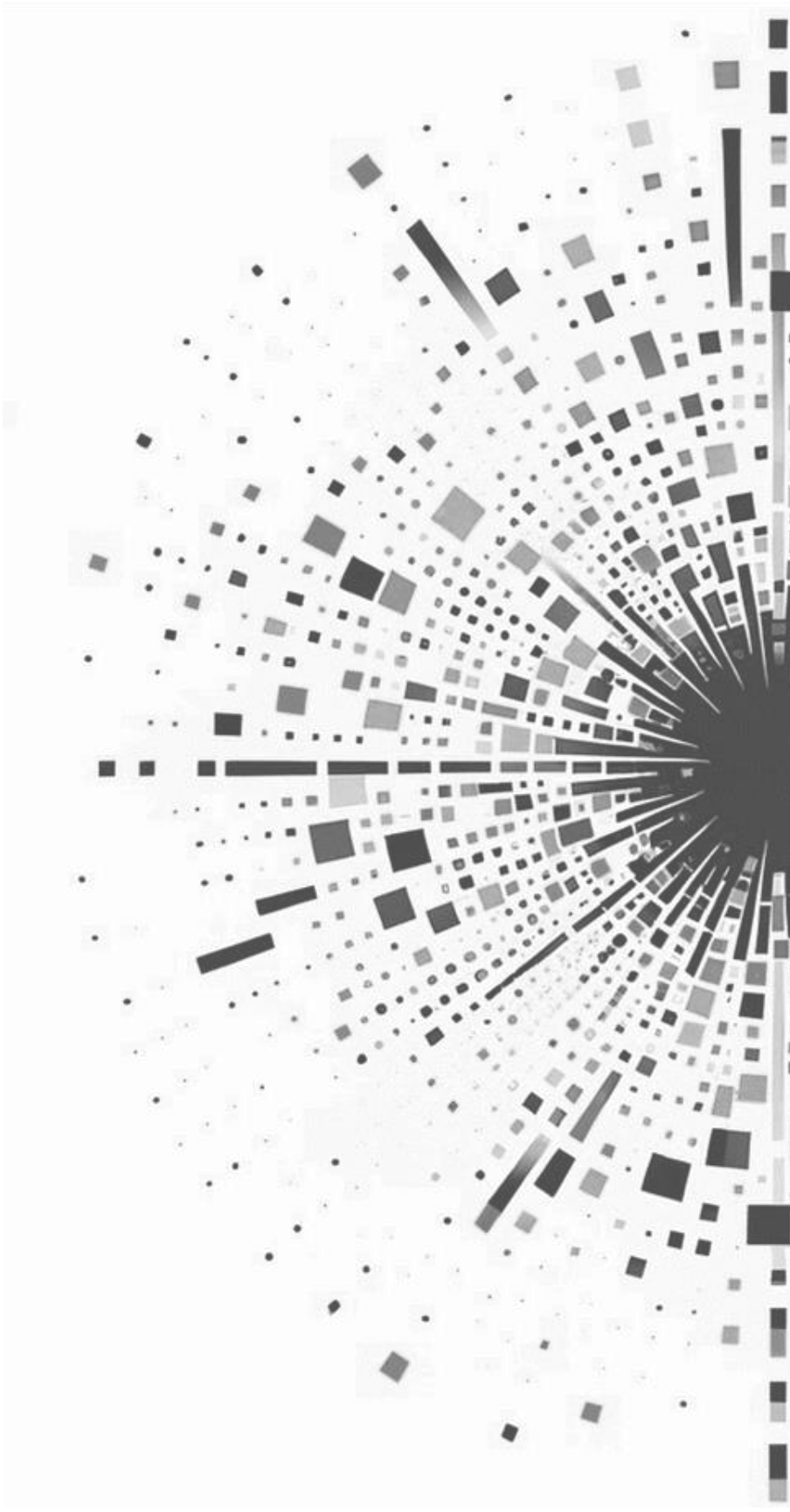

1. Ruiz-Ortega, M., Rayego-Mateos, S., Lamas, S., Ortiz, A. & Rodrigues-Diez, R. R. Targeting the progression of chronic kidney disease. *Nature Reviews Nephrology 2020 16:5* **16**, 269–288 (2020).
2. Go, A. S., Chertow, G. M., Fan, D., McCulloch, C. E. & Hsu, C. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med* **351**, 1296–305 (2004).
3. Matsushita, K. *et al.* Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. *The Lancet* **375**, 2073–2081 (2010).
4. Jankowski, J., Floege, J., Fliser, D., Böhm, M. & Marx, N. Cardiovascular Disease in Chronic Kidney Disease. *Circulation* **143**, 1157–1172 (2021).
5. Laget, J., Duranton, F., Argilés, À. & Gayrard, N. Renal insufficiency and chronic kidney disease – Promotor or consequence of pathological post-translational modifications. *Mol Aspects Med* **86**, 101082 (2022).
6. Düsing, P. *et al.* Vascular pathologies in chronic kidney disease: pathophysiological mechanisms and novel therapeutic approaches. *J Mol Med* **99**, 335–348 (2021).
7. Bikbov, B. *et al.* Global, regional, and national burden of chronic kidney disease, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet* **395**, 709 (2020).
8. Paris, G. R. Early history of uremia. *Kidney Int* **33**, 1013–1015 (1988).
9. Schreiner, G. E. & Maher, J. F. Uremia Biochemistry, Pathogenesis and Treatment. *J Med Educ* **37**, 408 (1962).
10. Pasternack, A., Kuhlbäck, B. & Tallgren, L. G. Serum Indican in Renal Disease. *Acta Med Scand* **176**, 751–756 (1964).
11. Leong, S. C. & Sirich, T. L. Indoxyl sulfate-review of toxicity and therapeutic strategies. *Toxins (Basel)* **8**, 358 (2016).
12. Lekawanvijit, S., Kompa, A. R. & Krum, H. Protein-bound uremic toxins: A long overlooked culprit in cardiorenal syndrome. *Am J Physiol Renal Physiol* **311**, F52–F62 (2016).
13. Florens, N., Yi, D., Juillard, L. & Soulage, C. O. Using binding competitors of albumin to promote the removal of protein-bound uremic toxins in hemodialysis: Hope or pipe dream? *Biochimie* **144**, 1–8 (2018).
14. Zare, F., Janeca, A., Jokar, S. M., Faria, M. & Gonçalves, M. C. Interaction of Human Serum Albumin with Uremic Toxins: The Need of New Strategies Aiming at Uremic Toxins Removal. *Membranes (Basel)* **12**, (2022).
15. Daneshamouz, S., Eduok, U., Abdelrasoul, A. & Shoker, A. Protein-bound uremic toxins (PBUTs) in chronic kidney disease (CKD) patients: Production pathway, challenges and recent advances in renal PBUTs clearance. *NanoImpact* **21**, 100299 (2021).
16. Saar-Kovrov, V. *et al.* Reduction of protein-bound uraemic toxins in plasma of chronic renal failure patients: A systematic review. *J Intern Med* **290**, 499–526 (2021).
17. Takamura, N., Maruyama, T. & Otagiri, M. Effects of uremic toxins and fatty acids on serum protein binding of furosemide: possible mechanism of the binding defect in uremia. *Clin Chem* **43**, 2274–2280 (1997).
18. Meijers, B. K. I. *et al.* p-cresyl sulfate and indoxyl sulfate in hemodialysis patients. *Clinical Journal of the American Society of Nephrology* **4**, 1932–1938 (2009).
19. Brunet, P., Dou, L., Cerini, C. & Berland, Y. Protein-bound uremic retention solutes. *Adv Ren Replace Ther* **10**, 310–320 (2003).
20. Tayyab, S. & Feroz, S. R. Serum albumin: clinical significance of drug binding and development as drug delivery vehicle. *Adv Protein Chem Struct Biol* **123**, 193–218 (2021).
21. Graboski, A. L. & Redinbo, M. R. Gut-Derived Protein-Bound Uremic Toxins. *Toxins (Basel)* **12**, (2020).
22. Fang, C. Y. *et al.* Selection of uremic toxin-reducing probiotics in vitro and in vivo. *J Funct Foods* **7**, 407–415 (2014).
23. Savira, F. *et al.* Molecular mechanisms of protein-bound uremic toxin-mediated cardiac, renal and vascular effects: underpinning intracellular targets for cardiorenal syndrome therapy. *Toxicol Lett* **308**, 34–49 (2019).
24. Gryp, T., Vanholder, R., Vaneechoutte, M. & Glorieux, G. p-Cresyl Sulfate. *Toxins 2017, Vol. 9, Page 52* **9**, 52 (2017).
25. Owada, A. *et al.* Effects of oral adsorbent AST-120 on the progression of chronic renal failure: a randomized controlled study. *Kidney Int Suppl* **63**, S188-90 (1997).
26. Asai, M., Kumakura, S. & Kikuchi, M. Review of the efficacy of AST-120 (KREMEZIN((R))) on renal function in chronic kidney disease patients. *Ren Fail* **41**, 47–56 (2019).

27. Koide, K. *et al.* A study of oral adsorbent in chronic renal failure. *Biomater Artif Cells Immobilization Biotechnol* **19**, 147–166 (1991).
28. Su, P. Y. *et al.* Efficacy of AST-120 for Patients With Chronic Kidney Disease: A Network Meta-Analysis of Randomized Controlled Trials. *Front Pharmacol* **12**, 1905 (2021).
29. Brettschneider, F. *et al.* Removal of Protein-Bound, Hydrophobic Uremic Toxins by a Combined Fractionated Plasma Separation and Adsorption Technique. *Artif Organs* **37**, 409–416 (2013).
30. Rifai, K. *et al.* Clinical feasibility of liver elastography by acoustic radiation force impulse imaging (ARFI). *Digestive and Liver Disease* **43**, 491–497 (2011).
31. Barreto, F. C. *et al.* Serum indoxyl sulfate is associated with vascular disease and mortality in chronic kidney disease patients. *Clinical Journal of the American Society of Nephrology* **4**, 1551–1558 (2009).
32. Meijers, B. K. I. *et al.* p-Cresol and cardiovascular risk in mild-to-moderate kidney disease. *Clinical Journal of the American Society of Nephrology* **5**, 1182–1189 (2010).
33. Meijers, B. K. I. *et al.* Free p-cresol is associated with cardiovascular disease in hemodialysis patients. *Kidney Int* **73**, 1174–1180 (2008).
34. Bammens, B., Evenepoel, P., Keuleers, H., Verbeke, K. & Vanrenterghem, Y. Free serum concentrations of the protein-bound retention solute p-cresol predict mortality in hemodialysis patients. *Kidney Int* **69**, 1081–1087 (2006).
35. Wu, I. W. *et al.* Serum free p -cresyl sulfate levels predict cardiovascular and all-cause mortality in elderly hemodialysis patients—a prospective cohort study. *Nephrology Dialysis Transplantation* **27**, 1169–1175 (2012).
36. Liabeuf, S. *et al.* Does P-Cresylglucuronide Have the Same Impact on Mortality as Other Protein-Bound Uremic Toxins? *PLoS One* **8**, e67168 (2013).
37. Meert, N. *et al.* Novel method for simultaneous determination of p -cresylsulphate and p -cresylglucuronide: clinical data and pathophysiological implications. *Nephrology Dialysis Transplantation* **27**, 2388–2396 (2012).
38. Vanholder, R., Gryp, T. & Glorieux, G. Urea and chronic kidney disease: the comeback of the century? (in uraemia research). *Nephrology Dialysis Transplantation* **33**, 4–12 (2018).
39. Kalim, S. *et al.* Longitudinal changes in protein carbamylation and mortality risk after initiation of hemodialysis. *Clinical Journal of the American Society of Nephrology* **11**, 1809–1816 (2016).
40. Kalim, S. Protein carbamylation in ESRD: Is there a mortality effect? *Curr Opin Nephrol Hypertens* **27**, 454 (2018).
41. Berg, A. H. *et al.* Carbamylation of serum albumin as a risk factor for mortality in patients with kidney failure. *Sci Transl Med* **5**, (2013).
42. Kalim, S., Karumanchi, S. A., Thadhani, R. I. & Berg, A. H. Protein Carbamylation in kidney disease: Pathogenesis and clinical implications. *American Journal of Kidney Diseases* **64**, 793–803 (2014).
43. Delanghe, S., Delanghe, J. R., Speeckaert, R., Van Biesen, W. & Speeckaert, M. M. Mechanisms and consequences of carbamoylation. *Nat Rev Nephrol* **13**, 580–593 (2017).
44. El-Gamal, D. *et al.* The urea decomposition product cyanate promotes endothelial dysfunction. *Kidney Int* **86**, 923–931 (2014).
45. Huang, Y. *et al.* An abundant dysfunctional apolipoprotein A1 in human atheroma. *Nat Med* **20**, 193 (2014).
46. Nagy, L., Tontonoz, P., Alvarez, J. G. A., Chen, H. & Evans, R. M. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARγ. *Cell* **93**, 229–240 (1998).
47. Reynolds, W. F., Kumar, A. P. & Piedrafita, F. J. The human myeloperoxidase gene is regulated by LXR and PPARalpha ligands. *Biochem Biophys Res Commun* **349**, 846 (2006).
48. Tan, Y. *et al.* Adipophilin Involved in Lipopolysaccharide-Induced Inflammation in RAW264.7 Cell via Extracellular Signal-Regulated Kinase 1/2-Peroxisome Proliferator-Activated Receptor Gamma Pathway. *DNA Cell Biol* **36**, 1159–1167 (2017).
49. Wang, J. *et al.* Lipopolysaccharide promotes lipid accumulation in human adventitial fibroblasts via TLR4-NF-κB pathway. *Lipids Health Dis* **11**, (2012).
50. Levitan, I., Volkov, S. & Subbaiah, P. V. Oxidized LDL: Diversity, Patterns of Recognition, and Pathophysiology. *Antioxid Redox Signal* **13**, 39 (2010).
51. Chawla, A. *et al.* A PPARγ-LXR-ABCA1 Pathway in Macrophages Is Involved in Cholesterol Efflux and Atherogenesis. *Mol Cell* **7**, 161–171 (2001).
52. Zhu, R., Ou, Z., Ruan, X. & Gong, J. Role of liver X receptors in cholesterol efflux and inflammatory signaling (Review). *Mol Med Rep* **5**, 895 (2012).
53. Xu, S. *et al.* Perilipin 2 and lipid droplets provide reciprocal stabilization. *Biophysics Reports 2019 5:3* **5**, 145–160 (2019).

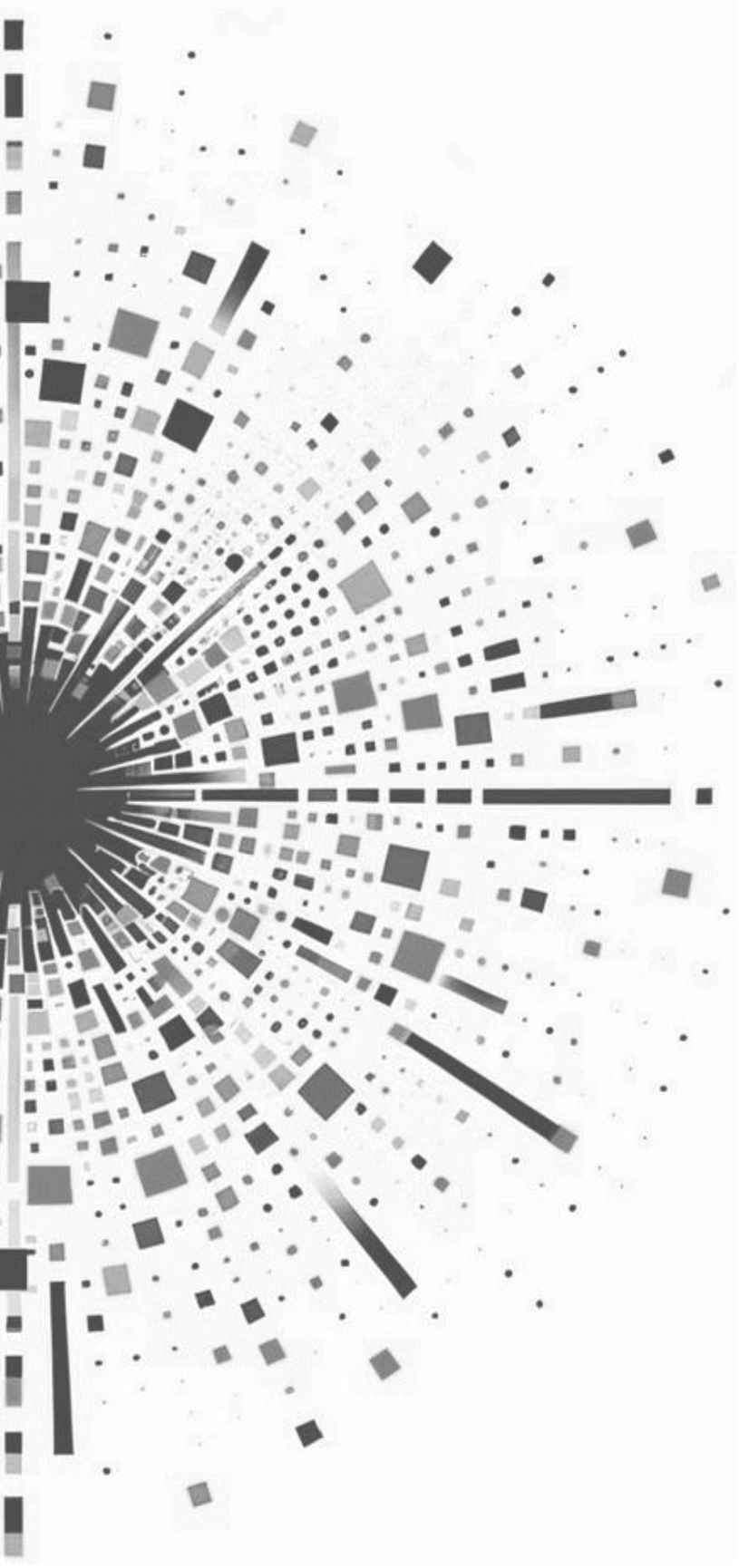
54. Larigauderie, G. *et al.* Adipophilin enhances lipid accumulation and prevents lipid efflux from THP-1 macrophages: potential role in atherogenesis. *Arterioscler Thromb Vasc Biol* **24**, 504–510 (2004).
55. Walczak, R. & Tontonoz, P. PPARadigms and PPARadoxes: expanding roles for PPARγ in the control of lipid metabolism. *J Lipid Res* **43**, 177–186 (2002).
56. Apostolov, E. O. *et al.* Carbamylated-oxidized LDL: proatherosclerotic effects on endothelial cells and macrophages. *J Atheroscler Thromb* **20**, 878–892 (2013).
57. Jomard, A. & Osto, E. High Density Lipoproteins: Metabolism, Function, and Therapeutic Potential. *Front Cardiovasc Med* **7**, 39 (2020).
58. Holzer, M. *et al.* Protein carbamylation renders high-density lipoprotein dysfunctional. *Antioxid Redox Signal* **14**, 2337–2346 (2011).
59. Holzer, M. *et al.* Uremia alters HDL composition and function. *Journal of the American Society of Nephrology* **22**, 1631–1641 (2011).
60. Battle, S. *et al.* The pattern of apolipoprotein A-I lysine carbamylation reflects its lipidation state and the chemical environment within human atherosclerotic aorta. *Journal of Biological Chemistry* **298**, 101832 (2022).
61. Chang, C. T. *et al.* PON-1 carbamylation is enhanced in HDL of uremia patients. *J Food Drug Anal* **27**, 542–550 (2019).
62. Sun, J. T. *et al.* Increased carbamylation level of HDL in end-stage renal disease: Carbamylated-HDL attenuated endothelial cell function. *Am J Physiol Renal Physiol* **310**, F511–F517 (2016).
63. Nilsson, L., Lundquist, P., Kågedal, B. & Larsson, R. Plasma cyanate concentrations in chronic renal failure. *Clin Chem* **42**, 482–483 (1996).
64. Doué, M. *et al.* Carbamylation of elastic fibers is a molecular substratum of aortic stiffness. *Scientific Reports 2021 11:1* **11**, 1–11 (2021).
65. Binder, V. *et al.* Impact of fibrinogen carbamylation on fibrin clot formation and stability. *Thromb Haemost* **117**, 899–910 (2017).
66. de Araújo Lira, A. L. *et al.* Serum albumin modified by carbamoylation impairs macrophage cholesterol efflux in diabetic kidney disease. **35**, 107969 (2021).
67. Garnotel, R., Sabbah, N., Jaisson, S. & Gillery, P. Enhanced activation of and increased production of matrix metalloproteinase-9 by human blood monocytes upon adhering to carbamylated collagen. *FEBS Lett* **563**, 13–16 (2004).
68. Jaisson, S. *et al.* Impact of Carbamylation on Type I Collagen Conformational Structure and Its Ability to Activate Human Polymorphonuclear Neutrophils. *Chem Biol* **13**, 149–159 (2006).
69. De Furia, F. G., Miller, D. R., Cerami, A. & Manning, J. M. The Effects of Cyanate In Vitro on Red Blood Cell Metabolism and Function in Sickle Cell Anemia. *J Clin Invest* **51**, 566–574 (1972).
70. Joshi, A. D., Mustafa, M. G., Lichti, C. F. & Elferink, C. J. Homocitrullination Is a Novel Histone H1 Epigenetic Mark Dependent on Aryl Hydrocarbon Receptor Recruitment of Carbamoyl Phosphate Synthase 1. *J Biol Chem* **290**, 27767 (2015).
71. Koeth, R. A. *et al.* Protein carbamylation predicts mortality in ESRD. *Journal of the American Society of Nephrology* **24**, 853–861 (2013).
72. Kalim, S. *et al.* Carbamylation of serum albumin and erythropoietin resistance in end stage kidney disease. *Clinical Journal of the American Society of Nephrology* **8**, 1927–1934 (2013).
73. Gorisse, L., Jaisson, S., Piétrement, C. & Gillery, P. Carbamylated Proteins in Renal Disease: Aggravating Factors or Just Biomarkers? *International Journal of Molecular Sciences 2022, Vol. 23, Page 574* **23**, 574 (2022).
74. Jankowski, V. *et al.* Carbamylated sortilin associates with cardiovascular calcification in patients with chronic kidney disease. *Kidney Int* **101**, 574–584 (2022).
75. Wang, Z. *et al.* Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nat Med* **13**, 1176–1184 (2007).
76. Johnson, M. L. *et al.* Eicosapentaenoic acid but not docosahexaenoic acid restores skeletal muscle mitochondrial oxidative capacity in old mice. *Aging Cell* **14**, 734–743 (2015).
77. Kalim, S. *et al.* The Effects of Parenteral Amino Acid Therapy on Protein Carbamylation in Maintenance Hemodialysis Patients. *Journal of Renal Nutrition* **25**, 388–392 (2015).
78. Shi, J. *et al.* Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. *Proceedings of the National Academy of Sciences* **108**, 17372–17377 (2011).
79. van Delft, M. A. M. & Huizinga, T. W. J. An overview of autoantibodies in rheumatoid arthritis. *J Autoimmun* **110**, (2020).

80. Turunen, S. *et al.* Rheumatoid arthritis antigens homocitrulline and citrulline are generated by local myeloperoxidase and peptidyl arginine deiminases 2, 3 and 4 in rheumatoid nodule and synovial tissue. *Arthritis Res Ther* **18**, (2016).
81. Verheul, M. K. *et al.* Anti-carbamylated protein antibodies: a specific hallmark for rheumatoid arthritis. Comparison to conditions known for enhanced carbamylation; renal failure, smoking and chronic inflammation. *Ann Rheum Dis* **75**, 1575–6 (2016).
82. Yee, A. *et al.* Anti-CarP antibodies as promising marker to measure joint damage and disease activity in patients with rheumatoid arthritis. *Immunol Res* **61**, 24–30 (2015).
83. Spinelli, F. R. *et al.* Association between antibodies to carbamylated proteins and subclinical atherosclerosis in rheumatoid arthritis patients. *BMC Musculoskelet Disord* **18**, 1–9 (2017).
84. Roux-Lombard, P., Pagano, S., Montecucco, F., Satta, N. & Vuilleumier, N. Auto-antibodies as Emergent Prognostic Markers and Possible Mediators of Ischemic Cardiovascular Diseases. *Clinical Reviews in Allergy & Immunology 2010 44:1* **44**, 84–97 (2010).
85. Jaisson, S. *et al.* Homocitrulline as marker of protein carbamylation in hemodialyzed patients. *Clinica Chimica Acta* **460**, 5–10 (2016).
86. Tan, K. C. B. *et al.* Carbamylated lipoproteins and progression of diabetic kidney disease. *Clinical Journal of the American Society of Nephrology* **15**, 359–366 (2020).
87. Dunphy, K., Dowling, P., Bazou, D. & O'Gorman, P. Current Methods of Post-Translational Modification Analysis and Their Applications in Blood Cancers. *Cancers (Basel)* **13**, 1930 (2021).
88. Chen, J. *et al.* Proteome analysis of gastric cancer metastasis by two-dimensional gel electrophoresis and matrix assisted laser desorption/lonization-mass spectrometry for identification of metastasis-related proteins. *J Proteome Res* **3**, 1009–1016 (2004).
89. Zhao, Y. & Jensen, O. N. Modification-specific proteomics: Strategies for characterization of post-translational modifications using enrichment techniques. *Proteomics* **9**, 4632 (2009).
90. Hattori, T. & Koide, S. Next-generation antibodies for post-translational modifications. *Curr Opin Struct Biol* **51**, 141–148 (2018).
91. Krahmer, N. *et al.* Organellar Proteomics and Phospho-Proteomics Reveal Subcellular Reorganization in Diet-Induced Hepatic Steatosis. *Dev Cell* **47**, 205-221.e7 (2018).
92. Zhang, D. *et al.* Spatial epigenome–transcriptome co-profiling of mammalian tissues. *Nature 2023 616:7955* **616**, 113–122 (2023).
93. Vickovic, S. *et al.* SM-Omics is an automated platform for high-throughput spatial multi-omics. *Nature Communications 2022 13:1* **13**, 1–13 (2022).
94. Kuppe, C. *et al.* Spatial multi-omic map of human myocardial infarction. *Nature* **608**, 766 (2022).
95. He, S. *et al.* High-plex imaging of RNA and proteins at subcellular resolution in fixed tissue by spatial molecular imaging. *Nature Biotechnology 2022 40:12* **40**, 1794–1806 (2022).
96. Vandereyken, K., Sifrim, A., Thienpont, B. & Voet, T. Methods and applications for single-cell and spatial multi-omics. *Nature Reviews Genetics 2023 24:8* **24**, 494–515 (2023).
97. Wang, X., Fan, D., Yang, Y., Gimple, R. C. & Zhou, S. Integrative multi-omics approaches to explore immune cell functions: Challenges and opportunities. *iScience* **26**, 106359 (2023).
98. Liu, M. *et al.* Cardiovascular disease and its relationship with chronic kidney disease. *Eur Rev Med Pharmacol Sci* **18**, 2918–2926 (2014).
99. Palit, S. & Kendrick, J. Vascular Calcification in Chronic Kidney Disease: Role of Disordered Mineral Metabolism. *Curr Pharm Des* **20**, 5829 (2014).
100. Kalra, S. S. & Shanahan, C. M. Vascular calcification and hypertension: Cause and effect. *Ann Med* **44**, S85–S92 (2012).
101. Raggi, P. Coronary artery calcification predicts risk of CVD in patients with CKD. *Nature Reviews Nephrology 2017 13:6* **13**, 324–326 (2017).
102. Pavik, I. *et al.* Secreted Klotho and FGF23 in chronic kidney disease Stage 1 to 5: A sequence suggested from a cross-sectional study. *Nephrology Dialysis Transplantation* **28**, 352–359 (2013).
103. Hu, M. C. *et al.* Klotho deficiency is an early biomarker of renal ischemia-reperfusion injury and its replacement is protective. *Kidney Int* **78**, 1240–1251 (2010).
104. Schurgers, L. J. *et al.* The circulating inactive form of matrix gla protein is a surrogate marker for vascular calcification in chronic kidney disease: A preliminary report. *Clinical Journal of the American Society of Nephrology* **5**, 568–575 (2010).
105. Saar-Kovrov, V., Donners, M. M. P. C. & van der Vorst, E. P. C. Shedding of Klotho: Functional Implications in Chronic Kidney Disease and Associated Vascular Disease. *Front Cardiovasc Med* **7**, 407 (2021).

106. Hu, M. C. *et al.* Klotho deficiency causes vascular calcification in chronic kidney disease. *Journal of the American Society of Nephrology* **22**, 124–136 (2011).
107. Mencke, R., Olauson, H. & Hillebrands, J. L. Effects of Klotho on fibrosis and cancer: A renal focus on mechanisms and therapeutic strategies. *Adv Drug Deliv Rev* **121**, 85–100 (2017).
108. Hui, H. *et al.* Klotho suppresses the inflammatory responses and ameliorates cardiac dysfunction in aging endotoxemic mice. *Oncotarget* **8**, 15663 (2017).
109. Hu, M. C., Shiizaki, K., Kuro-O, M. & Moe, O. W. Fibroblast growth factor 23 and klotho: Physiology and pathophysiology of an endocrine network of mineral metabolism. *Annu Rev Physiol* **75**, 503–533 (2013).
110. Bär, L., Stournaras, C., Lang, F. & Föller, M. Regulation of fibroblast growth factor 23 (FGF23) in health and disease. *FEBS Lett* **593**, 1879–1900 (2019).
111. Razzaque, M. S. The FGF23-Klotho axis: Endocrine regulation of phosphate homeostasis. *Nat Rev Endocrinol* **5**, 611–619 (2009).
112. Chen, C. Di, Podvin, S., Gillespie, E., Leeman, S. E. & Abraham, C. R. Insulin stimulates the cleavage and release of the extracellular domain of Klotho by ADAM10 and ADAM17. *Proc Natl Acad Sci U S A* **104**, 19796–19801 (2007).
113. Maekawa, Y. *et al.* Klotho suppresses TNF-α-induced expression of adhesion molecules in the endothelium and attenuates NF-κB activation. *Endocrine* **35**, 341–346 (2009).
114. Saito, Y. *et al.* Klotho protein protects against endothelial dysfunction. *Biochem Biophys Res Commun* **248**, 324–329 (1998).
115. Semba, R. D. *et al.* Plasma klotho and cardiovascular disease in adults. *J Am Geriatr Soc* **59**, 1596–1601 (2011).
116. Zhou, H. H., Pu, S., Zhou, H. H. & Guo, Y. Klotho as Potential Autophagy Regulator and Therapeutic Target. *Front Pharmacol* **12**, 2930 (2021).
117. Sachdeva, A. *et al.* Klotho and the Treatment of Human Malignancies. *Cancers (Basel)* **12**, 1–22 (2020).
118. Adema, A. Y., Vervloe, Blankenstein, M. A. & Heijboer, A. C. α-Klotho is unstable in human urine. *Kidney Int* **88**, 1442–1444 (2015).
119. Du, J. *et al.* Inhibition of CDKS by roscovitine suppressed LPS-induced •NO production through inhibiting NFκB activation and BH4 biosynthesis in macrophages. *Am J Physiol Cell Physiol* **297**, 742–749 (2009).
120. Pfänder, P., Fidan, M., Burret, U., Lipinski, L. & Vettorazzi, S. Cdk5 deletion enhances the anti-inflammatory potential of GC-mediated GR activation during inflammation. *Front Immunol* **10**, 1554 (2019).
121. Lee, H. Y., Lim, S. & Park, S. Role of Inflammation in Arterial Calcification. *Korean Circ J* **51**, 114 (2021).
122. Waring, O. J., Skenteris, N. T., Biessen, E. A. L. & Donners, M. M. P. C. Two-faced Janus: the dual role of macrophages in atherosclerotic calcification. *Cardiovasc Res* **118**, 2768 (2022).
123. Cicenas, J. *et al.* Roscovitine in cancer and other diseases. *Ann Transl Med* **3**, 135 (2015).
124. Bai, B. *et al.* Cyclin-dependent kinase 5-mediated hyperphosphorylation of sirtuin-1 contributes to the development of endothelial senescence and atherosclerosis. *Circulation* **126**, 729–740 (2012).
125. Do, P. A. & Lee, C. H. The Role of CDK5 in Tumours and Tumour Microenvironments. *Cancers 2021, Vol. 13, Page 101* **13**, 101 (2020).
126. Ohshima, T. *et al.* Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. *Proc Natl Acad Sci U S A* **93**, 11173 (1996).
127. Khongorzul, P., Ling, C. J., Khan, F. U., Ihsan, A. U. & Zhang, J. Antibody-drug conjugates: A comprehensive review. *Molecular Cancer Research* **18**, 3–19 (2020).
128. Dumontet, C., Reichert, J. M., Senter, P. D., Lambert, J. M. & Beck, A. Antibody–drug conjugates come of age in oncology. *Nat Rev Drug Discov* **22**, 641–661 (2023).
129. Taguchi, K. *et al.* Cyclin G1 induces maladaptive proximal tubule cell dedifferentiation and renal fibrosis through CDK5 activation. *J Clin Invest* **132**, (2022).
130. Bai, X., Hou, X., Tian, J., Geng, J. & Li, X. CDK5 promotes renal tubulointerstitial fibrosis in diabetic nephropathy via ERK1/2/PPARγ pathway. *Oncotarget* **7**, 36510 (2016).
131. Shupp, A. *et al.* Biological functions of CDK5 and potential CDK5 targeted clinical treatments. *Oncotarget* **8**, 17373–17382 (2017).



# Chapter 7



Summary

Samenvatting

Краткое Содержание

Kokkuvõte

## Summary

Cardiovascular disease (CVD) is the main cause of death worldwide. The major underlaying reason for CVD development is atherosclerosis, which is accumulation of lipids and inflammatory factors within the artery wall. It can lead to near occlusion of the vessel or to thrombosis, after rupture of the diseased artery wall, resulting in stroke or a heart attack. There are several risk factors for atherosclerosis, such as high blood pressure, smoking, obesity, sedentary lifestyle, as well as kidney function decline. Kidneys fulfill numerous activities in the organism, but most notably they help sustain a healthy mineral balance and excrete toxic waste products. Therefore, it is not surprising that worsening of kidney function negatively affects that of the heart and vessels. In fact, kidney disease patients often die because of cardiovascular problems and chronic kidney disease (CKD) is an independent risk factor for CVD[1]. The interplay between CKD and subsequent cardiovascular complications is called the "cardiorenal syndrome". However, the molecular processes behind this complex syndrome are not entirely understood.

This thesis is dedicated to understanding of several processes involved in cardiorenal syndrome. In **Chapter 2**, we look at small molecules and toxic waste products (the so-called protein bound uremic toxins, or PBUTs), that accumulate in the organism during kidney function decline, with the goal to identify the best approach for their reduction. In a healthy organism PBUTs are removed by the kidneys. However, with the start of CKD, the levels of these molecules begin to rise imposing severe toxicity on the cells, causing cardiovascular complications, and increasing the risk of CVD. A large part of PBUTs is strongly attached to plasma proteins making them difficult to remove with conventional dialysis that is based on filtering out small, water-soluble compounds. We conducted a systematic review of literature published between 1980 and 2020 to identify the best possible approach for reduction of PBUTs' blood levels in patients on dialysis. Our analysis revealed that there is currently no "one-size-fits-all" technique for effective reduction of all 33 known compounds. Nevertheless, certain methods, such as use of oral supplement AST-120 (Kremezin®), or implementation of fractionated plasma separation and adsorption dialysis, showed the highest degree of reduction in long-term and single treatment trials, respectively, for such well-studied PBUTs as indoxyl sulphate and *p*-cresyl sulphate. Further research is needed to pinpoint techniques for most effective removal of PBUTs from blood in an individual CKD patient.

Next, in **Chapter 3**, we studied a common protein modification found in CKD patients, carbamylation, and its role in atherosclerosis. We used conventional and fluorescent staining to map the extent and localization of carbamylation in atherosclerotic plaque of different stages and compare staining of plaques from patients with kidney failure to those with healthy kidneys. We discovered that plaque carbamylation significantly increases with atherosclerosis progression in patients with CKD. We also identified the uptake of carbamylated low density lipoprotein (carbLDL) by plaque macrophages as a major contributor to this increase. Our cell experiments showed, however, that the uptake of carbLDL is less toxic to the cells than

oxidized LDL, a well-studied modified cholesterol carrier known to induce atherosclerosis. Further studies are required to elucidate the physiological effects of carbamylation on atherosclerosis in CKD.

In **Chapter 4** we conducted a mini literature review to discuss an important anti-calcification factor that is negatively affected by CKD, Klohto. Calcification is a major complication of kidney disease and is strongly associated with worsening of cardiovascular health. Klotho is one of the protective factors that regulates blood phosphate levels and prevents vascular calcification in a healthy organism. However, its levels are significantly reduced already at the early stages of CKD. Klotho is produced in the kidney and needs to be cleaved from the cell surface before it can enter the circulation and fulfill its functions throughout the body. Depending on the cleavage mechanism, different variants of Klotho protein can be produced which also potentially possess different functional properties. Our review identifies the knowledge gap in different Klotho protein variants research highlighting the importance of further studies that could potentially reveal new mechanisms for preventing calcification and accompanying cardiovascular complications in patients with CKD.

Thanks to the advancement in gene expression profiling and data analysis, novel players can be identified within cardiovascular field that were previously left unnoticed. Thus, in **Chapter 5**, we used a new technique, weighted gene co-expression network analysis, to discover the association of macrophage cyclin-dependent kinase 5 (CDK5) with plaque calcification. CDK5 was known previously for its regulatory and proinflammatory functions, however the link with calcification has not been established. Our study demonstrated that specific deletion of CDK5 in a specific type of white blood cells (i.e. myeloid cells) resulted in less advanced atherosclerotic plaques with lower calcification levels in mice. These findings make CDK5 an attractive target for drug development for atherosclerosis.

To summarize, this thesis tackled several aspects of cardiorenal syndrome: from small molecules accumulating with kidney function decline, to protein modifications caused by these toxins and associated with CVD, to calcification-promoting proteins that hold potential for promising drug targets. It is important to understand the complexity and all the players involved in development of such multifactorial condition as cardiorenal syndrome to be able to effectively prevent and treat it in future.

# Samenvatting

Hart- en vaatziekten (HVZ) zijn wereldwijd de belangrijkste doodsoorzaak. De belangrijkste onderliggende oorzaak van hart- en vaatziekten is de ontwikkeling van atherosclerose, een ophoping van vetten en ontstekingsfactoren in de slagaderwand die kan leiden tot afsluiting van het bloedvat of trombose na openscheuren van de plaque, met een beroerte of hartaanval als gevolg. Er zijn verschillende oorzaken en verergerende factoren voor atherosclerose, zoals hoge bloeddruk, roken, obesitas, een zittende levensstijl en een verminderde nierfunctie. Nieren vervullen tal van functies in het organisme, zoals het in stand houden van een gezonde mineralenbalans en het uitscheiden van giftige afvalstoffen. Dus het is niet verwonderlijk dat een verslechtering van de nierfunctie een negatieve invloed heeft op hart en bloedvaten. Het is zelfs aangetoond dat nieraandoeningen de kans om te overlijden aan hart- en vaatziekten sterk verhoogd[1]. Het samenspel tussen de ontwikkeling van een chronische nierziekte (CKD) en daaropvolgende HVZ wordt het “cardiorenaal syndroom” genoemd. De moleculaire mechanismen achter dit syndroom worden echter niet volledig begrepen.

Dit proefschrift is gewijd aan het begrijpen van verschillende processen die betrokken zijn bij het cardiorenaal syndroom. In **Hoofdstuk 2** kijken we naar kleine moleculen, of eiwitgebonden uremische toxinen (PBUT's), die zich ophopen in het organisme tijdens de achteruitgang van de nierfunctie, met als doel het identificeren van de beste aanpak voor de vermindering ervan. PBUT's zijn een groep toxische afvalproducten die in een gezond organisme door de nieren worden verwijderd. Als gevolg van CKD zullen de bloedspiegels van deze moleculen echter stijgen, wat toxisch kan zijn voor de cellen in het lichaam en wat uiteindelijk kan bijdragen tot het ontstaan van HVZ. Een groot deel van de PBUT's is sterk gebonden aan plasma-eiwitten waardoor ze moeilijk te verwijderen zijn met conventionele nierdialyse, die gebaseerd zijn op het filtreren van kleine, in water-oplosbare verbindingen. We hebben een systematisch overzicht uitgevoerd van de literatuur die tussen 1980 en 2020 is gepubliceerd om de best mogelijke aanpak te identificeren voor het verlagen van de bloedspiegels van PBUT's bij patiënten die dialyse ondergaan. Uit onze analyse bleek dat er momenteel geen "one-size-fits-all" techniek bestaat die alle 33 bekende verbindingen effectief kan verwijderen. Niettemin bleken bepaalde methoden, zoals het gebruik van het orale supplement AST-120 (Kremezin®), of de toepassing van gefractioneerde plasmascheiding en adsorptiedialyse, op dit moment het effectiefste te zijn, bij langdurig gebruik en na eenmalige toepassing, respectievelijk, voor goed onderzochte PBUT's als indoxylsulfaat en p-cresylsulfaat. Er is ongetwijfeld verder onderzoek nodig om effectieve technieken te identificeren voor het aanpakken van de toename van PBUT's bij CKD-patiënten, die waarschijnlijk gebaseerd zouden zijn op de kenmerken van de individuele patiënt.

Vervolgens bestudeerden we in **Hoofdstuk 3** een veel voorkomende eiwitmodificatie bij CKD-patiënten, carbamylering, en de rol hiervan bij atherosclerose. Kleuring van de plaque met behulp van (fluorescerende) antilichamen stelde me in staat om de mate en lokalisatie van carbamylering in verschillende stadia van het ziekteproces in kaart te brengen en plaques van

patiënten met nierinsufficiëntie te vergelijken met die van patiënten met een behouden nierfunctie. We ontdekten dat carbamylering toeneemt met het voortschrijden van de ziekte met name in patiënten die ook aan nierfalen leiden. We hebben daarnaast de opname van gecarbamyleerd LDL, een cholesterol vervoerend eiwit in bloed (carbLDL) door macrofagen in de plaque als een belangrijke oorzaak van deze toename. Onze in vitro experimenten toonden echter aan dat de opname van carbLDL minder toxische effecten op de cellen had dan de opname van geoxideerd LDL, een goed bestudeerd gemodificeerd deeltje waarvan bekend is dat het atherosclerose induceert. Verdere studies zijn nodig om de fysiologische gevolgen van carbamylering op atherosclerose bij CKD op te helderen.

In **Hoofdstuk 4** hebben we een mini literatuuroverzicht uitgevoerd om een belangrijke antikalkfactor te bespreken die negatief beïnvloed wordt door CKD, Klohto. Verkalking is een belangrijke complicatie van nierinsufficiëntie die sterk geassocieerd is met cardiovasculaire morbiditeit. Klotho is een van de beschermende factoren die de fosfaatspiegels in het bloed reguleert en vaatverkalking voorkomt in een gezond organisme. De niveaus ervan zijn echter al in de vroege stadia van CKD aanzienlijk verlaagd. Klotho wordt geproduceerd in de nieren en moet worden gesplitst van het celoppervlak voordat het de bloedsomloop kan binnentreden om zijn rol elders in het lichaam te vervullen. Afhankelijk van het splitsingsmechanisme kunnen verschillende varianten van het Klotho eiwit geproduceerd worden die mogelijk ook verschillende functionele eigenschappen bezitten. Onze review identificeert de kennisleemte in het onderzoek naar verschillende Klotho eiwitvarianten en benadrukt het belang van verdere studies die mogelijk nieuwe mechanismen kunnen onthullen voor het tegengaan van verkalking en de bijbehorende cardiovasculaire complicaties bij patiënten met CKD.

Dankzij de recente vooruitgang in de analyse van (gen)expressieprofielen kunnen nieuwe spelers inde ontwikkeling of escalatie van HVZ worden geïdentificeerd, die voorheen onopgemerkt bleven. Zo hebben we in **Hoofdstuk 5** een nieuwe techniek, gewogen gen-coexpressienetwerkanalyse, toegepast en daarmee cycline-afhankelijk kinase 5 (CDK5) ontdekt als mogelijke speler in de aderverkalking. CDK5 was al eerder bekend om zijn regulerende en ontstekingsbevorderende functies, maar het verband met verkalking was nog niet aangetoond. Onze studie toonde aan dat specifieke verwijdering van CDK5 in bepaalde (myeloïde) witte bloedcellen in de muis resulteerde in een verminderd voortschrijden van de plaquevorming en een verlaagde verkalkingsgraad. Deze bevindingen maken CDK5 een aantrekkelijk doelwit voor de ontwikkeling van medicijnen tegen atherosclerose.

Samenvattend behandelde dit proefschrift verschillende aspecten van het cardiorenaal syndroom: van kleine moleculen die zich ophopen bij een afname van de nierfunctie, tot eiwitmodificaties veroorzaakt door deze toxines en geassocieerd met CVD, tot verkalking bevorderende eiwitten die potentieel veelbelovende doelwitten voor geneesmiddelen zijn. Het is belangrijk om de complexiteit en alle spelers te begrijpen die betrokken zijn bij de ontwikkeling van zo'n multifactoriële aandoening als het cardiorenaal syndroom om het in de toekomst effectief te kunnen voorkomen en behandelen.

# Краткое Содержание

Сердечно-сосудистые заболевания (ССЗ) являются первостепенной причиной смертности во всем мире. Основным источником развития ССЗ является атеросклероз - накопление липидов и воспалительных факторов в стенке артерии. Атеросклероз может вызвать закупорку сосудов или тромбоз, образованный разрывом стенки пораженной артерии, что неминуемо приводит к инсульту или инфаркту. Существует несколько факторов риска развития атеросклероза, таких как повышенное артериальное давление, курение, ожирение, малоподвижный образ жизни, а также почечная недостаточность. Почки выполняют множество функций в организме, но прежде всего они способствуют поддержанию здорового минерального баланса и выведению токсичных продуктов жизнедеятельности. Поэтому неудивительно, что ухудшение функции почек негативно сказывается на работе сердца и сосудов. Более того, пациенты с заболеваниями почек часто умирают из-за сердечно-сосудистых проблем, а хроническая болезнь почек (ХБП) является независимым фактором риска развития ССЗ[1]. Взаимосвязь между ХБП и последующими сердечно-сосудистыми осложнениями получила название "кардиоренальный синдром". Однако молекулярные процессы, лежащие в основе этого сложного синдрома, до конца не изучены.

Данная диссертация посвящена исследованию ряда процессов, участвующих в развитии кардиоренального синдрома. В **Главе 2** мы рассматриваем токсичные малые молекулы (так называемые связанные с белками уремические токсины, или PBUTs), которые накапливаются в организме при ухудшении функции почек, с целью определения оптимального клинического метода для выведения их из организма. В здоровом организме PBUTs выводятся почками. Однако с началом ХПН уровень этих молекул начинает расти, оказывая сильное токсическое воздействие на клетки, вызывая сердечно-сосудистые осложнения и повышая риск развития ССЗ. Значительная часть PBUTs прочно связана с белками плазмы, что затрудняет их удаление традиционным диализным методом, который основан на отфильтровывании мелких водорастворимых частиц. Мы провели систематический обзор литературы, опубликованной в период с 1980 по 2020 год, с целью выявления оптимального подхода к снижению уровня PBUTs в крови у пациентов, находящихся на диализе. Проведенный анализ показал, что в настоящее время не существует универсальной методики эффективного снижения уровня всех 33 известных токсинов. Тем не менее, некоторые методы, такие как использование пероральной добавки АСТ-120 (Кремезин®), проведение фракционированного разделения плазмы и адсорбционного диализа, показали наибольшую степень снижения уровня таких хорошо изученных PBUTs, как индоксилсульфат и *п*-крезилсульфат, в долгосрочном и однократном исследовании соответственно. Необходимы дальнейшие исследования для определения методов наиболее эффективного удаления PBUTs из крови основываясь на индивидуальных показателях пациентов с почечной недостаточностью.

В **Главе 3**, мы изучили распространенную модификацию белков, встречающуюся у больных ХПН, карбамилирование, и её роль в развитии атеросклероза. При сравнении окрашивания бляшек на разной стадии развития взятых у пациентов с почечной недостаточностью и пациентов со здоровыми почками обычным и флуоресцентным методами была определена степень и локализация карбамилирования, что в свою очередь выявило значительное увеличение карбамилирования прогрессивных бляшек у пациентов с ХПН. Мы также определили, что основной вклад в это увеличение вносит поглощение карбамилированного липопротеина низкой плотности (carbLDL) макрофагами бляшки. Однако, клеточные эксперименты показали, что поглощение carbLDL менее токсично для клеток, чем поглощение окисленного LDL - хорошо изученного модифицированного носителя холестерина, который, как известно, вызывает атеросклероз. Для выяснения физиологических эффектов карбамилирования на развитие атеросклероза при ХПН необходимы дальнейшие исследования.

В **Главе 4** был проведён мини-обзор литературы, где обсуждается важный антикальцификационный фактор, подверженный негативному влиянию ХПН, Klotho. Кальцификация является одним из основных осложнений заболевания почек и тесно связана с ухудшением сердечно-сосудистого здоровья. Klotho - один из защитных факторов, регулирующий уровень фосфатов в крови и предотвращающий кальцификацию сосудов в здоровом организме. Однако уже на ранних стадиях ХПН его уровень значительно снижается. Чтобы попасть в кровообращение и выполнять свои функции в организме Klotho, который вырабатывается непосредственно в почках, должен пройти процесс отщепления с поверхности клетки. В зависимости от механизма отщепления могут образовываться различные варианты белка Klotho, которые также потенциально обладают различными функциональными свойствами. Наш обзор выявляет пробел в знаниях о различных вариантах белка Klotho, подчеркивая важность дальнейших исследований, которые в свою очередь могут раскрыть новые механизмы предотвращения кальцификации и сопутствующих сердечно-сосудистых осложнений у пациентов с ХПН.

Благодаря последним развитиям в сфере анализа экспрессии генов появилась возможность обнаружить ранее незамеченные факторы, играющие важную роль в работе сердечно-сосудистой системы. Так, в **Главе 5** представлены результаты выявления связи макрофагальной циклин-зависимой киназы 5 (CDK5) с кальцификацией бляшек при помощи использования нового метода, такого как анализ взвешенной сети коэкспенсии генов. Ранее было известно, что CDK5 выполняет регуляторные и провоспалительные функции, однако её связь с кальцификацией не была установлена. Наше исследование на мышцах показало, что специфическая делеция CDK5 в определенном типе лейкоцитов (миелоидных клетках) приводит к образованию менее развитых атеросклеротических бляшек с более низким уровнем кальцификации. Полученные оптимистические результаты показывают необходимость

продолжения исследования CDK5 и её возможном применении при разработке препаратов против атеросклероза.

Подводя итог, можно сказать, что в данной диссертации рассмотрены несколько аспектов кардиоренального синдрома: от малых молекул, накапливающихся при снижении функции почек, до модификаций белков, вызываемых этими токсинами и связанных с ССЗ, а также белков, способствующих кальцификации, потенциально являющимися целями для разработки лекарственных препаратов. Важно понимать всю сложность и характер развития такого многофакторного заболевания как кардиоренальный синдром для улучшения эффективности методов его предотвращения и повышении результативности лечения.

# Kokkuvõte

Südame-veresoonkonnahaigused (SVH) on peamine surma põhjus kogu maailmas. Südame-veresoonkonna haiguste peamiseks tekitajaks on ateroskleroos, mis on lipiidide ja põletikufaktorite kogunemine arteri seintesse. See võib viia veresoone järkjärgulise sulgumiseni või tromboosini kui haigestunud arteri sein rebeneb ning mille tulemuseks on insult või südameinfarkt. Ateroskleroosil on mitmeid riskifaktoreid, näiteks kõrge vererõhk, suitsetamine, ülekaalulisus, istuv eluviis, samuti neerufunktsiooni langus. Neerud täidavad organismis arvukaid ülesandeid, kuid eelkõige aitavad nad säilitada tervislikku mineraalide tasakaalu ja väljutada mürgiseid jääkaineid. Seetõttu ei ole üllatav, et neerufunktsiooni halvenemine mõjutab negatiivselt südame ja veresoonte funktsiooni. Paraku surevad neeruhaiged sageli SVH probleemide tõttu ja krooniline neeruhaigus on iseseisev riskifaktor südame-veresoonkonna haiguste tekkeks. Neeruhaigus ja hilisemate SVH tüsistuste vastastikust mõju nimetatakse kardiorenaalseks sündroomiks (KRS). Selle keerulise sündroomi taga olevad molekulaarsed protsessid ei ole siiani täielikult uuritud ja mõistetud.

Käesolev doktoritöö on pühendatud mitmete kardiorenaalse sündroomiga seotud protsesside mõistmisele. **Peatükis 2** vaatleme valkudega seotud väikese molekulkaaluga toksilisi jääkaineid (nn valkudega seotud ureemilisi toksiine ehk PBUT), mis akumuleeruvad organismis neerufunktsiooni languse tagajärjel, eesmärgiga leida parim lähenemine nende taseme alandamiseks veres. Terves organismis eemaldavad valkudega seotud ureemilisi toksiine neerud. Kui aga tekib neeruhaigus, hakkab nende molekulide tase veres tõusma, omades rakkudele tõsist toksilist toimet, suurendades südame-veresoonkonna haiguste riski ning tekitades SVH tüsistusi. Suur osa PBUT-dest on tugevalt seotud plasmavalkudega, mistõttu on neid raske eemaldada tavalise dialüüsi abil, mis keskendub väikeste, vees lahustuvate ühendite verest väljafiltreerimisele. Viisime läbi süstemaatilise kirjanduse ülevaate 1980 ja 2020 aasta vahel avaldatud materjalides, et leida parim võimalik meetod PBUT-de verest eemaldamiseks dialüüsipatsientidel. Meie analüüs näitas, et praegu ei ole olemas üht meetodit kõigi 33 teadaoleva valkudega seotud toksiini tõhusaks eemaldamiseks. Siiski näitasid teatud meetodid, nagu suukaudse toidulisandi AST-120 (Kremezin®) kasutamine või fraktsioneeritud plasma eraldamine ja adsorptsioondialüüs, pikaajalistes ja ühekordse ravi uuringutes vastavalt kõige suuremat vere taseme alanemist selliste põhjalikult uuritud PBUT-de nagu indoksüülsulfaat ja *p*-kresüülsulfaat puhul. Täiendavad uuringud on vajalikud, et määrata kindlaks meetodid, mis võimaldavad kõige tõhusamalt eemaldada PBUT-e kroonilise neeruhaigusega patsientide verest .

Järgmisena uurisime **Peatükis 3** neeruhaigusega patsientidel tihti esinevat valkude modifikatsiooni, karbamüleerimist, ja selle rolli ateroskleroosis. Kasutasime tavapärast ja fluorestsentsvärvimist, et kaardistada karbamüleerimise ulatust ja lokatsiooni eri staadiumi aterosklerootilistes naastudes ning võrrelda neerupuudulikkusega patsientide ja tervete uuritavate naaste. Avastasime, et naastu karbamüleerimine suureneb oluliselt ateroskleroosi progresseerumisega neeruhaigusega patsientidel. Samuti tuvastasime, et selle suurenemise

peamiseks põhjuseks on karbamüleeritud madala tihedusega lipoproteiini (carbLDL) vastuvõtmine naastu makrofaagide poolt. Meie rakukatsed näitasid siiski, et carbLDL-i omastamine on rakkude jaoks vähem toksiline kui oksüdeeritud LDLi omastamine, mis on rohkelt uuritud modifitseeritud kolesteroolikandja, mis teadaolevalt põhjustab ateroskleroosi. On vaja täiendavaid uuringuid, et selgitada karbamüleerimise füsioloogiline mõju ateroskleroosile kroonilise neeruhaiguse korral.

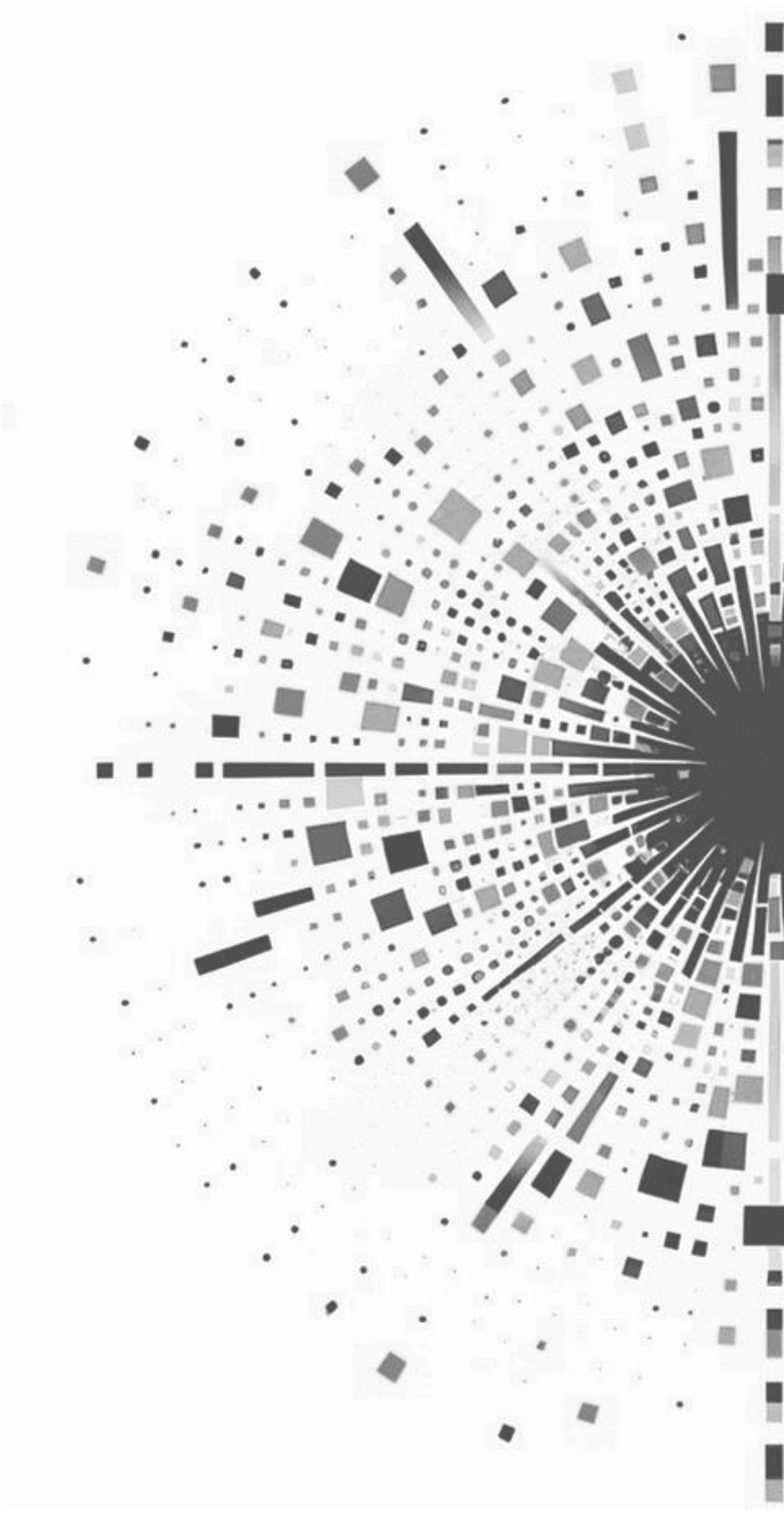
**Peatükis 4** teostasime väiksemahulise kirjanduse ülevaate, et kirjeldada ja diskuteerida Klothot kui olulist kaltsifikatsioonivastast tegurit, mida neeruhaigus negatiivselt mõjutab. Kaltsifikatsioon on neeruhaiguse peamine tüsistus ja on tugevalt seotud südame-veresoonkonna olukorra halvenemisega. Klotho on üks kaitsvatest teguritest, mis reguleerib vere fosfaadi taset ja takistab veresoonte kaltsifikatsiooni terves organismis. Selle tase väheneb aga oluliselt juba kroonilise neeruhaiguse varajases staadiumis. Klothot toodetakse neerudes ja see peab raku pinnalt lahustuma, enne kui see satub vereringesse ja täidab oma funktsioone kogu organismis. Sõltuvalt lõhustumismehhanismist võivad tekkida erinevad Klotho valgu variandid, millel on potentsiaalselt ka erinevad funktsionaalsed omadused. Meie ülevaates tuuakse välja teadmiste lünk erinevate Klotho valgu variantide uurimisel, rõhutades edasiste uuringute tähtsust, mis võivad potentsiaalselt paljastada uusi mehhanisme kaltsifikatsiooni ja sellega kaasnevate SVH tüsistuste ennetamiseks neeruhaigusega patsientidel.

Tänu edusammudele geenieksрessiooni profiilide koostamisel ja andmete analüüsimisel saab SVH valdkonnas tuvastada uusi tegureid, mis varem jäid märkamatuks. Seega kasutasime **Peatükis 5** uut tehnikat, kaalutud geenide koekspressioonivõrgustiku analüüsi, et avastada makrofaagide tsükliinist sõltuva kinaasi 5 (CDK5) seos naastude kaltsifikatsiooniga. CDK5 oli varem tuntud oma regulatiivsete ja proinflammatoorsete funktsioonide poolest, kuid seost kaltsifikatsiooniga ei olnud uuritud. Meie uuring näitas, et CDK5 spetsiifiline deletsioon teatud tüüpi valgelibledes (st müeloidrakkudes) põhjustas hiirtel vähem arenenud ateroskleroотilisi naastusid, mille kaltsifikatsioonitase oli madalam. Need leiud muudavad CDK5 atraktiivseks sihtmärgiks ateroskleroosi ravimite arendamisel.

Kokkuvõttes käsitleti käesolevas väitekirjas mitmeid kardiorenaalse sündroomi aspekte: alates neerufunktsiooni langusega akumuleeruvatest väikestest molekulidest ja nende toksiinide põhjustatud südame-veresoonkonna haigustega seotud valkude modifikatsioonist kuni kaltsifikatsiooni soodustavate valkudeni, millel on potentsiaal olla paljulubavate ravimite sihtmärkideks. Oluline on mõista kardiorenaalse sündroomi kui multifaktoriaalse haiguse arengu keerukust ja kõiki tegureid, et seda tulevikus tõhusalt ennetada ja ravida.

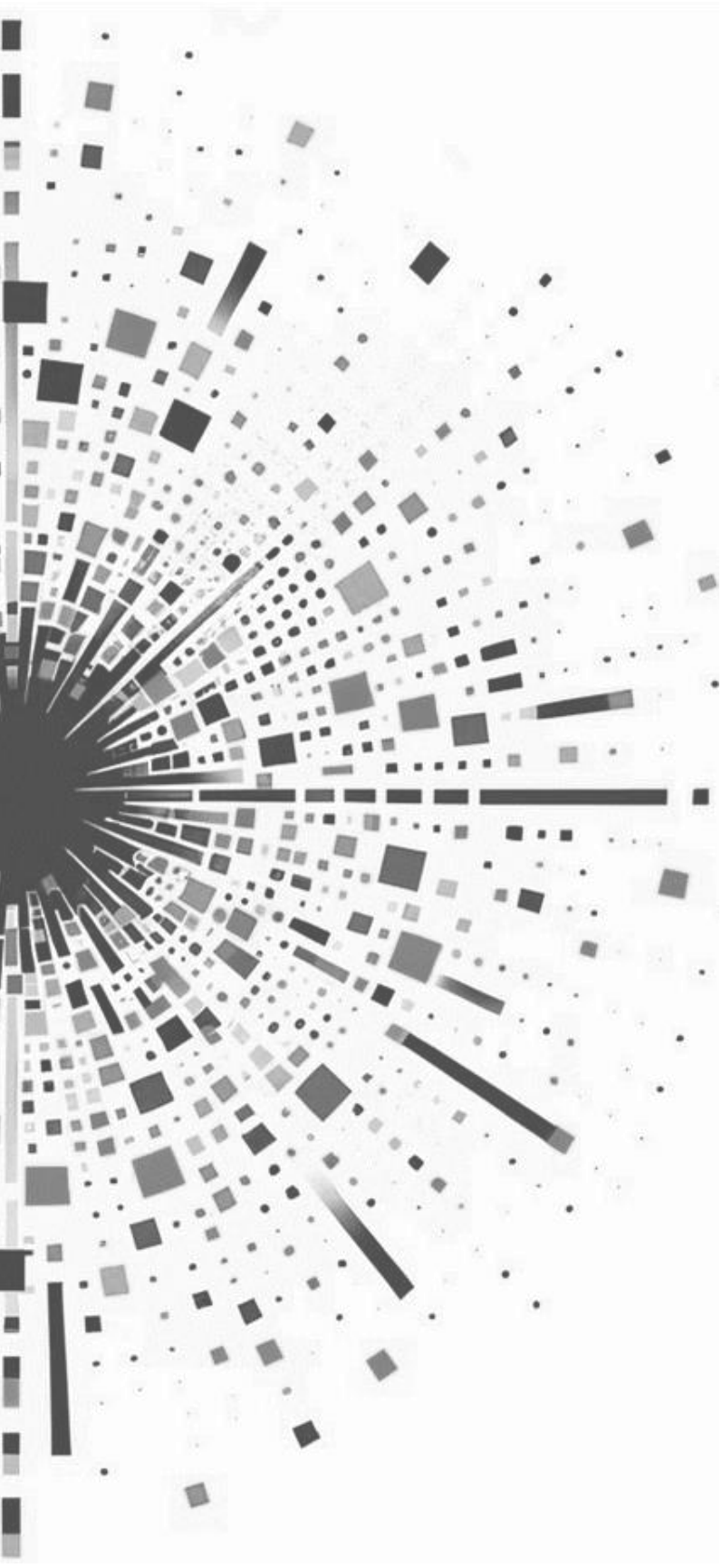
# References

1. Matsushita, K. *et al.* Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. *The Lancet* **375**, 2073–2081 (2010).



# Chapter 8

Impact



## Unmet need in dialysis research

This thesis is dedicated to studying the mechanisms underlying cardiorenal syndrome type IV, which describes cardiovascular complications caused by renal insufficiency[1]. Increased cardiovascular morbidity is, amongst others, associated with elevated concentrations of the uremic milieu in patients with chronic kidney disease (CKD)[2]. In **Chapter 2** of this thesis, we provide evidence that there is currently no universally suitable technique for reduction of all known 33 protein-bound uremic toxins (PBUTs), yet several approaches showed promising results for certain compounds, e.g. supplementation of haemodialysis patients with oral adsorbent AST-120 (Kremezin®) for reduction of indoxyl sulphate and *p*-cresyl sulphate in long-term trials. The systematic analysis made it clear that, due to the high heterogeneity of PBUTs, it is unlikely to achieve optimal reduction of all the compounds with a single therapy. Our work identifies the need for bigger longitudinal multicentre trials to study the effectiveness of combination therapies targeting compounds of different nature. Additionally, the review demonstrates the evident lack of research for reduction of certain PBUTs, e.g. 2-methoxyresorcinol, hydroquinone, putrescine and others, and serves as a helpful starting point for future studies targeting management of these understudied toxins. In a healthy kidney PBUTs are removed predominantly though tubular secretion, which is challenging to replicate with conventional dialysis. Thus, preservation of residual kidney function by, for instance, controlling blood pressure and minimizing use of nephrotoxic agents (e.g. radiocontrast dyes), as well as dietary interventions such as restriction of protein and increase of fibre and complex carbohydrates intake, can help to deceleration CKD progression and improve patients' survival[3,4].

## Role of post-translational protein modifications

Another factor that may link CKD to cardiovascular disease (CVD) risk increment is protein carbamylation, one of the first identified post translational protein modification (PTM) shown to be associated with uremia[5,6]. Its major role in cardiovascular disease was first brought to light by the group of Prof. S.L. Hazen with their work published in 2007 in Nature Medicine showing protein carbamylation resulting from myeloperoxidase activity in atherosclerotic plaque and blood carbamylation levels being predictive for patient death[7]. Leaving aside anti-carbamylation antibodies that are recently becoming recognized as a promising biomarker for rheumatoid arthritis and other diseases[8–10], carbamylation research received modest interest for the past decade with 20-30 articles per year. With our work in **Chapter 3**, we show that this type of protein modification is worth attention demonstrating its abundance in late stage hemorrhaged plaques of patients with kidney insufficiency and association with foam cells. CKD patients are known to have increased burden of atherosclerosis with plaques showing elevated risk of rupture[11,12]. It remains to be determined if plaque carbamylation is causally related to its vulnerability in patients with CKD. Additionally, we have seen differential cellular effects of carbLDL uptake by human macrophages compared to oxLDL uptake. This raises many questions such as: what the exact mechanism is of carbLDL trafficking, processing, and

storage by the cells compared to that of oxLDL; to which extent these processes are affected by the severity of the modification, and if they can be interfered with or, on the contrary, taken advantage of to influence the cells' faith and, as a result, plaque progression. Moreover, it is known that LDL proteins are not the only targets of carbamylation. In our results, a big portion of the extracellular matrix material in plaque, e.g. collagens, were also seen to stain positive for carb-lys. How this affects activity of the surrounding cells in the plaque as well as biomechanical properties of the protein itself and, consequently, plaque phenotype, remains to be determined. Finally, our confocal images revealed a strong nuclear carb-lys signal present in human macrophages. The mechanisms and significance of this nuclear carbamylation are yet to be dissected but may potentially hold a key to yet unknown gene expression regulation processes. To summarise, **Chapter 3** of this thesis highlights the necessity for future PTM studies and unveils possibilities for new discoveries of carbamylation-related cellular mechanisms.

PTM formation is not only an adverse process. In fact, practically all proteins require PTMs for proper functioning. In a mini review in **Chapter 4** we highlight the importance of post-translational cleavage of Klotho protein for its systemic function pointing out the difference between various forms of secreted Klotho, e.g. KL1, KL2, and full-length proteins. As can be seen from the review, higher levels of soluble Klotho in human were shown to be associated with lower risk of kidney disease while beneficial effects of its administration or overexpression have been plentifully demonstrated in mice, e.g. reduction of renal injury, improved recovery and survival, as well as prevention of calcification. However, no distinction has been made between the roles of full vs shorter Klotho forms while no information is available on the functions of KL2 thus far due to the lack of specific antibodies. This highlights the need for further studies to elucidate the molecular mechanisms behind generation of Klotho variants and the lack of knowledge regarding their, possibly, distinct functions. Better understanding of soluble Klotho generation and functioning could allow future drug discovery and generation of more precise therapies to target calcification and age-related complications.

## Novel drug targets in atherosclerosis

The identification of novel drug targets holds paramount importance in the development of efficacious therapeutic interventions across various pathological conditions. In this context, computational methodologies, such as weighted gene co-expression network analysis, offer a transformative solution allowing researchers to uncover hidden relationships between genes and their functions and providing insights into the molecular mechanisms underlying disease. Notably, our discovery of CDK5 as a previously unrecognized contributor to calcification (**Chapter 5**), underscores this potential. By analysing large sets of genetic data, macrophage CDK5 emerged as a central regulator of a gene program linked to calcification. Indeed, its deficiency led to reduced vascular calcification possibly by dampening inflammation *in vitro* and *in vivo*. This demonstrates how computational methods can reveal novel targets that

might have been overlooked using traditional approaches. The comprehension of the intricate molecular interactions allows discovery of innovative therapeutics tailored towards these new factors, thereby promoting development of enhanced and targeted interventions for cardiovascular disease and accompanying morbidities. Our work shows that myeloid-specific inhibition of CDK5 would potentially reduce plaque burden through decreasing inflammation and, subsequently, calcification.

However, a few hurdles remain to be addressed. Firs, to the best of our knowledge, there is only one specific CDK5 inhibitor described in the literature so far that still requires testing *in vivo*[13] while there are currently no CDK5-specific inhibitors being tested in clinical trials. Additionally, the therapeutic benefits of CDK5 inhibitors for atherosclerosis alleviation need to be investigated, as CDK5 is ubiquitously expressed by many other cell types, e.g. pancreatic β cells and neurons, and shown to play a vital role in neuronal development and cell survival[14,15]. Thus, a more targeted approach might be required to avoid possible side effects of systemic CDK5 inhibition. This could be achieved through, for example, implementation of antibody-drug conjugate technology for plaque macrophage targeting. However, such system remains to be developed. It would be also interesting to study the downstream targets of CDK5 in the plaque to possibly reveal a more precise targets for attenuation of plaque inflammation and calcification. Taken together, our study sheds light on possibilities for novel drug targets for treatment of atherosclerosis.

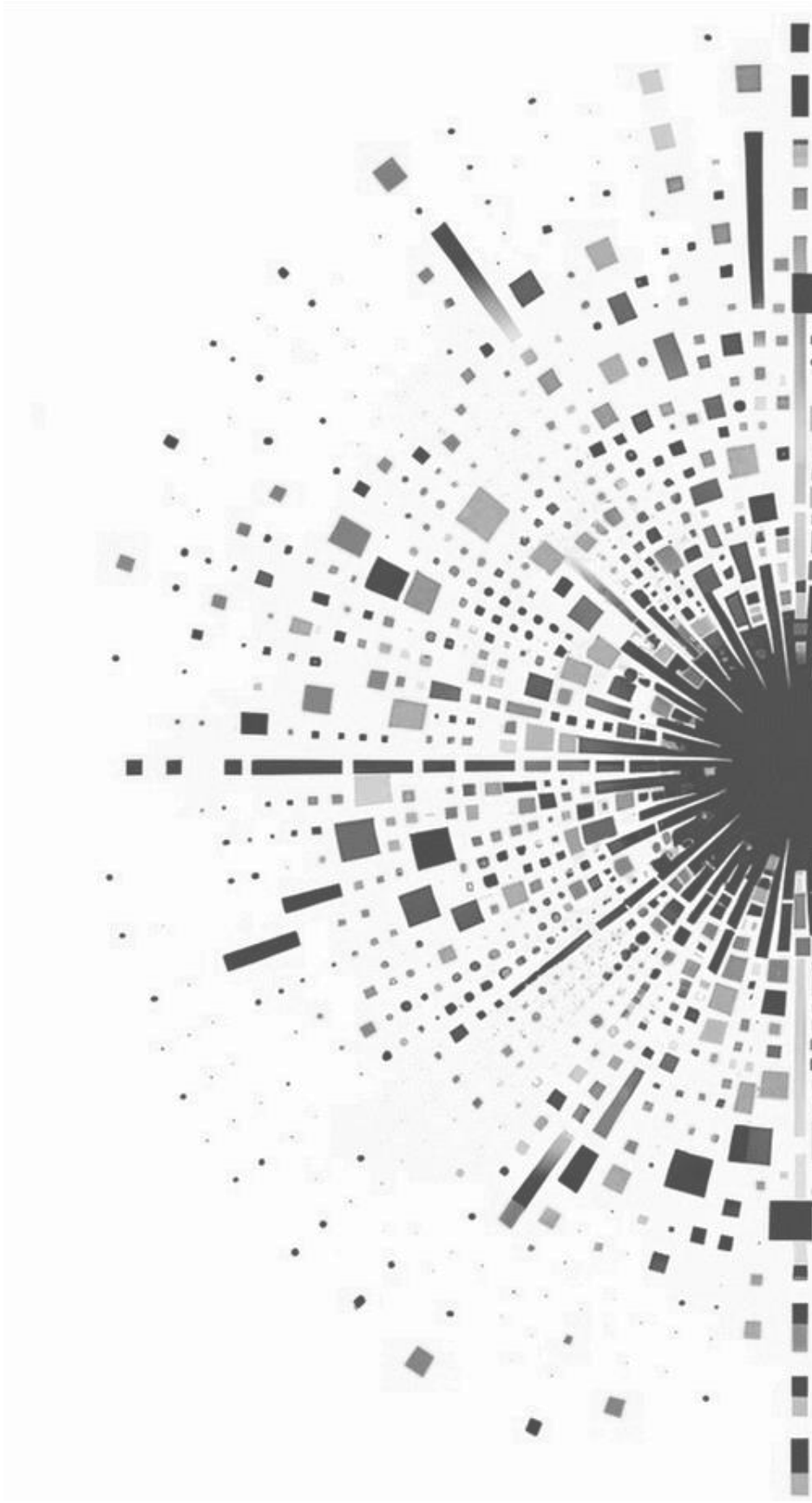
## Importance of scientific collaboration

In the environment of high competition and scares financial resources, researchers should be encouraged and incentivised to put aside their personal interests and engage in transparent exchange of knowledge and discoveries for the sake of societal progress. Hence, this work would have not been possible without collaborative effort from several parties. With this thesis we established diverse collaboration network between University of Maastricht and a number of other of universities in Germany, UK, Sweden, and Italy. Projects initiated with this work continue to develop beyond the scope of the thesis bringing a possibility of promising high impact publications and valuable scientific contribution in the future.

Taken together, this thesis broadens our understanding of complex processes of cardiorenal syndrome opening up opportunities for future drug discovery and personalized therapies to reduce the burden of CDK-associated cardiovascular disease.

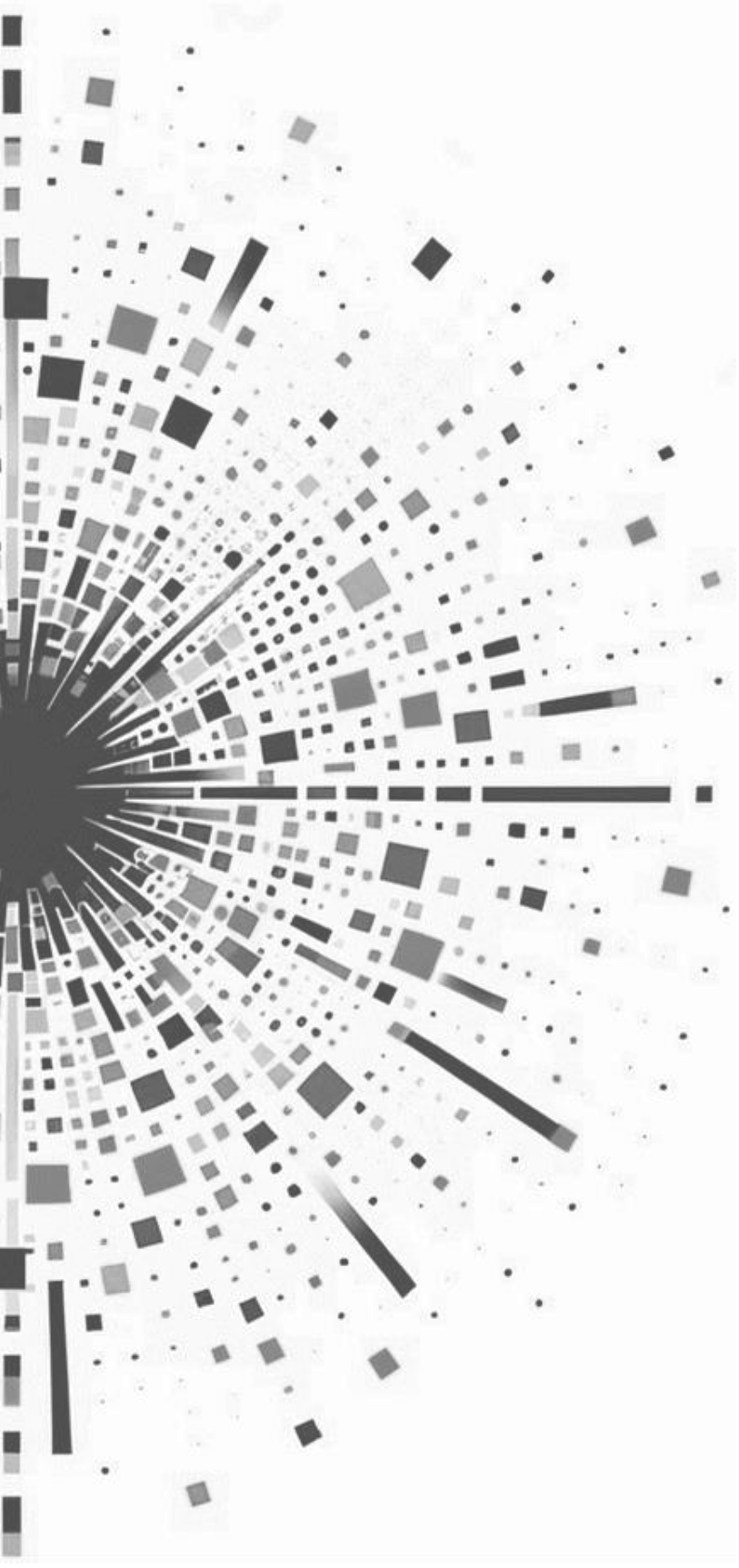
# References


1. Rangaswami, J. *et al.* Cardiorenal Syndrome: Classification, Pathophysiology, Diagnosis, and Treatment Strategies: A Scientific Statement From the American Heart Association. *Circulation* **139**, E840–E878 (2019).
2. El Chamieh, C., Liabeuf, S. & Massy, Z. Uremic Toxins and Cardiovascular Risk in Chronic Kidney Disease: What Have We Learned Recently beyond the Past Findings? *Toxins (Basel)* **14**, (2022).
3. Faria, M. & de Pinho, M. N. Challenges of reducing protein-bound uremic toxin levels in chronic kidney disease and end stage renal disease. *Transl Res* **229**, 115–134 (2021).
4. Mathew, A. T., Fishbane, S., Obi, Y. & Kalantar-Zadeh, K. Preservation of Residual Kidney Function in Hemodialysis Patients: Reviving an Old Concept for Contemporary Practice. *Kidney Int* **90**, 262 (2016).
5. Stark, G. R., Stein, W. H. & Moore, S. Reactions of the Cyanate Present in Aqueous Urea with Amino Acids and Proteins. *J Biol Chem* **235**, (1960).
6. Erill, S., Calvo, R. & Carlos, R. Plasma protein carbamylation and decreased acidic drug protein binding in uremia. *Clin Pharmacol Ther* **27**, 612–618 (1980).
7. Wang, Z. *et al.* Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nat Med* **13**, 1176–1184 (2007).
8. Dong, R. *et al.* Distribution and clinical significance of anti-carbamylation protein antibodies in rheumatological diseases among the Chinese Han population. *Front Immunol* **14**, (2023).
9. Calixto, O. J. *et al.* Posttranslational modifications in psoriatic arthritis: A systematic literature review. *Autoimmun Rev* **22**, (2023).
10. Liem, S. I. E. *et al.* Anti-carbamylated protein antibodies in systemic sclerosis. *Arthritis Res Ther* **25**, (2023).
11. Valdivielso, J. M. *et al.* Atherosclerosis in Chronic Kidney Disease. *Arterioscler Thromb Vasc Biol* **39**, 1938–1966 (2019).
12. Pelisek, J. *et al.* Impact of chronic kidney disease on carotid plaque vulnerability. *J Vasc Surg* **54**, 1643–1649 (2011).
13. Daniels, M. H. *et al.* Discovery and Optimization of Highly Selective Inhibitors of CDK5. *J Med Chem* **65**, 3575–3596 (2022).
14. Kanungo, J., Zheng, Y. L., Amin, N. D. & Pant, H. C. Targeting Cdk5 Activity in Neuronal Degeneration and Regeneration. *Cell Mol Neurobiol* **29**, 1073 (2009).
15. Cheung, Z. H., Gong, K. & Ip, N. Y. Cyclin-Dependent Kinase 5 Supports Neuronal Survival through Phosphorylation of Bcl-2. *The Journal of Neuroscience* **28**, 4872 (2008).



# Addendum

Abbreviations

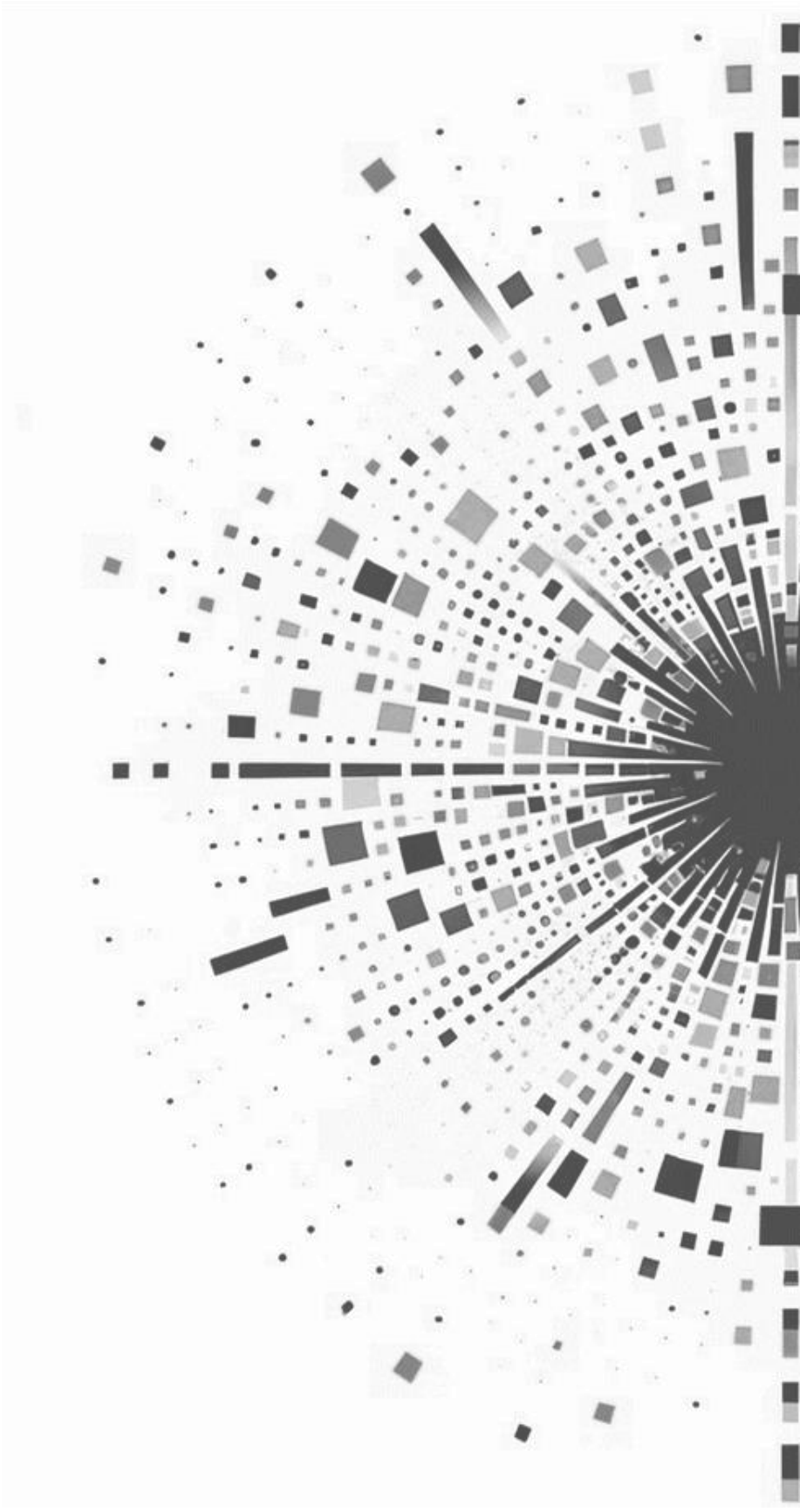


| | |
|---|---|
| 3-DG | 3-deoxyglucosone |
| AAV | Adeno-associated virus |
| ADAM | A disintegrin and metalloprotease |
| AKI | Acute kidney injury |
| AN69 | Acrylonitrile-69 |
| anti-CarP | Anti-carbamylated protein |
| APD | Automated peritoneal dialysis |
| ApoB | Apolipoprotein B |
| BACE | Beta-site APP cleaving enzyme |
| BSA | Bovine serum albumin |
| CAPD | Continuous ambulatory peritoneal dialysis |
| carbLDL | Carbamylated low density lipoprotein |
| CDK5 | Cyclin-dependent kinase 5 |
| CKD | Chronic kidney disease |
| CM | Calcium supplemented medium |
| CML | Ne-(carboxymethyl)lysine |
| CMPF | 3-Carboxy-4-methyl-5-propyl-2-furanpropionate |
| CRP | C-reactive protein |
| CRS | Cardiorenal syndrome |
| CTA | Cellulose triacetate |
| CTCF | Correlated total cell fluorescence |
| CURAY | Cuprammonium rayon |
| CVD | Cardiovascular disease |
| CVVH | Continuous venovenous hemofiltration |
| CVVHD | Continuous venovenous hemodialysis |
| DCFDA | 2’,7’-dichlorodihydrofluorescein diacetate |
| DHD | Daily short hemodialysis |
| DIAC | Cellulose diacetate |
| DMSO | Dimethylsulfoxide |
| DOP | Dopamine |
| DVB-PVP | Polyvinylpyrrolidone-coated divinylbenzene |
| ECM | Extracellular matrix |
| eGFR | Estimated glomerular filtration rate |
| EndoMT | Endothelial-to-mesenchymal transition |
| EUTox | European uremic toxins work group |
| FCA | Fibrous cap atheroma |
| FCS | Fetal calf serum |
| FGFR | Fibroblast growth factor receptors |

| | |
|---|---|
| FPAD | Fractionated plasma separation, adsorption, and dialysis |
| FPSA | Fractionated plasma separation and adsorption |
| FUR | Furosemide |
| GO | Glyoxal |
| H&E | Hematoxylin-eosin |
| HA | Hippuric acid |
| HCO | High-cut-off |
| Hcy | Homocysteine |
| HD | Hemodialysis |
| HDF | Hemodiafiltration |
| HDL | High density lipoproteins |
| HEMO | Hemophan |
| HF | Hemofiltration |
| HF-CTA | High-flux cellulose triacetate |
| HF-HD | High-flux hemodialysis |
| HF-PS | High-flux polysulphone |
| HFR | Hemo-filtrate-reinfusion |
| HICB | Hexadecyl-immobilized cellulose beads |
| HP | Hemoperfusion |
| HPLC | High-performance liquid chromatography |
| IAA | Indole-3-acetic acid |
| IBU | Ibuprofen |
| ICAM-1 | Intercellular adhesion molecule 1 |
| IDG | Indoxyle-b-D-glucuronide |
| IF | Immunofluorescent |
| IHC | Immunohistochemistry |
| IND | Indican |
| IPH | Intraplaque hemorrhage |
| IPIS | Increased plasma ionic strength |
| IRI | Ischemia-reperfusion injury |
| IS | Indoyl sulfate |
| KA | Kynurenic acid |
| KDIGO | Kidney disease: improving global outcomes |
| KoA | Mass transfer area coefficien |
| KY | Kynurenine |
| LDL | Low density lipoprotein |
| LF-CTA | Low-flux cellulose triacetate |
| LF-HD | Low-flux hemodialysis |
| LF-PS | Polysulphone |

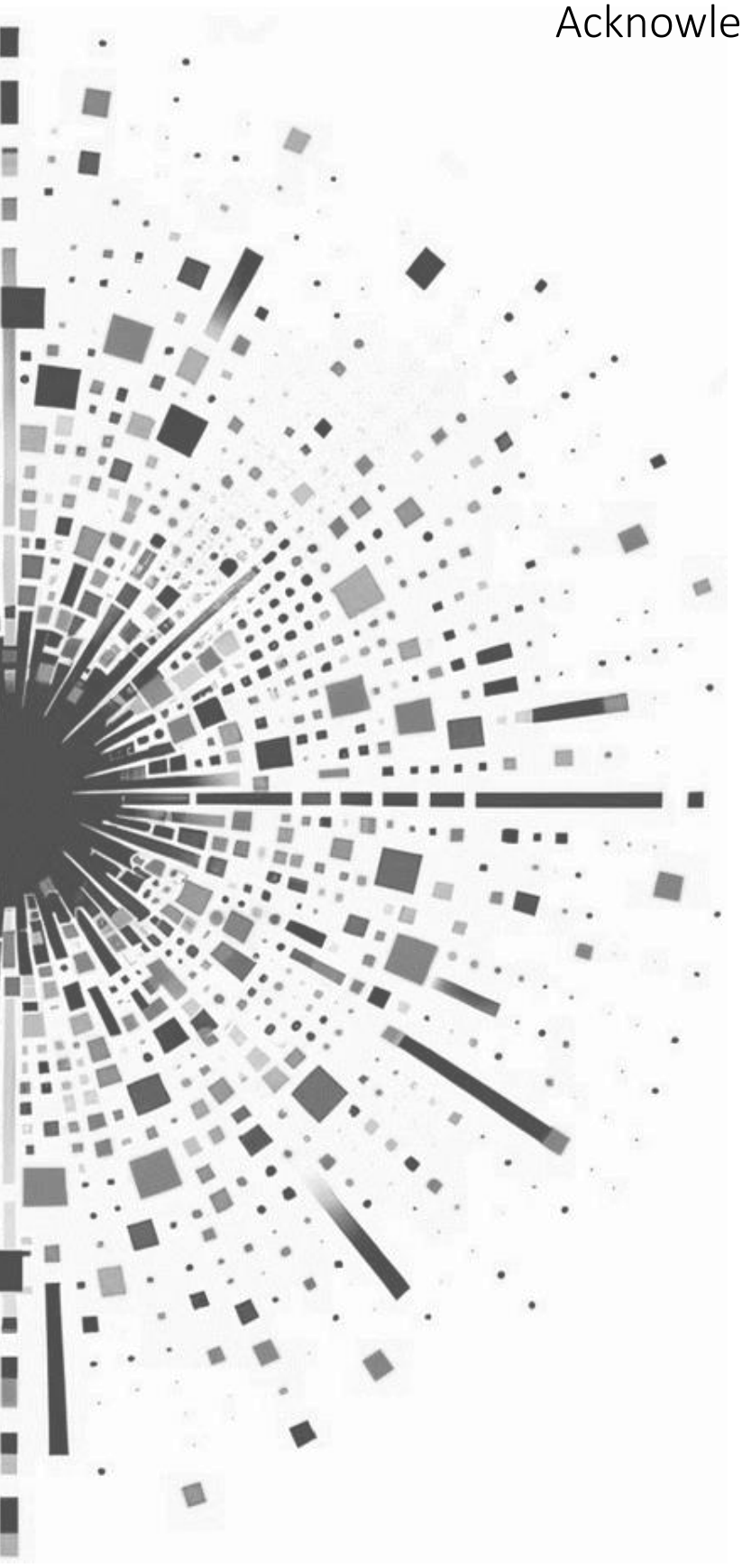
| | |
|---|---|
| LOX-1 | Lectin-like oxidized LDL receptor-1 |
| LPS | Lipopolysaccharide |
| LXRα | Liver X receptor alpha |
| MAHD | Magnetically-assisted hemodialysis |
| M-CSF | Macrophage colony-stimulating factor |
| MDM | Monocyte derived macrophages |
| MEL | Melatonin |
| MFI | Mean fluorescence intensity |
| MG | Methylglyoxal |
| MMM | Mixed matrix membrane |
| MOFs | Zirconium-based metal−organic frameworks |
| MPHD | Multipass hemodialysis |
| MPO | Myeloperoxidase |
| MS | Mass spectrometry |
| NAC | N-acetylcysteine |
| Nx | Nephrectomy |
| OH-HPA | Hydroxyhipuric acid |
| OLHDF | Online hemodiafiltration |
| oxLDL | Oxydised low density lipoprotein |
| P | Phenol |
| PAA | Phenylacetic acid |
| PAG | Phenylacetylglutamine |
| PAH | Para-aminohippuric acid (HA derivate) |
| PAN | Polyacrylonitrile |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate-buffered saline |
| PBUT | Protein-bound uremic toxin |
| PC | Positive control |
| PCG | P-cresylglucuronide |
| pCS | Para-cresyl sulfate |
| PD | Peritoneal dialysis |
| PEI | Polyether imide |
| PENT | Pentosidine |
| PES | Polyethersulfone |
| PIT | Pathological intima thickening |
| PLD | Protein-leaking dilayzer |
| PMA | Phorbol 12-myristate 13-acetate |
| PMMA | Polymethylmethacrylate |
| PPARγ | Peroxisome proliferator-activated receptor gamma |

| | |
|---|---|
| PRISMA | Preferred Reporting Items of Systematic Reviews |
| PRR | Pattern recognition receptor |
| PS | Polysulphone |
| PTM | Post-translational modification |
| Qd | Dialyzate flow |
| RBP | Retinol-binding protein |
| RFP | Fluorescent protein |
| ROS | Reactive oxygen species |
| RR | Reduction ratio |
| SF-CTA | Super-flux cellulose triacetate |
| SF-PS | Super-flux polysulphone |
| SUD | Sequential ultrafiltration/dialysis |
| TC | Thiocyanate |
| TLR-4 | Toll-like receptor 4 |
| TMAO | Trimethylamine N-oxide |
| TRP | Tryptophan |
| UF | Ultrafiltration |
| UUO | Unilateral ureteral obstruction |
| VCAM-1 | Vascular cell adhesion molecule-1 |
| VSMC | Vascular smooth muscle cells |
| WGCNA | Weighted gene co-expression network analysis |
| WT | Wild type |



# Addendum

## Acknowledgements| Благодарность



Standing on the shoulders of giants.

First of all, I want to thank my supervisors: **Prof. Erik Biessen**, **Dr. Marjo Donners**, **Prof. Joachim Jankowski**, and **Dr. Pieter Goossens**. Without you and your trust in me, this thesis work would not have existed, at least not in the same shape.

**Erik**, thank you for giving me this chance and believing in me. You were one of the main reasons I chose this project at the first place. I remember how at the first interview you asked what my weaknesses were, which I failed to answer back then. Sure enough, there were plenty to discover throughout the PhD. Thank you for your support and patience (I know it must have not been so easy with me at times). Thank you for not letting us down during the pandemic, keeping us informed and making feel supported. And even when times were difficult for you, you still kept a high spirit and never let negativity to overtake. Indeed, your positivity and optimism are something to be admired. And thank you very much for trusting me with the multiplex experiments and supporting the collaboration with Charité. That was one of the major highlights of my PhD. Thank you for your significant contribution to this fantastic journey that changed my life. **Marjo**, thank you for always being there when I had any kind of sophisticated, or not-so-much, question regarding science or things in general. You were always honest and open and shared your thoughts with me. Your wisdom and care helped me to keep going. Thank you for believing in me and trusting along the way. I will miss our insightful conversations. **Joachim**, thank you very much for welcoming me to your lab and allowing me to be a part of CaReSyAn project. This work would have not been possible without you. I also want to thank you for suggesting the protein-bound uremic toxins study to me, which resulted in a great publication and became a significant contribution to this thesis. Thank you for your support and advice along the way. **Pieter**, you were not only a supervisor but also a friend and provided me with support and advice when I needed it most. Thank you for that. And thank you for being a loyal Twitter follower! You always celebrated my wins with me. And I am extremely happy and grateful that one of your achievements resulted also in mine and is now hanging there on the office wall framed in a dark wood. Thank you for the great times and I am sure you have an amazing scientific career ahead of you!

**Dr. Heidi Noels**, I want to thank you as one of the key persons behind my project since you were the very first person I talked to about it. Thank you for your confidence in me and for supporting my candidature.

**Prof. Vera Jankowski**, thank you for your guidance and patience during my first months in your lab. I learned a lot from you and your contribution was invaluable for the success of this thesis. Thank you for that.

I want to thank the members of my assessment committee, **Prof. Casper Schalkwijk**, **Dr. Maarten Christiaans**, **Prof. Miranda van Eck**, and **Prof. Wilhelm Jahnen-Dechent** for kindly dedicating their time for careful assessment of my thesis.

I would like to thank **Prof. Axel zur Hausen**, for allowing me to conduct research for the PhD thesis and to be a part of the Department of Pathology team. I also want to thank **Prof. Leon Schurgers** for supporting me and allowing to perform experiments at the Department of Biochemistry.

I want to thank **Dr. Barend Mees** and **Julien Luyten** for assisting in the atherosclerotic plaque studies, as well as all the **patients** for providing this valuable material for research.

I would like to thank **Prof. Frank Tacke** for welcoming me to the lab and allowing me to perform the multiplex imaging study that made a paramount contribution to this thesis and beyond. I also want to thank **Dr. Adrien Guillot** for the dedication and commitment. Regardless if it was late in the evening or early morning, I could always count on your support. Thank you for that.

I would like to thank my paranymphs and friends, **Olivia** and **Elias**, thank you both for accepting this role and for being by my side on this important day. **Olivia**, you were one of the first people I met during my PhD. There were plenty of fun times and a lot of valuable scientific work that we created together. Thank you for your significant professional and personal contribution to my PhD. **Elias**, I am happy that we had a chance to work together. Your positivity, friendliness, and kindness are something I believe we all greatly appreciate in you. Thank you for your help with the imaging experiments and for our inspiring conversations, not only about science but also about movies and culture. Thank you for all the fun times and positive energy.

I want to thank my "first lab" of this project, the Uniklinik RWTH Aachen team – **Wendy**, **Jana**, **Sofia**, **Giulia**, **Sandra**, and others. You we highly important for me during the start of the PhD and became my friends. Thank you, **Wendy**, for helping me and teaching me. Thank you, **Giulia**, for still being a strong support for me. Thank you, **Jana**, for your help and contribution to the thesis. I am happy we have this interesting friendship that started in Germany and continues back to the home country.

I want to thank my Pathology team, everyone who helped me with my work, for your advice, your time, your commitment, and care – **Judith**, **Lieve**, **Mat**, **Gregorio**, **Marion**, **Jacques**, **Clairy**, **Emiel**, **Jack**, **Audrey**, **Monique**, **Andy**, and **Dietbert**. This thesis would not have been possible without you. **Judith**, I admire your dedication and strength, thank you for your advice and for being an amazing example of a female leader. **Lieve**, thank you for your positivity and advice. **Marion**, thank you for your help with the plaques, and I really appreciated your directness! **Clairy**, thank you for helping me with the cohorts and your significant contribution to this thesis. **Gregorio**, thank you for so many hours spent on my project and for your help with the histology. **Mat**, you were the first one to teach me a few tricks in the lab, qPCR, ELISA, plaque collection to name a few, and the one that we could wholeheartedly rely on in any circumstances, even when the lab gets flooded. Thank you for that.

I would like to thank all my friends and colleagues in Maastricht lab that we spent so much time with and have so many cool memories together, **Adele**, **Renée**, **Sebastiaan, Chang**, **Zhenyu**, **Dlzar, Lele**, **Baixue**, **Stan**, and **Laura.** Thank you, **Adele**, for your help with this thesis, for choosing me as paranymph at your defence, and for our interesting conversations. I will miss it. **Renée**, your dedication and skill are something I've always admired. You helped me in the lab, not only to save it from drowning but also with the science-related questions. Thank you for that. **Sebastiaan**, thank you for the fun and for inspiring entrepreneurial spirit! **Chang**, thank you for all your help with the data analysis and for trusting me the important role of a paranymph at your defence. Also, I want to thank everyone who maybe left earlier but still are very vivid in my memory, **Han**, **Jan**, **Margaux**, **Kim**, **Jenny**, **Jasper**, **Ruud**, **Suzan**, and **Danielle**. Thank you, guys, for the awesome times before COVID!

I want to thank members of **I'MCARIM**, thank you for welcoming me to your team and for all the wonderful times we had together. It was an important experience for me, and I am grateful we could contribute to the life of the university together. I also want to thank **Prof. Tilman Hackeng** for this fantastic opportunity.

I would like to thank my students, **Ola Pawlowska**, **Amien Abid**, and **Stan Laaper** for their dedication and contribution to this thesis.

I want to thank all members of the **CaReSyAn** project, as well as all the **ESRs**. It was amazing to spend time with you guys! Sad we could not have done it more often in-person, but it is great that we can still stay in touch.

I am deeply thankful to my friends, **Lee-Ann**, **Gosia**, **Bastian**, **Lena**, **Thais**, and **Berta**. **Lee-Ann**, I want to thank you for your support and advice, for not letting me down during difficult times, for always being there regardless of for celebration or to listen to me complaining. It is incredible how it started all the way back in Sweden and now look at us here in the Netherlands, still close friends. There is a lot we have been through together and I want to say thank you for being such a loyal and selfless friend. **Gosia**, thank you for your support, for helping me with the cell experiments, for your advice, for our chats and wonderful nature walks. Я также хочу сказать спасибо своим друзьям со школы, **Ире**, **Элине**, **Кате**, **Маше**, **Илоне**, и **Яне**. Спасибо за вашу поддержку и верную дружбу! Я хочу поблагодаритй свою "русскую рулетку", **Вику**, **Таню**, **Лизу**, **Катю**, и **Оксану** за ту радость и вдохновение которые наши встречи всегда мне дарили.

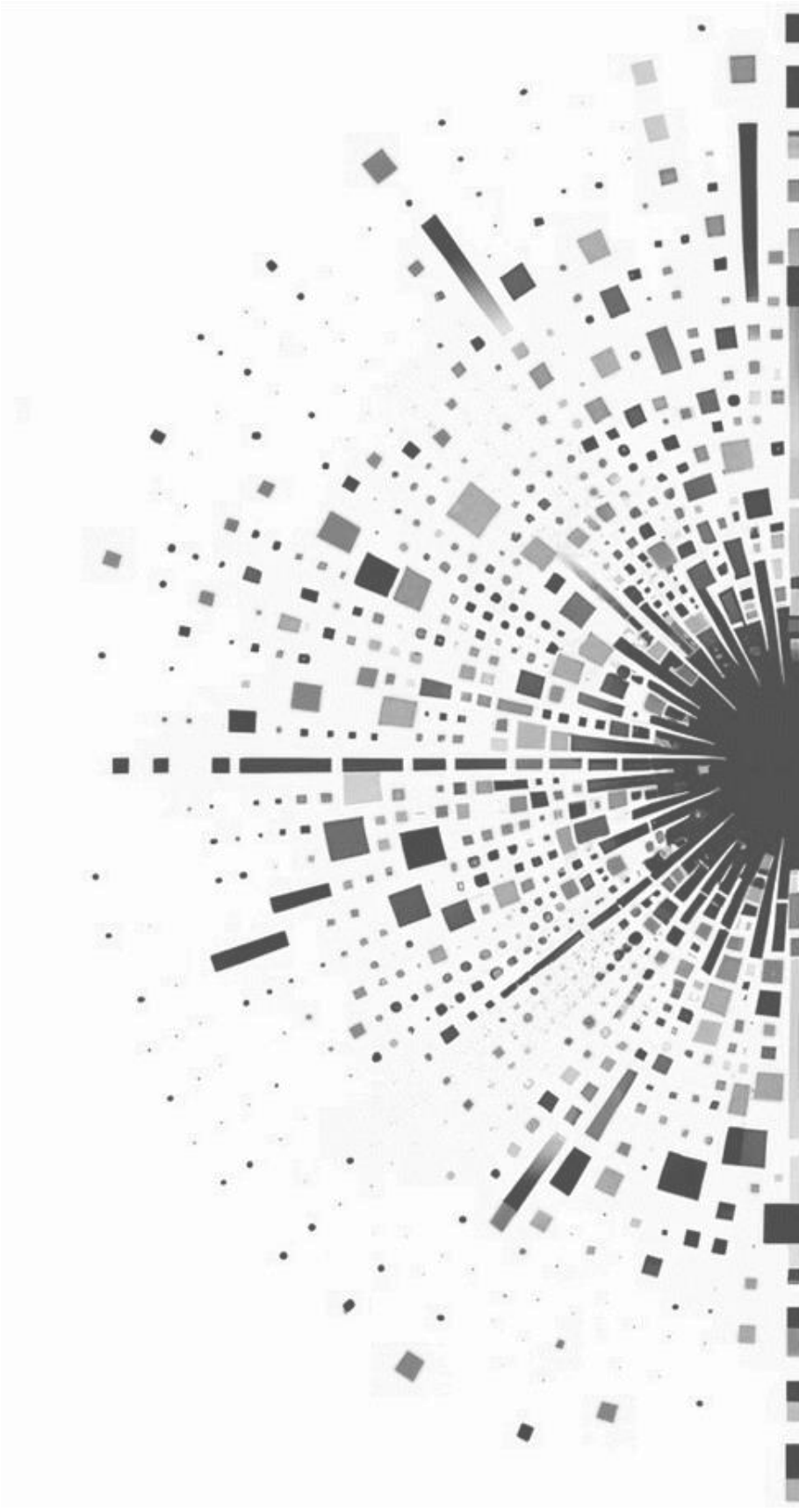
Я хочу от всей души поблагодарить свою семью, **маму**, **Юру**, **бабушку**, **Артура**, **Арю**, и **папу**. Спасибо что вы верили и поддерживали меня всё это время. Эта книга, звание доктора, и вся эта работа была бы невозможна без вашей поддержки и любви. Вы вдохновляли меня и давали силы для новых изысканий и смелых поступков. Спасибо, что мы остаемся близки несмотря на большие расстояния. Для меня очень важно осознавать, что есть люди которые мною гордятся и для которых мои маленькие победы также важны как и для меня самой. Спасибо огромное за вашу любовь. **Мама**, сложно представить как бы оно было бы по-другому. Без твоей поддержки и доверия эта книга

бы не существовала. Я хочу чтобы ты знала, как мне дороги наши с тобой регулярные звонки. Спасибо тебе за всё.

Я хочу также сказать спасибо моей семье со стороны Олега, **Тане**, **Юрию**, **Тоне**, **Ане**, **Bruno**, **Галие**, и **Алевтину**. Вы всегда поддерживали и переживали за нас обоих и мы всегда чувствовали вашу заботу и любовь независимо от того в какой стране мы находились. Спасибо вам за то что я чувствую себя с вами как дома и что вы так тепло и открыто приняли меня в свою семью. Я хочу также поблагодарить свою расширенную семью, **Жанну**, **Максима** с семьей, и **Ладу** с семьей. Спасибо вам за поддержку.

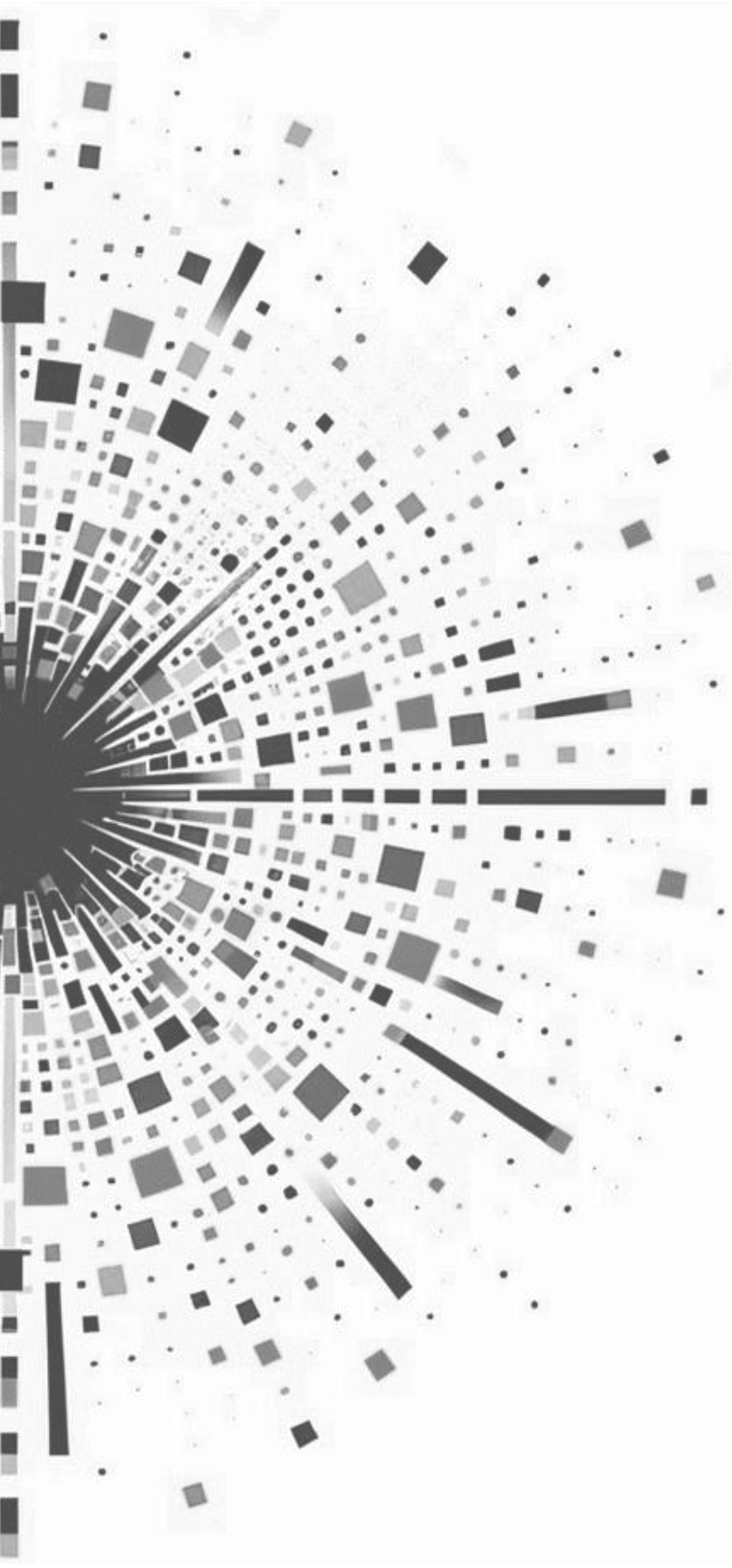
И наконец, **Олег**, сложно передать словами насколько важную роль ты играешь в моей жизни. Я хочу сказать тебе спасибо за все что ты делаешь для меня, когда у меня что-то не ладится или был плохой день, я всегда знаю что дома я найду поддержку и утешение. Спасибо за твое доверие и терпение, я понимаю, иногда они необходимы. При этом я многому у тебя учусь. Меня восхищает как ты развиваешься и становишься мудрее и всегда можешь дать полезный совет. Многие из моих успехов были бы невозможны без твоей поддержки. As I said during your defence, it requires bravery to make the leap of faith and take a step into the unknown, and I couldn't have been more grateful that we are taking these steps together. Я счастлива что мы вместе все эти годы, и не важно в какой мы стране или как сложно бы не было, я уверена мы всё можем преодолеть, дом – это там где мы вместе. Спасибо тебе огромное за то что ты со мной. А ещё недавно появился **Timi**! And we became a cat family. Теперь у меня дома не один, а два милых мужичка ждущих пока их покормят (шучу), что меня невероятно радует. Спасибо вам за терпение, любовь и преданность! I love you ♡

Well, this was fun. Time to move forward and conquer new heights. Adelante!



# Addendum

Curriculum vitae



# Valeria Saar-Kovrov

05.04.1991
Tallinn, Estonia

## EDUCATION

**PhD training in Biomedical Sciences** 2018 – 2023
Department of Pathology, Maastricht University, the Netherlands
Supervisors: Prof. Erik AL Biessen, Prof. Joachim Jankowski
Co-supervisors: Dr. Marjo MPC Donners, Dr. Pieter Goossens
*Tackling the Complexity of CKD-associated Cardiovascular Disease: From Small Molecules to Proteins*

**Master of Science in Molecular Biology** 2012 – 2015
Umeå Center of Molecular Medicine, Umeå University, Sweden
Supervisor: Prof. Simon Tuck
Co-supervisors: Dr. Cecilia Sarmiento Guerin, Dr. Garry Wong
*Investigating Regulatory Elements in the C. elegans mir-124 Gene*

**Bachelor of Science in Gene Technology** 2009 – 2012
Department of Gene Technology, Tallinn University of Technology, Estonia
Supervisor: Dr. Maria C Sarmiento Guerin
*Characterization of the Promoter Region of Caenorhabditis elegans mir-124*

## WORK EXPERIENCE

**Biotech Venture Capital Fellow** 2023 – 2024
M Ventures, Amsterdam, the Netherlands

**Valorization Intern** 2023
Dutch Cardiovascular Alliance, the Netherlands

**Research Assistant** 2016 – 2018
Lipigon Pharmaceuticals AB, Umeå, Sweden
Department of Medical Biosciences, Umeå University, Sweden

**Research Assistant** 2015 – 2016
Faculty of Health Sciences, University of Macau, China

**Student Research Assistant** 2014 – 2015
Department of Medical Biosciences, Umeå University, Sweden

## OTHER EXPERIENCE

**Conferences and events**

- Flash Talk Presentation, DAS, 2023, Amersfoort, the Netherlands
- Poster presentation, 90th EAS Congress 2022, Milan, Italy
- Rapid fire talk, Virtual Cardiorenal Winter School, 2021, online
- Volunteered, 87th EAS Congress 2019, Maastricht, the Netherlands
- *Lipids In 21 Century: Where we are* International workshop 2017, SaoPaulo, Brazil
- Poster presentation, 2nd Macau Symposium on Biomedical Sciences 2015, Macau, China
- Poster presentation, 6th International Conference on Nutrition and Physical Activity 2015, Taipei, Taiwan
- Volunteer, TEDx University of Macau, 2015, Macau, China

**Courses**

- Next Level Cardiovascular Course (DHF courses), 2021, Papendal, the Netherlands
- Time-and Project Management for PhD Candidates and Postdocs, 2021, online
- Business Entrepreneurship and Commercialization, 2020, online
- Proteomics Bioinformatics, 2019, Cambridge, United Kingdom
- International Atherosclerosis Research School 2016, Hamburg, Germany

**Tutoring**

- Threats and Defense Mechanisms, Maastricht University, 2021
- Threats and Defense Mechanisms, Maastricht University, 2020
- Homeostasis and Organ Systems, Maastricht University, 2020

**Grands and awards**

- Best Flash Talk Presentation award, Dutch Atherosclerosis Society, 2023
- CARIM Commitment award, for the contribution through I'MCARIM, 2022
- Erasmus+ staff mobility grand, 2022

**Other positions**

- PhD representative, I'MCARIM, Maastricht University 2021 - 2023
- Event host and organizer, GGI Maastricht 2020 - 2023
- Outreach Committee Member, H2020-MSCA-ITN 2018 - 2021

# PUBLICATIONS

**Myeloid-specific CDK5 knockdown reduces atherosclerotic plaque calcification.**
Waring OJ, **Saar-Kovrov V**, Jin H, Lu C, Ruder A, Gijbels MJJ, Nagenborg J, Temmerman L, Giacca M, Vettorazzi S, Schalkwijk CG, Mees BME, Jaminon AMG, Schurgers LJ, Stoll M, Goossens P, Matic L, Donners MMPC, and Biessen EAL.
*In preparation*

**Plaque protein carbamylation is associated with atherosclerosis progression and uremia and accumulates in foam cells.**
**Saar-Kovrov V**, A Pawlowska A, Guillot A, Gijbels MJJ, Fazzi GE, Sluimer JC, Mees BME, Tacke F, Jankowski J, Donners MMPC, and Biessen EAL.
*In preparation*

**Lipoprotein size is a main determinant for the rate of hydrolysis by exogenous LPL in human plasma.**
Kovrov O, Landfors F, **Saar-Kovrov V**, Näslund U, and Olivecrona G.
2022, *J Lipid Res* 63(1):100144

**Shedding of Klotho: functional implications in chronic kidney disease and associated vascular disease.**
**Saar-Kovrov V,** Donners MMPC, and van der Vorst EPC.
2021, *Front Cardiovasc Med* 28;7:617842

**Reduction of protein-bound uraemic toxins in plasma of chronic renal failure patients: a systematic review.**
**Saar-Kovrov V**, Zidek W, Orth-Alampour S, Fliser D, Jankowski V, Biessen EAL, Jankowski J.
2021, *J Intern Med* 290(3):499-526

**Human amyloid β peptide and tau co-expression impairs behavior and causes specific gene expression changes in *Caenorhabditis elegans*.**
Wang C, **Saar V**, Leung KL, Chen L, and Wong G.
2018, *Neurobiol Dis* 109(Pt A):88-101

**Structure-activity relationships for lipoprotein lipase agonists that lower plasma triglycerides *in vivo*.**

Caraballo R, Larsson M, Nilsson SK, Ericsson M, Qian W, Nguyen Tran PN, Kindahl T, Svensson R, **Saar V**, Artursson P, Olivecrona G, Enquist PA and Elofsson M.

2015, *Europ J Med Chem* 103:191-209