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CHRONIC KIDNEY DISEASE – A CLINICAL MODEL OF PREMATURE VASCULAR AGING

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Lu Dai

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CHRONIC KIDNEY DISEASE - A CLINICAL MODEL OF PREMATURE VASCULAR AGING
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ABSTRACT

Patients with chronic kidney disease (CKD) are prone to develop an accelerated vascular aging phenotype characterized by vascular calcification (VC), a major culprit of cardiovascular complications and premature death. While VC has been recognized as an active pathophysiologic process with involvement of specific mediators and effectors, the co-existence of traditional risk factors (i.e., high age, diabetes, hypertension, dyslipidemia), inflammaging stimuli and pharmacological interventions (e.g., phosphate binders, warfarin and statin therapy) adds to the complexity of the course and consequences of different types of VC (e.g., intima and media VC, micro- and macrocalcification) in the context of CKD. This work attempts to further explore the prognostic value, predictive markers as well as collateral therapeutic consequence of VC in uremic milieu.

Study I explores the associations of the composites of coronary artery calcium (CAC) score, i.e., CAC density and CAC volume, with mortality risk in patients with CKD stage 5 (CKD G5). We found that while mortality risk increases with higher CAC score and CAC volume, CAC density shows an inverse-J shaped pattern, with the crude mortality rate being highest in the middle tertile of CAC density.

Study II evaluates the overlapping presence of aortic valve calcium (AVC) and CAC and the prognostic value of AVC in CKD5 patients. We found a more common overlap of AVC and CAC in CKD G5 than that observed in general population. High AVC score is associated with increased all-cause mortality independent of presence of CAC, traditional risk factors and inflammation.

Study III investigates phenotypic factors associated with the presence of biopsy-verified media VC in CKD G5 patients using the relaxed linear separability feature selection model. We identified through a mapping and ranking process, 17 features including novel biomarkers and traditional risk factors that can differentiate patients with media VC from those without. These results, if confirmed, may inform future investigations on media VC without the need of arterial biopsies.

Study IV assesses the association of commonly prescribed phosphate binder sevelamer with gut microbial metabolites in CKD G5 patients. We found that sevelamer therapy associates with increased gut-derived uremic toxins and poor vitamin K status, suggesting potential trade-offs of sevelamer therapy in CKD.

Study V explores the plausible association between plasma dephosphorylated-uncarboxylated matrix Gla-protein (dp-ucMGP, a circulating marker of functional vitamin K deficiency), VC and mortality in CKD G5 patients. We found an independent association between high dp-ucMGP levels and increased mortality risk that is not modified by presence of CAC and AVC in CKD G5.

LIST OF SCIENTIFIC PAPERS

I. Mukai H, **Dai L**, Chen Z, Lindholm B, Ripsweden J, Brismar TB, Heimbürger O, Barany P, Qureshi AR, Söderberg M, Bäck M, Stenvinkel P. Inverse J-shaped relation between coronary arterial calcium density and mortality in advanced chronic kidney disease. Nephrol Dial Transplant. 2020;35(7):1202-1211.

II. **Dai L**, Plunde O, Qureshi AR, Lindholm B, Brismar TB, Schurgers LJ, Söderberg M, Ripsweden J, Bäck M, Stenvinkel P. Aortic Valve Calcium Associates with All-Cause Mortality Independent of Coronary Artery Calcium and Inflammation in Patients with End-Stage Renal Disease. J Clin Med. 2020;9(2):607.

III. **Dai L**, Debowska M, Lukaszuk T, Bobrowski L, Barany P, Söderberg M, Thiagarajan D, Frostegård J, Wennberg L, Lindholm B, Qureshi AR; Waniewski J, Stenvinkel P. Phenotypic features of vascular calcification in chronic kidney disease. J Intern Med. 2020;287(4):422-434.

IV. **Dai L**, Meijers BK, Bammens B, De loor H, Schurgers LJ, Qureshi AR, Stenvinkel P, Evenepoel P. Sevelamer Use in End-Stage Kidney Disease (ESKD) Patients Associates with Poor Vitamin K Status and High Levels of Gut-Derived Uremic Toxins: A Drug–Bug Interaction? Toxins (Basel). 2020;12(6):351.

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LIST OF ABBREVIATIONS

ACEi/ARB	Angiotensin converting enzyme inhibitors/Angiotensin II receptor antagonists
AE	Apparent error
AUC	Area under the curve
BMI	Body mass index
CAC	Coronary artery calcium
CI	Confidence interval
CKD	Chronic kidney disease
CKD G5	Chronic kidney disease stage 5
CKD-MBD	Chronic kidney disease - mineral bone disorders
CT	Computed tomography
CTX	Carboxy-terminal collagen crosslinks
CVE	Cross-validation error
Dp-ucMGP	Dephosphorylated uncarboxylated matrix Gla protein
FRS	Framingham risk score
fT3	Free triiodothyronine
HD	Hemodialysis
HGS	Handgrip strength
hsCRP	High sensitivity C-reactive protein
IgM antiMDA	IgM antibodies against malondialdehyde
IgM antiPC	IgM antibodies against phosphorylcholine
IndS	Indoxyl sulfate
iPTH	Intact parathyroid hormone
KRT	Kidney replacement therapy
LD-RTx	Living donor kidney transplant
MGP	Matrix Gla protein
MOTS-c	Mitochondrial open-reading-frame of the twelve S rRNA-c
OPG	Osteoprotegerin
OR	Odds ratio
PAG	Phenylacetylglutamine

pCS	p-Cresyl sulfate
PD	Peritoneal dialysis
RLS	Relaxed linear separability
ROC	Receiver operating characteristic
SD	Standard deviation
SGA	Subjective global assessment
sHR	Sub-hazard ratio
sRANKL	Soluble receptor activator of nuclear factor-κB ligand
TRAP 5a	Tartrate resistant acid phosphatase 5a
TMAO	Trimethylamine N-oxide
VSMCs	Vascular smooth muscle cells
VC	Vascular calcification

1 INTRODUCTION

1.1 CHRONIC KIDNEY DISEASE AND PREMATURE VASCULAR AGING

Patients with chronic kidney disease (CKD) are characterized by an accelerated aging process, including multiple cardiovascular complications, muscle wasting, osteoporosis and frailty [1,2]. In particular, the arterial vasculature in CKD patients undergoes changes typical of aging and atypical of the chronological age [2,3]. This premature vascular aging phenotype, accompanied by progressive vascular calcification (VC), along with chronic inflammation, persistent oxidative stress and deficient anti-aging systems [2], is considered as a major culprit of unfavorable cardiovascular complications in CKD.

Current clinical strategies aiming at counteracting VC are focused on controlling atherosclerosis and CKD - mineral bone disorders (CKD-MBD), using among others statin therapy, inhibition of calcium-phosphate depositions by employing phosphate binders, and calcimimetics, calcitriol, or vitamin D analogues while while vitamin K, magnesium, and crystallization inhibitors (e.g., pyrophosphate and sodium thiosulfate) are also being considered. Established and some emerging treatments have been evaluated in interventional studies, yet results are inconclusive and it remains ambiguous whether they are efficient in ameliorating VC progression in patients with CKD [4]. Additionally, although the unraveling of molecular targets of VC (e.g., intravenous myo-inositol hexaphosphate [5], serum- and glucocorticoid-inducible kinase 1 inhibitor [6] and apabetalone [7]) has brought either preclinical or clinical evidence for potential novel therapies of VC, no pharmaceutical treatments have so far proven to avert or thoroughly reverse VC progression in CKD.

Recent data from the European Renal Association-European Dialysis and Transplant Association (ERA-EDTA) Registry on trends of excess mortality - in relation to the general population - among 280,075 adult patients who started kidney replacement therapy (KRT) between 2002 and 2015 indicate an overall improvement of survival that was most prominent in dialysis patients who showed a decrease in excess mortality risk per five years by 28% for atheromatous cardiovascular disease (CVD), 10% for non-atheromatous CVD and 10% for infections [8]. This may reflect developments in dialysis treatment as well as the introduction and implementation of guideline-recommended treatments to improve cardiovascular health in the CKD population. However, the implications and impact of these newer treatment strategies on ameliorating cardiovascular complications including vascular aging (e.g., atheroma and arteriosclerosis) in this population are not fully deciphered.

It is plausible that the concurrence of traditional risk factors, CKD-MBD, gut dysbiosis, persistent oxidative stress, uremic inflammation and cellular senescence involved in the pathological VC process, together with the diverse consequent forms of VC may bewilder its actual clinical relevance and importance as a therapeutic target in the context of CKD. More profound knowledge of phenotypic features, clinical relevance and prognostic value of VC

remains to be clarified in this enigmatic scenario to develop and orientate efficient preventive and therapeutic strategies to counteract VC.

1.2 VASCULAR CALCIFICATION IN CKD - A COMPLEX SCENARIO

In CKD, premature VC can occur in different vascular layers (i.e., intima and media VC) distributed at divergent anatomical sites (i.e., different vascular trees) with distinct nature (i.e., microcalcification and macrocalcification). The divergent forms of VC obstruct the utilization of VC in risk prediction and adds complexity to its role in risk prediction and disease prognosis in clinical settings. The development of Agatston scoring as a semi-quantitative measure of coronary artery calcium (CAC) [9] has allowed screening, quantification and follow-up of large study groups to compute the presence and progression of atherosclerotic calcification, which was found to be a predictor of future CVD events beyond traditional Framingham risk score (FRS) [10]. However, the appropriateness of upweighting CAC for density has been questioned with emerging data indicating a disparate trajectory of CAC volume and density in CVD risk prediction in different populations [11–15].

Moreover, the distribution of intima and media calcification may differ in different artery segments, e.g., with more limited media calcification but more prominent atherosclerotic plaques in coronary arteries. Given the possible profound media calcification in CKD, quantification of calcification at coronary artery sites represented by CAC may fail to give a full coverage of VC burden in CKD population. Since current imaging techniques are not able to differentiate between intima and media calcification, and it is uncertain whether intimal and medial layers of calcification are of equal weight in risk prediction, the prognostic value of CAC scoring of atherosclerotic plaque in CKD may not be as representative as observed in the general population. In addition, the nature of atherosclerotic calcification, i.e., micro- and macrocalcification, and the role of inflammation in the evolution of these two entities, largely remains to be illuminated. Taken together, the persistent uremic inflammation concomitant with other traditional and non-traditional risk factors may complicate the role of atherosclerosis in risk prediction, and the unequal susceptibility of intima and media calcification in different vascular segments could further challenge a holistic evaluation of VC in the context of CKD.

1.3 CLINICAL RELEVANCE AND PROGNOSTIC VALUE OF VC COMPONENTS

The presence of VC is a common outcome of different types of vessel wall injury resulting from numerous stimuli and cellular insults, such as oxidative stress and inflammation. The consequent diverse forms of VC might be considered as a reflection of the heterogeneous VC pathogenesis on a cellular level. In the clinical setting, it is therefore crucial to differentiate the phenotypic features and clinical spectrums of VC, as to better understand the clinical relevance of VC and to improve interventional strategies.

In the early stages of atherosclerosis, initial hydroxyapatite deposition in response to pro-inflammatory stimuli induces the formation of microcalcification nuclei, which can in turn exacerbate the progression of inflammation and calcium precipitation, causing propagation of vessel impairment [16]. Ultimately, this vicious interplay promotes plaque rupture as a result

of the progressive thinning of the fibrous cap and the detrimental mechanical effect of microcalcification [17]. Nevertheless, if an adaptive and repairing response prevails, vascular smooth muscle cells (VSMCs) would undergo osteogenic phenotypic differentiation and mineralization, prompting the formation of macrocalcification, which can stabilize the plaque by acting as a barrier restricting the spread of inflammation [16]. Postmortem analysis of coronary artery biopsies from victims of acute myocardial infarction presented more extensive VC than those from non-cardiac victims, and the extent of VC was less advanced in unstable than in stable plaques, suggesting an inverse correlation between calcium deposition and cap inflammation [18].

From a histological perspective, calcifications are defined as *microcalcification* (≥0.5 μm, typically <15 μm in diameter), *speckled calcification* (≤2 mm), collectively referred to as "microcalcification" or spotty calcification based on the granular pattern of calcium deposition, *fragmented calcification* (2-5 mm), and *diffuse calcification* (≥5 mm segment of continuous calcium) or "macrocalcification" based on the sheet-like conformation of the calcified tissue. The last three histological classifications correspond to the radiographic categories as speckled, fragmented, and diffuse calcification, respectively. The earlier stages of microcalcifications or spotty calcification may be associated with risks of plaque rupture. In cases of sudden coronary death, 65% of acute plaque ruptures were characterized by exclusive speckled calcification and over 50% of thin-cap fibro atheroma showed either an absence of calcification or speckled calcification by computed tomography (CT) imaging [19]. Additionally, routine lipid lowering strategies (i.e., statin use) targeting CAC have yielded conflicting results, with previous studies suggesting an impact on CAC regression and contemporaneous data indicating rather the opposite [20–22]. Of note, while CAC progression might be attributed to plaque development into a more unstable morphology with accumulative spotty and focal calcifications, CAC progression in the context of lipid lowering treatment might be pertinent to a shift towards plaque stabilization with a more stable fibro-calcific morphology [23].

This CAC paradox may be attributed to the limitation of conventional CT whereby micro-calcification nodules smaller than 15 μm and speckled calcification <0.5 mm are undetectable and classified as "absent" in the radiographic examination, which may also partially explain the inconclusive results regarding the predictive value of CAC components (i.e. CAC density and volume). While CAC volume was found to be positively and CAC density negatively associated with CVD events across all levels of CAC volume, as well as multiple strata of other risk factors in general population [13], Bellasi et al. [14] reported a positive association among plaque density and risk of all-cause mortality in hemodialysis (HD) patients, implying that high density calcium by conventional CT does not illuminate a favorable plaque stabilization in the context of CKD. The prognostic value of CAC components in uremia is further explored in *Study I.* The prevalence of calcification at different vascular sites and the prognosis of aortic valve calcium (AVC) is further investigated in *Study II.*

Aside from intima calcification and atherosclerosis, patients with CKD display a typical pattern with media calcification characterized by pathological deposition of hydroxyapatite in the

medial layer of the arteries [24]. Media calcification is centered around VSMC calcification that shares similarities with developmental osteogenesis/chondrogenesis [25]. Under physiological conditions, an endogenous defensive system would protect VSMCs from phenotypic transdifferentiation and ectopic calcification. Multiple lines of evidence indicate that the uremic milieu (e.g. uremic toxins retention and other pathological cellular stress) triggers key pathways of VSMCs calcification and induces tissue damage via various modifications in proteins and DNA [26–29]. The inhibitory defense pathways, which tend to be suppressed in CKD by factors such as hyperphosphatemia, hypercalcemia, hyperparathyroidism and hypomagnesemia, with coexistence of inflammation and oxidative stress, are further challenged to counteract the VC burden [28,30]. Media calcification causes arterial stiffness, a hallmark of early vascular aging (EVA), and the extent of media calcification can be taken as an estimation of biological vascular age. This is in line with the notion that CKD may serve as a clinical model of EVA whereby cellular senescence is possibly involved in the VC process [31].

As mentioned previously, media calcification also accounts for cardiovascular comorbidity and mortality in CKD, similar with intima calcification. However, the exact causality between media calcification and poor clinical outcome remains to be established as most of the clinical research conclusions are based on observational studies. Well-designed prospective follow-up studies are warranted to illustrate whether the progression of media calcification is crucial in determining clinical outcomes. However, such studies have been hampered by the lack of specific and valid imaging and quantification techniques of media calcification. Clinical investigations have focused most often on coronary arteries and the aortic arteries, where both media and atherosclerotic calcification can occur in parallel.

While carotid-femoral pulse wave velocity (PWV) has been considered as an established marker of arterial stiffening [32,33] and a reflection of media calcification, it is a functional measurement rather than a direct quantification of calcification. Precise quantification of media calcification can only be achieved from arterial beds exclusively devoid of atherosclerosis, where media calcification takes place with high sensitivity. The research exploration of mammograms in breast arteries has provided new insights into the detection of media calcification as atherosclerosis does not occur in breast arteries [34]. According to mammographic data, it was recently shown that the progression of media calcification in breast arteries accelerated significantly in advanced CKD, and diminished to control levels after kidney transplantation [34]. While this might indeed provide a novel and easy method to evaluate the presence and progression of media calcification, these studies were conducted in a small sample of female patients, and the validity in large-size cohorts of CKD patients including men remains to be testified. In fact, no fundamental breakthroughs have been made in the diagnosis (i.e., visualization of the extent and severity of media calcification) or therapeutics that directly target media VC.

Hence, aside from expanding mechanistic knowledge in preclinical studies, advanced data analysis methodology and machine-learning algorithms including integrating biomarkers,

mechanistic and imaging data so as to predict and to diagnose the presence of media calcification and more importantly, to discover novel therapeutic targets, are urgently needed. In *Study III*, we attempt to explore the predictive features of media calcification using an advanced statistical model.

1.4 THERAPEUTIC STRATEGIES, GUT MICROBIAL METABOLISM AND VC

Current clinical strategies targeting VC or aiming at improving clinical outcomes linked to VC, range from lipid-lowering drugs, management of CKD-MBD, to inhibition of calcium phosphate deposition. Hyperphosphatemia, one of the most common metabolic disorders in CKD, seems to be amplified in advanced CKD and associates with adverse clinical outcome across all stages of the disease [35,36]. Even without predominant hyperphosphatemia and hypercalcemia, the phosphate and calcium load tends to drive VC in both CKD and in the general population [37]. Also, in combination with elevated parathyroid hormone levels and frequent use of calcitriol, phosphate overload and hyperphosphatemia may further induce development of kidney and parathyroid glands resistance to fibroblast growth factor 23 (FGF23), eventually resulting in a paradigm of hyperphosphatemia, excessive FGF23 levels and secondary hyperparathyroidism in advanced CKD. The direct or indirect role of FGF23 in VC development has been discussed and it remains to be determined whether FGF23 acts as a protective or detrimental factor in uremic calcification [38–41]. Targeting FGF23 alone is clearly not an optimal strategy to combat VC as the pathophysiologic trajectory is still elusive. Phosphate binders targeting hyperphosphatemia, which also reduce FGF23 levels, hence become a cornerstone in improving clinical outcome in uremic patients.

Currently, both sevelamer hydrochloride and sevelamer carbonate are used in clinical practice and are first-line phosphate binders in many dialysis units [42]. Despite not being fully established, some randomized clinical trials suggested that compared with calcium-containing phosphate binders, sevelamer ameliorated VC progression and conferred a survival benefit in HD patients [43,44]. However, sevelamer treatment may have trade-offs. First of all, both experimental and clinical data have indicated that sevelamer may bind essential nutrients, such as vitamin D and K [45–47]. Secondly, gastrointestinal symptoms are common among sevelamer users, albeit the underlying pathophysiology is poorly addressed. Thirdly, depositions of mucosal sevelamer crystals along the gastrointestinal tract [48–50] can alter the influx of minerals and nutrients and influence the transit time in the colon. Altogether, they may induce gut dysbiosis, ultimately resulting in an increased generation of toxins originating from protein and choline fermentation. It is worth noting that like sevelamer, many pharmaceutical agents often have gastrointestinal side effects, yet the role of the gut microbiome in these processes is rarely examined. Along the recent increased awareness of drug-microbiome interactions [51], the association between sevelamer use and gut microbial metabolism is explored in *Study IV*.

1.5 VITAMIN K AND VC

The role for vitamin K in VC has been fairly well established in preclinical studies, mainly through carboxylation of vitamin K-dependent protein matrix Gla protein (MGP), which is primarily synthesized and secreted by VSMCs [52]. Preceding work on MGP biology was conducted in a knockout mouse model, where MGP-deficient mice developed extensive calcification both in arteries and cartilage within two months [53]. MGP inhibits VC *in vivo* [53–55], possibly by a direct binding with hydroxyapatite in the arterial walls [56] and by a downregulation of the activation of bone morphogenetic proteins [57,58]. Activation of MGP requires two post-translational modifications, including serine phosphorylation and γ-glutamate carboxylation [59,60]. Vitamin K serves as a cofactor of MGP carboxylation by converting glutamate residues into γ-carboxyglutamate [59,61]. Vitamin K deficiency, thus limits the carboxylation of MGP in VSMCs, leading to a subsequent high secretion of dephosphorylated uncarboxylated MGP (dp-ucMGP). High circulating dp-ucMGP levels, as a marker of functional vitamin K deficiency, have been linked with mortality in various study subjects, including patients with CVD [62–64], CKD [65–67], diabetes[68], as well as in the general population [69,70]. Whether this association can be attributed to its role in inhibiting VC is being debated. The association between vitamin K status and VC from observational studies are equivocal [71–75]. Results from the recent K4Kidneys trial revealed that 12-month vitamin K2 supplementation generally failed to improve parameters of vascular health in patients with CKD [76]. Given the multifaceted etiology of VC, it is plausible that one single intervention is insufficient to rescue VC process and the exact weight of vitamin K in VC progression is to be elucidated.

In addition, a large body of epidemiological studies suggests that vitamin K is involved in age-related disease phenotypes other than vascular health. Results from the he Health, Aging and Body Composition Study (Health ABC) indicated that vitamin K deficiency was associated with diminished lower-extremity function over 4-5 years of follow-up [77]. The prospective Longitudinal Aging Study Amsterdam (LASA) cohort study showed that a lower baseline vitamin K status (indicated by higher dp-ucMGP levels), was associated with a higher frailty index score among the elderly with 13-year follow-up [78]. Though the underlying mechanism behind these associations has not been clearly illustrated, the epidemiological evidence suggests a beneficial role of vitamin K above its role as a nutritional supplement or remedy. Vitamin K deficiency is prevalent in CKD and progresses with the decline of renal function [65,74,79,80]. Given the role of vitamin K insufficiency in VC and age-related disease, it would be interesting to evaluate the association between vitamin K status, VC, and overall clinical outcome in CKD - a paradigm of profound aging process. This issue is further examined in *Study V*.

2 RESEARCH AIMS

The overall aim of the present thesis is to expand our understanding of risk factors and clinical relevance of vascular calcification in CKD.

More specifically, the objectives were to:

- Investigate the prognostic value of atherosclerotic calcification, represented by CAC density and CAC volume, and the role of AVC in risk stratification in patients with advanced CKD (***Study I and II***)

- Identify and characterize risk factors associated with histologically verified media calcification in arterial biopsies obtained from patients with advanced CKD (***Study III***)

- Study the possible link between sevelamer use and gut microbial metabolism in patients with advanced CKD (***Study IV***)

- Explore the plausible associations between vitamin K deficiency, vascular calcification, and all-cause mortality in patients with advanced CKD (***Study V***)

3 PATIENTS AND METHODS

The work presented in this thesis has been conducted using data from three cohorts of patients that have been/are collected and coordinated by the Division of Renal Medicine, Karolinska University Hospital at Huddinge, Stockholm, Sweden and from one cohort of patients at University Hospital Leuven, Belgium. At the Karolinska University Hospital, Huddinge, patients with CKD were enrolled in the following studies: the prospective MIA study of incident CKD stage 5 (CKD G5) patients started in 1994 with ongoing recruitment and follow-up; MIMICK 2 study of prevalent peritoneal dialysis (PD) patients initiated in 2008 with a median follow-up of 32 months; and the prospective Kärltx (RTx-LD) study of living donor kidney transplant recipients commenced in 2009 with ongoing patient recruitment and follow-up. At University Hospital Leuven, data were obtained from kidney transplant recipients who had consented to participate in a prospective kidney allograft biopsy program. Hence, the patient material was processed using *post hoc* analysis of collected data. The number of patients included and the primary parameters or clinical endpoints in the different sub-studies of this thesis are summarized in **Table 1**.

Table 1. Cohort information and main investigation in each study.

	Study I	**Study II**	**Study III**	**Study IV**	**Study V**
N	296	259	152	423	493
Cohort					
MIA*	107	94	-	-	270
MIMICK2*	55	53	-	-	82
Kärltx *	134	112	152	76	141
Leuven cohort #	-	-	-	347	-
Age, years	55	55	46	54	55
Male, %	67%	67%	66%	66%	66%
Primary parameters or clinical endpoint (if applicable)	Total CAC, CAC density, CAC volume; all-cause mortality	AVC, CAC, all-cause mortality	Baseline clinical features; media VC	Sevelamer use, IndS, pCS, TMAO, PAG, dp-ucMGP	Dp-ucMGP, CAC, AVC; all-cause mortality

* Cohorts based and established at Karolinska University Hospital, Stockholm, Sweden; # Patients from University Hospital Leuven, Belgium.

3.1 SUBJECTS

The **Malnutrition, Inflammation and Atherosclerosis (MIA)** cohort is a patient cohort consisting of incident patients with CKD G5 (GFR<15 ml/min) sampled close to the start of

KRT at the Department of Renal Medicine, Karolinska University Hospital, Stockholm, Sweden. Patients are further followed up till death or transplantation. Patients were invited to attend additional visits after one year and two years on dialysis. This ongoing prospective cohort study started in 1994, and a descriptive protocol has been reported previously [81]. The study exclusion criteria were: age below 18 years, clinical signs of acute infection, active vasculitis or liver disease at the time of recruitment, or unwillingness to participate in the study. In the MIA study, patients have a median age of 56 years at inclusion, 63% are male, 36% have CVD and 30% have diabetes. Causes of CKD were chronic glomerulonephritis in about 22% of patients, diabetic nephropathy in about 26% of patients, autosomal dominant polycystic kidney disease (ADPKD) in about 12% patients and other or unknown etiologies in about 40% of patients. The vast majority of patients started dialysis therapy (either HD or PD) shortly after enrollment. Most patients were prescribed with commonly used drugs in CKD, e.g., phosphate- and potassium-binders, diuretics, erythropoiesis-stimulating agents, iron substitution and vitamin B, C and D supplementation. In addition, 97% of patients received antihypertensive treatment (62% were prescribed with angiotensin-converting enzyme inhibitors and/or angiotensin II receptor antagonists (ACEi/ARB), 64% with beta blockers and 52% with calcium-channel blockers) and 28% of the patients used statins. The Swedish Ethical Review Authority approved the study (Dnr 2016/1470-31/4). This cohort constitutes the patient materials included in *Studies I, II and V*.

The **Mapping of Inflammatory Markers in Chronic Kidney Disease 2 (MIMICK2)** is a cohort consisting of prevalent patients undergoing PD at Karolinska University Hospital at Huddinge and Danderyds Hospital, Stockholm, Sweden. This study originally aimed at investigating the variability of inflammatory parameters in prevalent PD patients over time. Recruitment of patients occurred from March 2008 through April 2011. The median age of patients is 64 years, 68% are males, 29% have CVD and 24% have diabetes. Causes of CKD were chronic glomerulonephritis in about 14% of patients, diabetic nephropathy in about 12% of patients, polycystic kidney disease in about 6% patients and other, or unknown etiologies in about 68% of patients. The Swedish Ethical Review Authority approved the study (Dnr. 2007/1663-31)

The **Kärltx** cohort is a prospective study cohort consisting of living donor kidney transplant (LD-RTx) recipients. Since March 2009, CKD patients undergoing LD-RTx at the Department of Transplantation Surgery of the Karolinska University Hospital are invited to participate in this study, which is designed to deepen the knowledge on inflammatory markers and proteins that affect bone turnover and vascular calcification in CKD patients. Blood and urine are collected prior to the transplantation procedure as well as at follow-up after 12 and 24 months. Artery biopsies and biopsies from fat and muscle are obtained from patients during the transplant surgery. Samples are utilized for a wide range of analyses, including assessment of biomarkers of inflammation, metabolism and atherosclerosis, as well as tissue staining and DNA and RNA assays. In addition, the vascular tissue undergoes histopathological examination to assess the degree of media calcification. As of the investigations reported in the thesis, a total of 199 patients were recruited. Their median age is 46 years, 69% are males, 15%

have CVD and 9% have diabetes. Causes of CKD were chronic glomerulonephritis in about 33% of patients, diabetic nephropathy in about 6% of patients, ADPKD in about 10% of patients and other or unknown etiologies in about 51% of patients. The most commonly used medications before RTx were erythropoiesis-stimulating agents (82%), phosphate binders (90%), antihypertensive medications (55% with ACEi/ARB, 58% with betablockers and 55% with calcium-channel blockers) and statin use (35%). About 55% of the patients received dialysis treatment for a median period of 1.1 years before undergoing LD-RTx; 24% of the patients received PD, 28% received HD and 3% had both PD and HD, as two patients switched from PD to HD and three patients switched from HD to PD treatment. The Swedish Ethical Review Authority approved the study (no. 2008/1748-31/2, 2016/1790-32).

The **Leuven cohort** is a prospective observational study comprised of CKD G5 patients referred for single kidney transplant at the University Hospital Leuven. The aim of this study is to investigate the natural history of bone histomorphometry and vascular calcification in dialysis-dependent patients before and after kidney transplantation. Adult patients (>18 years) eligible for kidney transplantation at the University Hospital Leuven are invited to participate in a kidney allograft protocol biopsy program. Exclusion criteria are: use of bisphosphonates within 6 months before the study entry, clinical signs of acute infection and unwillingness to participate in the study. Blood samples and bone biopsies (prior to kidney transplantation) and epigastric inferior artery biopsies (during kidney transplantation procedure) are collected and stored for a range of analyses. The ethical committees of University Hospitals Leuven approved the study (S52091).

3.2 CLINICAL AND PHYSICAL EXAMINATION

Each patient's medical chart was reviewed and relevant data including underlying kidney disease, history of CVD, diabetes, other comorbid conditions, common medications, and survival were extracted.

CVD was defined by clinical history of signs of ischemic cardiac disease, and/or presence of peripheral vascular disease and/or cerebrovascular disease. Smoking habits were recorded as current smokers, former smokers, and non-smokers. In the present studies, patients with current smoking habits are defined as smokers. The estimated glomerular filtration rate (eGFR) in the MIA and Kärltx cohort was estimated by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula [82]. In MIMICK2 cohort, total time on PD was defined as vintage time.

FRS, an estimate of 10-year risk of developing CVD, was calculated from sex- and age-stratified formulas with scores for systolic blood pressure, diabetes, anti-hypertensive medication, total cholesterol, high-density lipoprotein (HDL) cholesterol and smoking status [83].

Survival was determined from the day of examination and sample collection, with no loss to follow-up of any patients. Cardiovascular death was identified by physicians and was defined as a result of sudden death, coronary heart disease, stroke or complicated peripheral vascular

disease. Causes of death were registered by a nephrologist blind to patient data and to the study objectives.

Nutritional status

Nutritional status was assessed using the subjective global assessment (SGA), with a four point scale grading system consisting of six components: three subjective assessments answered by the patients concern the patient's history of weight loss, incidence of anorexia and vomiting, and three assessments performed by evaluators based on the subjective grading of muscle wasting, presence of edema and loss of subcutaneous fat [84]. On the basis of these assessments, each patient received a nutritional status score, 1 = normal nutritional status, 2 = mild malnutrition, 3 = moderate malnutrition and 4 = severe malnutrition. For the purpose of the study, poor nutritional status was defined as SGA score >1 and normal nutritional status was defined as SGA score = 1.

Anthropometric evaluation

At time of recruitment, body weight, body mass index (BMI, kg/m^2), and other anthropometric measurements were obtained. Lean body mass and fat mass were calculated by anthropometry with measurements of biceps, triceps, sub-scapular and supra-iliac skinfold thickness using the Durnin and Womersley caliper method [85], and by equations proposed by Siri [86]. Lean body mass index and fat body mass index were calculated according to the method of Kyle et al [87] and expressed as kg/m^2. Handgrip strength (HGS) was measured both in the dominant hand or in the hand without fistula (in the prevalent HD patients) using a Harpenden Handgrip Dynamometer (Yamar, Jackson, MI, USA). Each measurement was repeated three times for the measured hand, and the highest value was noted. For the analyses in the thesis, HGS was converted into percentage of sex-matched healthy subjects (% HGS).

Augmentation index

Assessment of arterial stiffness was performed non-invasively by SphygmoCorVR System (AtCor Medical, Sydney, Australia), using tonometry-based and cuff-based SphygmoCor Devices. The peripheral pulse waveform (PPW) was recorded from the radial artery at the wrist in non-fistula arm using applanation tonometry with a sensor probe. PPW and brachial blood pressure measurements were used to estimate central aortic pressure waveform calculated by the transfer function. Using the cuff-based SphygmoCor Device, brachial artery compression waveforms were obtained by partially inflating a cuff over the brachial artery between shoulder and elbow joint. Brachial waveforms were calibrated using cuff-measured brachial systolic and diastolic blood pressures, and then used to generate central aortic pressure waveforms by transfer function. Augmentation pressure (AP) and augmentation index (AIx) were derived based on pulse wave analysis. The merging of incident and the reflected wave (the inflection point) were identified on the generated central aortic pressure waveform. AP was defined as maximum systolic pressure minus pressure at the inflection point. AIx was defined as AP divided by pulse pressure and expressed as a percentage. In addition, because AIx is influenced

by heart rate, an index normalized for heart rate of 75 beats per minute (bpm) was used. SphygmoCor adjusts the AIx at an inverse rate of 4.8% for each 10 bpm increment.

Skin autofluorescence

Advanced glycation end-products autofluorescence was measured using an Autofluorescence AGE reader (DiagnOptics Technologies BV, Groningen, The Netherlands). Patients with tattooed and dark skin were not investigated. The AGE reader illuminates a skin surface of 1 cm^2, guarded against surrounding light, with an excitation light source between 300 and 420 nm. Emission light (fluorescence in the wavelength range between 420 and 600nm) and reflected excitation light (with a wavelength between 300 and 420nm) from the skin are measured with a spectrometer. SAF was calculated as the ratio between the emission light and reflected excitation light, multiplied by 100, and expressed in arbitrary units (AU). All measurements were performed at room temperature in a semi-dark environment.

3.3 BIOCHEMICAL MEASUREMENTS

Blood samples were collected after an overnight fast or before dialysis session in HD patients after the longest interdialytic period. Plasma was separated within 30 min and was kept frozen at -70 °C if not analyzed immediately. Determinations of creatinine, albumin (bromcresol purple), calcium, phosphate, intact parathyroid hormone (iPTH), total cholesterol, low-density lipoprotein (LDL) and HDL cholesterol, triglyceride, hemoglobin, and high-sensitivity C-reactive protein (hsCRP, high-sensitivity nephelometry assay) were measured by routine methods at the Division of Clinical Chemistry, Department of Laboratory Medicine, Karolinska University Hospital, Huddinge. The rest of biochemical parameters were mostly performed at Renal Lab of Division of Renal Medicine, Karolinska Institutet, or elsewhere in designated labs. Plasma interleukin-6 was analyzed by immunometric assays on an Immulite 1000 Analyzer (Siemens Healthcare Diagnostics, Los Angeles, CA, USA) using commercial kits (coefficient of variation, CV 4%). Total osteocalcin (N-MID; Immunodiagnostic Systems, Boldon, UK) and inactive/active carboxylated osteocalcin (Takara Bio, Otsu, Shiga, Japan) were analyzed with Commercial ELISA Kits. Klotho was measured by Human solubleα-Klotho ELISA Assay from IBL International (Hamburg, Germany) and human FGF23 (C terminal) was measured by ELISA Kit from Immutopics International (San Clemente, CA). Total alkaline phosphatase (ALP) activity was measured with Commercial Reagent Kit (Alkaline Phosphatase (IFCC) Plus; Thermo Fisher Scientific Oy, Vantaa, Finland) by an automatic chemical analyzer (Konelab 20XTi; Thermo Electron Corporation, Vantaa, Finland) and bone ALP (BALP) was measured using Ostase BAP ELISA kit (Immunodiagnostic Systems, Boldon, UK).

Vitamin K status was indirectly evaluated by measuring plasma dp-ucMGP levels in a single run by the Laboratory of Coagulation Profile (Maastricht, the Netherlands) using the commercially available IVD CE-marked chemiluminescent InaKtif MGP assay on the IDS-iSYS system (Immunodiagnostic Systems, Boldon, UK) [88]. In brief, plasma samples and internal calibrators were incubated with magnetic particles coated with murine monoclonal

antibodies against dp-MGP, acridinium-labelled murine monoclonal antibodies against ucMGP and an assay buffer. The magnetic particles were captured using a magnet and washed to remove any unbound analyte. Trigger reagents were added, and the resulting light emitted by the acridinium label was directly proportional to the level of dp-ucMGP in the sample. The analytical range was between 300 and 12,000 pmol/L and was linear up to 11,651 pmol/L. The within-run and total variations of this assay were 0.8–6.2% and 3.0–8.2%, respectively.

Serum levels of indoxyl sulfate (IndS), p-Cresyl sulfate (pCS), trimethylamine N-oxide (TMAO), phenylacetylglutamine (PAG) were quantified using a dedicated ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method as described elsewhere [89].

3.4 CORONARY ARTERY CALCIUM AND AORTIC VALVE CALCIUM

Coronary artery calcium (CAC) and aortic valve calcium (AVC) were measured by 64-channel detector scanner (LightSpeed VCT; General Electric (GE) Healthcare, Milwaukee, WI, USA) in cine mode. Scans were ECG-gated and a standard non-contrast media protocol was applied using a tube voltage 100 kV, tube current 200 mA, rotation time 350 ms, slice thickness 2.5 mm and a display field of view of 25 cm. CAC data were processed and analyzed using an Advantage Workstation (GE Healthcare, Milwaukee, Wisconsin, USA). CAC was assessed as a lesion with an area > 1 mm^2 and a peak intensity >130 Hounsfield Units (HU) based on the Agatston method and expressed in Agatston units (AU) [9]. The Agatston score is calculated using a weighted measurement to the highest density of calcification in a coronary artery. The density is expressed in Hounsfield units, and graded as 1 = (130-199 HU), 2 = (200-299 HU), 3 = (300-399 HU), and 4 = ($\geqslant$400 HU). The Agatston score is a product of density and area (mm^2) of coronary calcification. The Agatston score of each plaque is then summed for all image slices of the heart (left main artery, the left anterior descending artery, the left circumflex artery and the right coronary artery) to determine the total CAC score. The total volume score (CAC volume, mm^3) is a product of total area score (CAC area) and slice thickness (2.5mm)[13]. AVC-scores were computed using the Agatston CAC-scoring method from non-contrast cardiac CT scans. AVC was defined as the sum of calcium in aortic valve area including calcium within valve leaflets as well in aortic wall immediately connected to the leaflets. Presence of AVC and CAC was defined as total AVC score>0 and CAC score>0, respectively.

3.5 ARTERY BIOPSIES AND MEDIA CALCIFICATION SCORING

Within 20 min after skin incision at start of surgery, one piece (1-2 cm in length) of the inferior epigastric artery was collected by sharp dissection. Samples were immediately placed in AllProtect Tissue Reagent (Qiagen, Hilden, Germany) or snap frozen and subsequently stored at −70 °C, or fixed in 4% phosphate-buffered formalin. Formalin-fixed tissues were embedded in paraffin. One- to two-µm-thick sections were stained with hematoxylin and eosin and von Kossa staining, respectively. The degree of media calcification was semi-quantified on von Kossa-stained sections and graded 0 to 3 by an experienced pathologist: score 0 indicates no

calcification, score 1 indicates minimal calcification, score 2 indicates moderate calcification and score 3 indicates extensive calcification. In *Study III*, patients with score 0 (n=25) and 1 (n=68) were combined into one group representing no-minimal media VC (n=93), while those having moderate (score 2; n=38) or extensive (score 3; n =21) signs of VC were combined into another group representing moderate-extensive media VC (n = 59).

3.6 STATISTICAL ANALYSES

Data are expressed as median (either 10^{th}-90^{th} percentile or 25^{th}-75^{th} percentile interquartile range (IQR)), mean (standard deviation, SD), number, or percentage, as appropriate. Statistical significance was set at the level of $p < 0.05$. Comparisons between two groups were assessed with the non-parametric Wilcoxon test for skewed continuous variables and t test for normally distributed continuous variables and Fischer´s exact test for nominal variables. Comparisons between more than two groups were assessed with Kruskal-Wallis test for the non-parametric continuous variables, one-way analysis of variance (ANOVA) for normally distributed variables and Chi-square test for nominal variables. Spearman rank correlation analysis was used to determine associations between two variables. Multivariate associations were performed by multiple linear regressions and multinomial logistic regression analyses.

In *Study III*, the relaxed linear separability (RLS) method was applied to select the subset of features associated with VC. The term "relaxed" in the name of the method means the deterioration of the linear separability (between two groups of patients) due to the gradual neglecting of selected features. Initially, in the RLS algorithm, the optimal hyperplane that separates patients from two groups, is determined. This hyperplane is usually described by a large number of features. The repeated minimization of criterion function with a gradual increase of regularization parameter of RLS method allows to generate in a deterministic manner the descending sequence of feature subsets. In the process of evaluation of each feature subset, the cross-validation (leave one out) procedure was used. The apparent error (AE) and the cross-validation error (CVE) determined the errors on the training and testing parts of the data, respectively, and both denote the proportion of misclassified patients. The feature subset with minimal CVE was selected as optimal and applied on data of all patients to determine receiver operating characteristic (ROC) curve and check classification accuracy. The details of RLS feature selection method were presented elsewhere [90,91]. Missing values were assigned using k-nearest neighbor algorithm at k = 1 using "knnimpute" function from Bioinformatics Toolbox (Matlab 2018b, Mathworks, Natick, MA, USA). In total 8% of missing values were imputed. The mean values of features in the resultant dataset differed on average by 1.21 ± 1.73% from the original data; however, for none of the features the difference was statistically significant.. The imputation of missing values did not involve the outcome variable. The RLS model was applied for the final dataset with complete set of values, whereas all the other methods operated on the original data set.

Survival analyses were conducted with Fine & Gray competing-risk regression models with kidney transplantation as a competing risk to establish cumulative incidence curves. The

relative risk for mortality was presented as sub-hazard ratio (sHR, 95% confidence interval (CI)).

Statistical analyses were performed using statistical software SAS version 9.4 (SAS Campus Drive, Cary, NC, USA), Stata 16.1 (Stata Corporation, College Station, TX, USA) and Matlab (2018b, Mathworks, Natick, MA, USA). Figures were created using GraphPad Prism (version 9.0 GraphPad Software, www.graphpad.com).

3.7 STUDY CONSIDERATIONS

Strengths of the studies

The access to relatively large and extensively phenotyped cohorts of CKD patients followed for several years - and with no patients lost during follow-up - that allowed analyses of long-term consequences of VC with many potential confounders being taken into account represents a major strength of our studies. While the observational nature of the investigations does not allow us to draw conclusions on causality, our studies yielded several novel observations representing in some cases the first reported associations in this research field that we hope will stimulate and guide future research activities aiming at elucidating disease etiology, diagnosis, prognosis and adverse effects of VC in CKD.

Limitations of the studies

a) study design

First, the studies presented in this thesis are of *post hoc* nature and therefore do not allow conclusions regarding causality. Secondly, during the long recruitment time window of the Kärltx (ongoing since 2009) and MIA (patients recruited between 1994-2014) cohorts, updated clinical guidelines with recommendations for therapeutic changes were introduced affecting the treatment of the patients. For example, use of non-calcium phosphate binders and statin therapy increased during recent decades. Thirdly, all investigated patients represent a selected group of those individuals surviving earlier stages of CKD and with no complications excluding them from participation. Among investigated patients, those from Kärltx and Leuven cohorts represent individuals eligible to undergo kidney transplantation that are younger and healthier than average incident patients receiving KRT in Sweden. This selection bias thus limits the generalizability of our results to the whole CKD population. On the other hand, it is a strength that investigated patients are not burdened by very high age and acute complications as these factors could have overshadowed the impact of typical pathways leading to VC.

b) clinical measurements

Many, if not all clinical assessments, are at least to some extent not objective. Data on diagnosis were obtained from medical charts, which provided diagnoses that not necessarily had been confirmed by detailed clinical investigations and also did not separate between different degrees of severity. For instance, a patient who had a history of aortic aneurysm, could have had one or several incidences of myocardial infarction, angina pectoris, cerebrovascular lesion

or peripheral arterial insufficiency; all were diagnosed as CVD. Clearly, this general grouping may fail to cover the severity of underlying etiologies of CVD that would influence the course and prognosis of the disease. Also, as we relied on medical charts, we cannot exclude that some previous events taking place at other hospitals were not appropriately recorded or overlooked; therefore, mostly likely, the overall presence of CVD may be underestimated in our patient materials. Also, causes of death are collected from the medical records and death certificates. Autopsy that may be required to establish the actual cause of death is usually not performed. Hence, the cause of death represents an opinion of the physician issuing the death certificate and for other reasons noted above the use of "cardiovascular mortality" as an endpoint of clinical investigations is likely to be biased. Thus, in some studies presented in this thesis, in which we test "cardiovascular mortality" as sensitivity analysis, we prefer using overall deaths, i.e., all-cause mortality as this unquestionably represents the most robust definition of the ultimate clinical endpoint.

SGA is by design subjective and thus subject to bias as it relies on subjective assessments including patients' self-reported answers to patient-related outcomes. Also, while the SGA assessment was conducted by trained nurses, we cannot rule out intra- and inter-individual variations [92]. Even so, we and others have reported that SGA is a strong predictor of clinical outcomes in CKD patients[93–95], suggesting that SGA provides a meaningful measurement of nutritional status. In *Study II*, aside from AVC, SGA also shows a strong association with mortality with multiple adjustments. In addition, anthropometric measurements, e.g., skinfold thickness and body weight (and BMI calculation), may be influenced by fluid retention and hydration status and shall be interpreted with caution in the setting of CKD.

c) biochemical measurements

Some of the biochemical measurements presented in this thesis have been measured *post hoc* from frozen samples. Thus, we cannot rule out the possibility of sample degradation due to long-term storage or sample alterations due to repeated thawing and refreezing processes. Also, it shall be noted that biochemical measurements in these studies are based on one single time point whereas the investigated molecules may vary over time influenced by various factors and conditions.

d) statistical methods

Due to the observational design of the studies and insufficient sample size, and, despite extensive phenotyping, we are not able to control for all possible confounders in these investigations, while, in some cases, we may have induced over-adjustment. However, we have attempted to remove factors in regression models suspected to have collinearity and to avoid adjusting for factors that are pathophysiologically related. Also, as some may hold against dichotomizing continuous variables in multiple regressions, we have sometimes done so given that a limited sample size did not allow determining associations per units of increase. In addition, though sex differences may be reflected in the course of age-related diseases, we did not perform sex-stratified statistical analyses mainly due to a limited sample size.

4 MAIN RESULTS AND DISCUSSIONS

4.1 CAC COMPONENTS AND AVC IN RISK PREDICTION

The studies presented in the thesis (*Study I and II*) further demonstrated the prognostic value of cardiac atherosclerosis, with a focus on coronary atherosclerosis (represented by CAC score and its components, i.e., CAC density and volume) and aortic valve calcium (AVC), in risk prediction in the context of uremic milieu.

The Agatston CAC score adds to FRS for CVD prediction and improves risk stratification in various study populations [10]. While higher CAC volume associates with worse outcomes in the general population [14,96–99], it has been proposed that increased CAC density in the arterial wall reflects plaque stabilization[100–103], leading to reduced risk of coronary events [104]. However, recent reports suggested that the density of calcium in the plaques was not associated with mortality in patients with type 2 diabetes[15] and a high density of calcified plaques was independently associated with increased all-cause mortality in HD patients [14]. Thus, in these complex disease scenarios, the role of CAC density in risk prediction and plaque stabilization is yet to be determined. In *Study I,* we reported an inverse J-shaped relationship between CAC density and mortality in advanced CKD G5 patients, with middle tertile of CAC density being associated with the highest mortality and highest tertile of CAC density forming an intermediate risk group (**Figure 1**). It is plausible that with the concurrence of traditional risk factors with uremia-related risk factors, such as hyperphosphatemia, hypercalcemia, hypomagnesemia, hyperparathyroidism together with a diminished effectiveness of factors within the VC inhibitory system (e.g., fetuin-A, MGP, osteoprotegerin (OPG)), CKD patients are predisposed to a more complex conundrum of vascular aging processes over and above single entities promoting VC. Our data indicate that high CAC volume associates with inflammation, malnutrition and low handgrip strength. The concurrent inflammation, sarcopenia and atherosclerotic calcification burden may reflect such a progressive aging process in CKD. Moreover, we observed that inflammation modifies the relationship between CAC density and mortality, supporting the catalytic effect of inflammation on cardiovascular risk factors in uremic milieu [105].

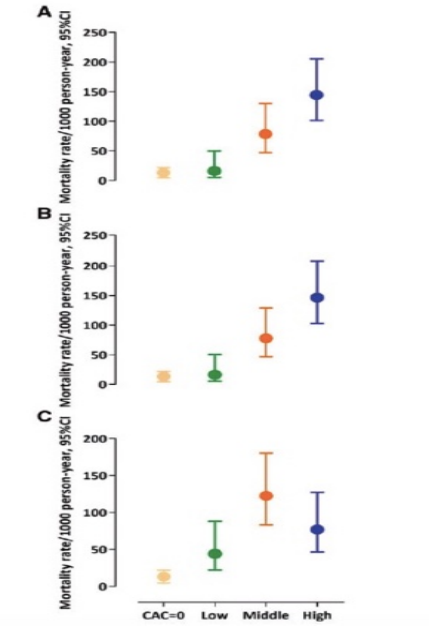

Figure 1. Crude mortality rate/1000 patient-years (95% CI) according to tertiles of (A) CAC score, (B) CAC volume and (C) CAC density (n=207) and in patients with a CAC score of 0 (n=89). Figure from *Study I* [106].

Both intima and media calcification can be co-existent in CKD [4]. In *Study I*, we report that the extent of arterial media calcification in epigastric arteries was significantly associated with both high CAC volume and high CAC density. Interestingly, while medial layer appears to be the major histological sites affected by calcification in epigastric arterial biopsies, 14% of patients with extensive media calcification were absent from coronary calcification. Hence, the magnitude and susceptibility for calcification can differ between divergent arterial sites [107]. Though CT scanning yielding CAC score does not differentiate between intima and media calcification, it is likely that CAC to a larger extent represents calcium in the intimal layer of coronary arteries which are more susceptible to atherosclerotic calcification. Since both intima and media calcification are associated with poor clinical outcomes, focusing on CAC density in single anatomical arteries trees (i.e., coronary arteries) may fail to represent overall calcification burden and its implications for risk prediction in CKD. Another complicating factor is that as conventional CT scanning cannot identify the calcified plaque pattern (microcalcification and macrocalcification), and thus it is a challenge to determine whether a high calcium density score or the aggravation of calcium score truly represents the underlying stabilization of calcified plaques.

In *Study II*, we further explore the prognostic value of AVC and report that the presence of AVC is associated with all-cause mortality, independent of coronary calcification indicated by CAC score, inflammation, malnutrition, and FRS in CKD G5 patients (**Figure 2**). Aortic valve calcification, another hallmark of premature vascular aging, is prevalent among 25% of individuals >65 years [108] and can progress into aortic valve stenosis causing left ventricular obstruction. Data from the Multi-Ethnic Study of Atherosclerosis (MESA) study found a 13% prevalence of AVC and 11% of overlap prevalence of AVC and CAC in the general population [109]. Moreover, adjusting for the presence of subclinical atherosclerosis (estimated by CAC score) and inflammation, the presence of AVC was independently associated with increased risks of coronary and cardiovascular events, suggesting a prognostic value of AVC in risk prediction beyond that of coronary atherosclerosis [109]. In dialysis patients, cardiac valve

calcification is 4-5 times more prevalent compared to the general population [110–113]. Similar to atherosclerosis, pathological factors involved in valve calcification include traditional risk factors, inflammation and disordered bio-mineralization [114]. Here, our study shows that AVC was present in 39% of the investigated CKD G5 patients and that overlapping presence of AVC and CAC was 37%, which is >3 times higher than that reported in the general population (11%) [109] (**Figure 3**). More importantly, as observed in the general population, our data support the strong prognostic role of AVC in risk prediction, which is beyond subclinical coronary atherosclerosis (CAC>0), traditional risk factors, inflammation, and nutritional status in advanced CKD.

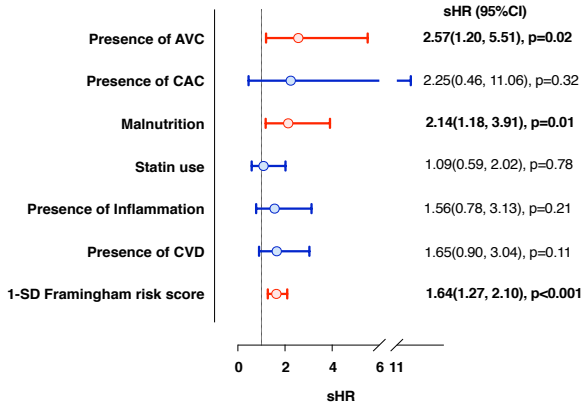

Figure 2. AVC associates with all-cause mortality independent of CAC, inflammation and other factors in CKD G5 patients.

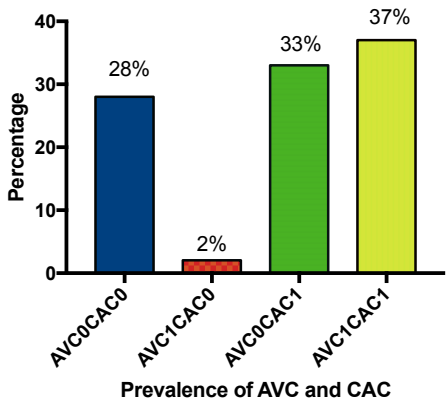

Figure 3. Prevalence of four groups of patients according to presence (+) or not (-) of aortic valve calcium (AVC) or coronary artery calcium (CAC). Figure from *Study II* [115].

Premature vascular aging (e.g. atherosclerotic VC, media VC and EVA) is highly prevalent in CKD [2,116,117] and calcification can take place with different histological and anatomical forms. This is further supported in our sub-analyses whereby 86% of patients were found to have calcification either in coronary arteries estimated by CAC, aortic valves estimated by AVC or/and media calcification in epigastric arteries estimated by histological scoring (**Figure 4A**). Whereas an overall similar trend of progression of AVC and CAC was observed in response to the severity of media VC, the proportions of AVC and CAC in different media VC groups differed remarkably (**Figure 4B-D**), suggesting that distinct mechanisms of calcification are involved in different vascular beds beyond the common risk profile.

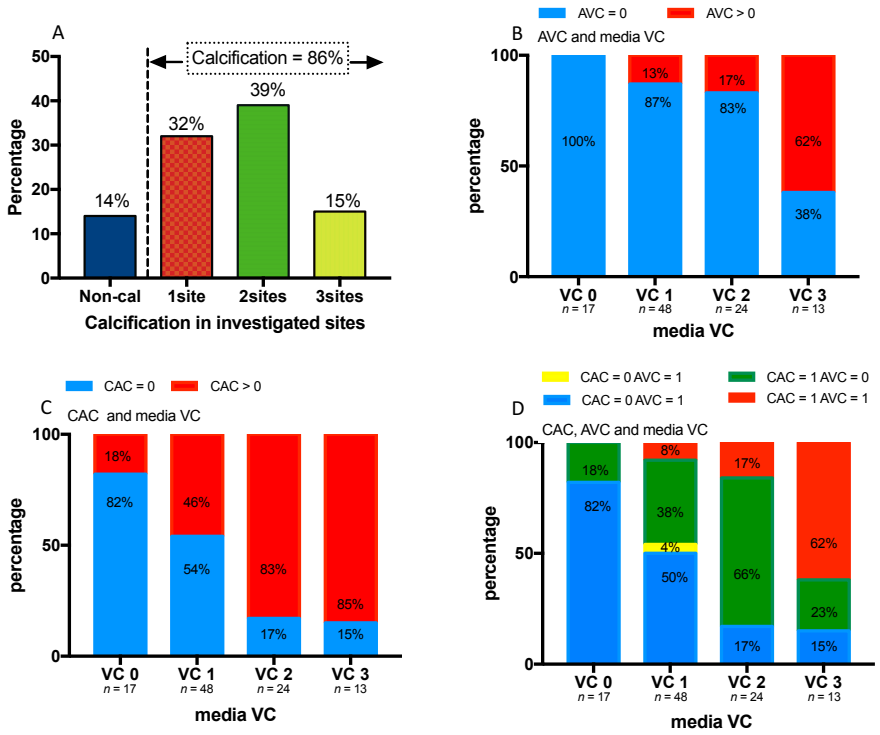

Figure 4. Prevalence of calcification at three sites, inferior epigastric artery, aortic valve (AVC) and coronary artery (CAC) among 102 CKD G5 patients who underwent both arterial biopsies and cardiac CT imaging. (A) Presence of calcification detected at three vascular sites. (B) Prevalence of AVC with severity of media VC. (C) Prevalence of CAC with severity of media VC. (D) Prevalence of combined presence of CAC and AVC with severity of media VC. Figure from *Study II* [115].

Given the cross-sectional nature of the study design, the true relation between CAC density and mortality may not be accurately reflected and the "inverse J-shaped" pattern observed in *Study I* further reflects a complex scenario of VC in the setting of CKD. Also, the strong predictive value of AVC for mortality suggests that AVC should be considered and included in the standard risk evaluation in advanced CKD. Long-term prospective studies are warranted to evaluate the joint impact of evolution of CAC components, AVC, concomitant media calcification and other potential risk factors on clinical outcome.

4.2 PHENOTYPIC FEATURES OF MEDIA CALCIFICATION

In *Study III*, we have identified 17 features including traditional risk factors and novel biomarkers associated with histologically verified media calcification in a clinical dataset of CKD G5 patients undergoing LD-RTx. The identification of risk factors promoting media calcification in patients with a complex disease condition (e.g. CKD), is challenging as it requires handling of a multifactorial panel of factors involved in VC. Here we applied multifactorial RLS model by taking all available factors into account concomitantly to minimize the risk of statistical errors as this may provide a biologically more relevant way of analyzing data. In contrast to traditional models, where factors are tested one by one or in pre-selected groups versus an outcome, the RLS method provides a holistic and less biased selection of risk factors that concomitantly associate with presence of VC in the uremic milieu.

Among 62 features, a feature set of 17 factors was obtained representing the best feature panel with the lowest CVE (0.16), which allows 89% of the subjects to be correctly classified to their respective groups (**Figure 5**). We identified that media calcification is largely associated with traditional risk factors, bone turnover markers, as well as with several novel biomarkers (**Table 2**). Also, by comparing performance of RLS with traditional logistic regression model, we found that four parameters identified as determinants of VC in the multivariate logistic regression model, i.e., age, sex, BMI and OPG, were also present among the 17 features identified by the RLS. Nevertheless, the predictive performance using logistic regression was lower than that achieved by RLS with 76% vs. 89% of subjects, respectively, correctly classified into their groups. In accordance, the ratio of true-positive to false-positive rate was less advantageous in logistic regression than in RLS model with the area under ROC curves being 0.80 in logistic regression and 0.91 in RLS selection (**Figure 6**).

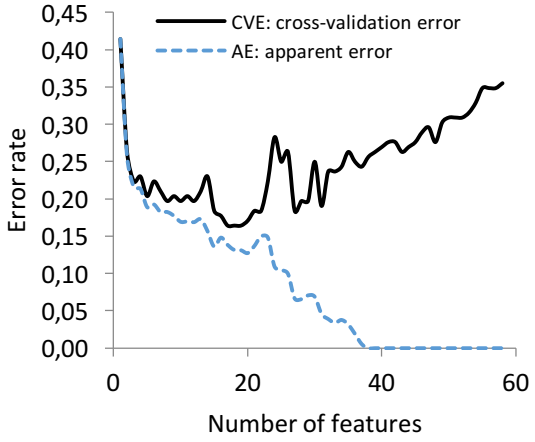

Figure 5. Cross-validation error (CVE) and apparent error (AE) during RLS procedure**.** RLS model was applied to separate patients with vascular calcification from non-calcified patients among 152 CKD G5 patients. A total of 63 features (including media vascular calcification score) were entered into the model. The lowest possible CVE was achieved with a subset of selected 17 features. Figure from *Study III* [118].

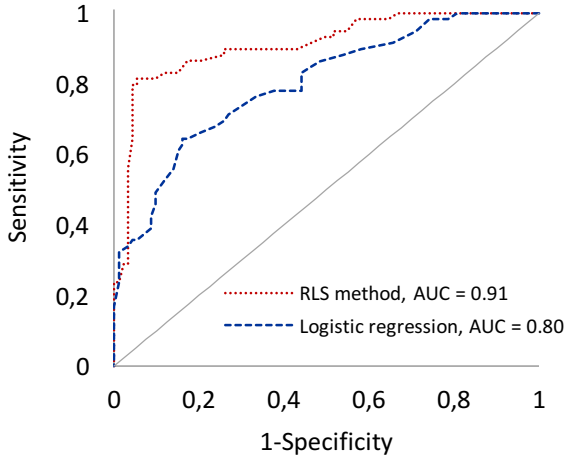

Figure 6. Receiver operating characteristic (ROC) curves with area under curve (AUC) for relaxed linear separability (RLS), red dotted curve, and multivariate logistic regression methods, blue dashed line. Figure from *Study III* [118].

Table 2. List of 17 features derived from RLS method as predictors of media calcification in 152 CKD G5 patients. Table modified from *Study III* [118].

No.	Feature Name	RLS factor	No.	Feature Name	RLS factor
1	sRANKL	4.05	10	fT3	-1.58
2	Diabetes	3.67	11	TRAP 5a	-1.51
3	Age	3.55	12	MOTSc	1.48
4	Angiopoietin 2	3.22	13	CTX	1.41
5	Cholesterol	3.15	14	IgM antiMDA	1.40
6	BMI	2.95	15	OPG	1.30
7	Sex, Male vs Female	2.54	16	iPTH	1.26
8	Uric acid	2.48	17	Betaine	-0.95
9	IgM antiPC	-1.82			

It shall be, however, acknowledged that this model is based on a data set of CKD patients undergoing LD-RTx who had specific clinical characteristics and phenotypic measurements unique to this cohort. Therefore, pre-selection of features was largely based on already available knowledge about potentially relevant factors of VC; *vice versa*, unmeasured markers proven to be related to media calcification were not included in the analysis. Also, as we discussed in the previous studies about the existence of various forms of VC in CKD, calcification in one single vasculature bed may not be sufficiently representative for the entire vascular system. Nevertheless, this study represents a novel approach that may have the capacity to identify unknown combined effects of individual phenotypic features. While the validation of phenotypic features associated with biopsy-verified vascular media calcification needs replication in other cohorts, our findings, if confirmed, may facilitate future investigations on EVA without the need of taking arterial biopsies.

4.3 SEVELAMER USE AND GUT MICROBIAL METABOLISM

With recent increased awareness of drug-microbiome interactions [51], in *Study IV*, we explored the possible link between sevelamer therapy and markers of gut microbial metabolism and show that sevelamer use is associated with increased exposure to gut-derived uremic toxins represented by circulating concentrations of IndS and PAG, and a poor vitamin K status, in CKD G5 patients. In CKD, the high prevalence of vitamin K insufficiency is mainly attributed to dietary restrictions [119] and impaired vitamin K recycling [120]. Multiple lines of evidence suggests that poor vitamin K status is involved in the pathogenesis of VC and bone fragility, both of which are common uremic features [121–123]. Here we found that patients treated with sevelamer had a poor vitamin K status, confirming and extending a recent finding from a smaller cohort study [46]. It is likely that sevelamer sequesters vitamin K through the gastrointestinal tract and abates its absorption. However, *in vitro* studies evaluating the binding of sevelamer and vitamin K (both phylloquinone and menaquinone) yielded conflicting results [47,124] and answers remains to be addressed as whether sevelamer interferes with vitamin K bioavailability. Intriguingly, we found an independent association between low vitamin K

status and high serum levels of PAG and TMAO. Though our observational study design precludes solid conclusions on the causality, this may suggest a possible link between gut dysbiosis and vitamin K deficiency. It is plausible that while gut dysbiosis may lead to decreased endogenous microbial synthesis of vitamin K, a low availability of vitamin K in the gut, may alternatively disrupt gut microbial metabolism (**Figure 7**). Indeed, it has been indicated that menaquinones are necessary growth factors to modulate human gut microbiome (e.g. *Faecalibacterium*) [125].

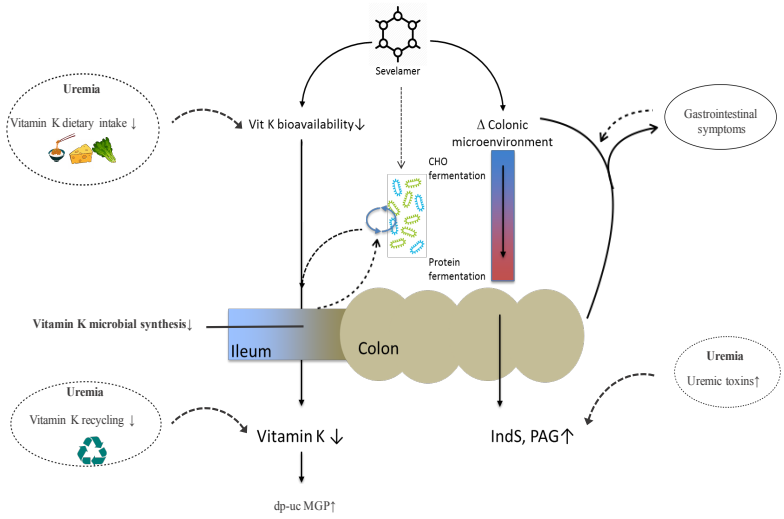

Figure 7. Sevelamer use and microbial metabolism in end-stage kidney disease. Figure from *Study IV* [126].

In addition, we observed that sevelamer treatment was associated with high IndS and PAG, independent of traditional and non-traditional factors such as age, sex, phosphate, creatinine and dialysis vintage. While this finding seems to be contradicted by some *in vitro* studies showing chelation of the precursor compounds by sevelamer [127,128], it is supported by a study [129] reporting that 8-week sevelamer hydrochloride treatment in HD patients did not alter serum IndS and Indole-3-acetic acid (IAA), but increase serum pCS. It can be speculated that sevelamer may alter the gut microenvironment, either by dragging nutrients and minerals into the colon and/or prolonging the colon transit time, and eventually result in accentuated protein fermentation (**Figure 7**).

As such, our observations that sevelamer use is associated with vitamin K deficiency and increased exposure to uremic toxins may indicate clinical trade-offs of sevelamer therapy and moreover, explain some of the neutral or negative findings in intervention studies [130,131]. The fact that pharmaceutical drugs have both beneficial and unfavorable effects is not restricted to sevelamer. A recent survey revealed that 24% of drugs may have an impact on the gut microbiome suggesting that a better understanding of drug-bug interactions can facilitate side effect control beyond the benefits [51]. Much research work remains to be performed in this area to illustrate such effects, including mechanistic and prospective studies to address possible causal links between sevelamer use and disturbed gut microbial metabolism. Also, our findings need to be testified in cohort studies with considerable information on diet and residual kidney function which are missing in the current investigation.

4.4 VITAMIN K STATUS, VC AND MORTALITY

In *Study V*, we investigated the clinical associations between functional vitamin K deficiency, VC, and all-cause mortality in advanced CKD. We observed an independent association between high dp-ucMGP and increased risk of all-cause mortality which is not modified by the presence of VC represented by CAC and AVC (**Figure 8**). Also, we found that dp-ucMGP is not an independent determinant of presence of CAC and AVC (**Table 3**). This comes as an unexpected finding but it shall be noted that whilst the role of MGP in VC is well established in animal models [53–55], data regarding the association between dp-ucMGP and VC remain inconclusive in the clinical setting. Whereas some observational studies showed a positive association between circulating dp-ucMGP and VC [65,72,74,132], others have failed to do so [71,75,133–135]. Corresponding to a *post hoc* analysis of a randomized trial showing that dp-ucMGP was not associated with baseline CAC, nor that the change of dp-ucMGP was associated with the change in CAC after 3-year vitamin K supplementation, our data also did not support dp-ucMGP as an independent determinant of CAC and AVC. One possible explanation can be the intrinsic contrast between high dp-ucMGP turn-over and slow progression of VC, making it difficult to observe a temporal association between dp-ucMGP and VC at one single observational time. Prospective studies investigating the dynamic changes of dp-ucMGP and VC development at multiple time windows may fulfill this gap in knowledge. In addition, as we have previously discussed that since cardiac CT scan of calcium score was determinant in differentiating macro- and micro-calcification, VC represented by CAC and AVC may underestimate the underlying microcalcification pattern. Therefore, the observed relation between dp-ucMGP and calcium score in our study and in most other current research work cannot mirror the true relation between dp-ucMGP and calcium score. Future studies investigating the role of dp-ucMGP in micro- and macrocalcification are in need as to delineate its impact on plaque remodeling and VC pattern.

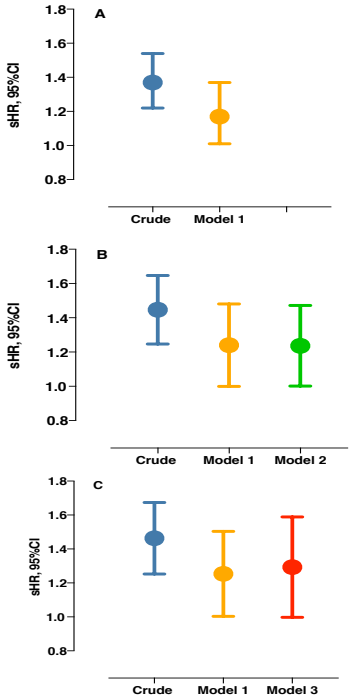

Figure 8. Associations of dp-ucMGP with all-cause mortality in all patients (A, n=493) and sub-group of patients with assessment of CAC (B, n=237) and AVC (C, n=223). Model 1, adjusted for age, sex, cardiovascular disease, diabetes, body mass index, inflammation, and dialysis treatment; Model 2: model 1 plus presence of CAC; Model 3, model 1 plus presence of AVC. Figure from *Study V* [136].

Table 3. Determinants of the presence of vascular calcification*. Table from *Study V* [136].

	Presence of CAC (n = 237, pseudo r^2 =0.45)		**Presence of AVC** (n = 223, pseudo r^2 =0.29)	
	OR	95% CI	OR	95% CI
Age, per 1-SD increase	8.10	4.43-14.81	3.83	2.39-6.14
Diabetes, yes/no	10.49	1.32-83.18	-	-
Beta-blocker, yes/no	-	-	2.23	1.03-4.78
Albumin, per 1-SD increase	-	-	0.59	0.41-0.85

* Multivariate logistic regression with stepwise backward selection of variables.

In agreement with pioneering work suggesting functional vitamin K deficiency as a risk factor of mortality and cardiovascular events in a variety of study populations [62–69,137], our study showed that higher dp-ucMGP is independently associated with increased all-cause mortality risk, after adjustments for relevant confounders (e.g. age, sex, BMI, CVD, diabetes, inflammation and dialysis treatment). Moreover, this association withstood further adjustment for presence of VC, lending support to the speculation that functional vitamin K deficiency may affect clinical outcome in CKD via pathways beyond one single entity of VC. Indeed, vitamin K deficiency may be linked to the "diseasome of aging" [138]. A large amount of clinical evidence posits the involvement of vitamin K deficiency in non-cardiovascular age-related diseases/complications including physical decline [139], frailty [78], osteoporosis [140], fractures [122,141] and depression [142]. Recently, a meta-analysis incorporating data from three large cohorts concluded that low circulating phylloquinone (reflecting dietary vitamin K intake) was associated with an increased risk of all-cause mortality, but not with CVD [143]. These results are in line with the findings in our study that circulating dp-ucMGP was not an independent determinant of VC, nor was the association between dp-ucMGP and all-cause mortality modified by the presence of VC. Nevertheless, our study is limited by the observational design and we are not able to explain this non-significant association between dp-ucMGP and VC with supportive evidence. Also, dp-ucMGP and VC were measured at one single time point (i.e. at baseline), and repeated measurements over time can be more reflective regarding the true interplay between dp-ucMGP and VC. However, this "non-positive" finding may motivate future research to explore more about the role of vitamin K deficiency in the pathophysiology of cardiovascular health, and more importantly, to illustrate its less explored mechanisms as a risk factor for non-cardiovascular causes of poor outcomes in the context of CKD.

5 CONCLUSIONS

I. The relationship between CAC density and mortality is "inverse J-shaped" in CKD G5 patients, with middle CAC density tertile being associated with the highest mortality and highest CAC density tertile forming an intermediate risk group.

II. The overlap of AVC and CAC (37%) was three times higher in advanced CKD than that reported in the general population. AVC associated with increased mortality risk independent of presence of CAC, traditional risk factors and inflammation.

III. The RLS model identified 17 features including traditional risk factors and novel biomarkers that each - when analyzed together - concomitantly associated with biopsy-verified media VC in advanced CKD.

IV. Sevelamer use is associated with disturbed gut microbial metabolism as indicated by high serum IndS and PAG levels and a poor vitamin K status in CKD G5, suggesting a possible drug-bug interaction whereby sevelamer therapy could alter gut microbiome as a potential trade-off of improving phosphate control.

V. Functional vitamin K deficiency is associated with increased mortality risk in CKD G5, independent of the presence of CAC and AVC. The results highlight the need for further studies illustrating the role of vitamin K deficiency in mediating cardiovascular and non-cardiovascular causes of poor outcomes in advanced CKD.

6 DIRECTIONS OF FUTURE RESEARCH

In the present work, we performed observational studies to expand the knowledge of risk factors and prognostic value of VC in the context of CKD. We observed several novel and intriguing associations that, as a next step, may guide the design of longitudinal explorations, interventional and mechanistic studies, aiming at establishing causal relationships and possibly leading to improved diagnostic, preventive and therapeutic strategies for this severe complication of CKD.

The current finding that the role of CAC density score in predicting clinical outcome in CKD is contradictive to the assumption of its role in the general population implies that more details of CAC scoring need to be delineated. Indeed, CAC density score provides only the average density of calcified lesions, not the density of each lesion; however, both low- and high-density calcified plaques can co-exist in the same patient. Explorations into the role of each individual lesion's CAC density would provide new insights into the prognostic value of CAC scoring. Also, to further understand the predictive role of plaque density in clinical outcome, it would be interesting to evaluate the dynamic effects of micro- and macro calcification plaques in risk prediction. The use of ^{18}F-sodium fluoride PET-CT imaging might be promising to detect microcalcification in vulnerable plaques; however, longitudinal follow-up studies evaluating the validity of ^{18}F-sodium fluoride PET-CT imaging are needed to establish its value as diagnostic tool to identify microcalcification in CKD. While we found a strong prognostic value of AVC in risk prediction, it is important to note that the susceptibility of being calcified at vascular sites is not uniformly distributed in the vasculature. Although all vessels may have the potential to calcify, only a certain portion of vascular trees develop typical atherosclerosis, e.g. coronary arteries, aortic arteries/branches, and arteries of the abdomen and lower extremities. By contrast, others appear fairly or thoroughly resistant to the atherogenesis, such as the arteries of the upper extremities. Further studies are needed to unveil mechanisms underlying the susceptibility to calcification in vascular trees and, from a clinical perspective, it is important to compare or combine the prognostic value of calcification at different sites in CKD population.

The RLS feature selection identified several less-explored novel risk factors of media calcification which may inform further investigations required to illustrate the underlying mechanisms that mediate the process of media VC in CKD. Also, the resulting feature set predicting media VC derived from this cohort needs to be further tested and validated in other CKD cohorts with similar phenotype pattern. Moreover, the clinical challenge to differentiate media calcification from intima calcification and to quantify media calcification non-invasively rather than by taking biopsies is to be tackled. The recent development of a nanoparticle-based test of the calcification propensity (T50) [144] may be of value as a clinical approach to evaluate the risk of VC in CKD; yet, the specificity and sensitivity is to be validated. Hopefully, the development of artificial intelligence and use of big data algorithms would help to provide advanced diagnostic models.

While we observed an association between sevelamer use and markers of disturbed microbial metabolism, the possible causal relationship between the two needs to be determined with longitudinal and interventional studies. Also, the direct role of sevelamer use as well as other common drugs (e.g. proton-pump inhibitors and iron supplementation) in gut microbial profiles/patterns is to be explored *in vitro* and *in vivo*. As such, a better knowledge of drug-bug interactions may provide solutions for side effect control. The observed association between vitamin K deficiency and increased risk of mortality, independent of CAC and AVC, requires further evidence-based explanations. Prospective studies with repeated measures over time would be more accurate in reflecting the long-term interplay between dp-ucMGP and VC. Also, as previously mentioned, the different susceptibility to calcification propensity and different patterns of micro- and macro-calcification in different vascular trees could potentially be incorporated in the construction of new measures of the actual presence and evolution of VC. Our observations on calcification, represented by CAC and AVC, may be biased measures underestimating the true presence and missing out the true identity of VC. Future studies incorporating anatomical (i.e. different vascular sites), histological (i.e. media and intima VC) and dynamic patterns of calcification (i.e. micro- and macro-calcification) with long-term follow up are warranted to address and tackle the potentially biased research findings.

It is worth to note that while we normally focus on identifying risk factors as potential interventional targets to reduce disease progression, the protective mechanisms have not been equally investigated in cardio-metabolic research. A recent concept of the opposite extreme of EVA, i.e., supernormal vascular aging (SUPERNOVA) [145,146], may introduce a new perspective to explore protective mechanisms of EVA by studying subjects who exhibit an exceptional resistance to arterial stiffness for their age and sex. This concept can be further extended to investigate subjects with other extreme phenotypes with absent or minimal signs of vascular aging including premature atherosclerosis and media calcification. By contrasting extremes, it may be possible to discover novel biomarkers and preventative/therapeutic targets based upon novel understanding of the protective pathways.

7 SOCIAL IMPACT

In 2017, approximately 860 million people were estimated to have kidney disease, including CKD stage 1-5 (843.6 million), acute kidney injury (13.3 million) and kidney failure requiring KRT (3.9 million), an intimidating figure that is twice the estimated amount of people with diabetes on a global scale [147]. According to The Global Burden of Diseases, Injuries, and Risk Factors Study (GBD), 1.4 million people died from CKD in 2019 with an increase of 28.8% since 2010, rendering CKD as the 11th leading cause of deaths globally [148]. While kidney dysfunction as such is a major contributor to morbidity and mortality, by increasing the risks associated with other leading causes of death worldwide (including CVD, cancer and diabetes), CKD *per se* is projected to be the 5th leading cause of global death by 2040 [149]. Global fatality from all kidney diseases could be even higher, reaching 5 million annually, and the lack or limited access to life-saving KRT is contributing to a rising fraction of deaths in low-middle-income countries (LMICs) [150]. In 2010, some 2.6 million people worldwide were receiving KRT with kidney failure whilst 2.3 to 7.1 million more people, mostly in LMICs, required KRT but died in need for it [151]. Kidney disease is also associated with tremendous health expenditures. According to the United States Renal Data System (USRDS) 2020 annual report, total Medicare-related expenditures in USA for CKD in 2018 were $81 billion (representing 22.3% of Medicare fee-for-service) including $49.2 billion due to kidney failure [152]. Timely identification and management of risk factors involved in CKD progression aiming at reducing its concerning fatality rate and growing global health care burden can clearly have a great social and economic impact.

Of note, the major culprits of high morbidity and mortality in kidney dysfunction are attributed to its high prevalence of cardiovascular complications, which can be several-fold higher than in age-matched subjects without kidney failure. According to USRDS 2020 annual report [152], the prevalence of CVD was 76.5 % in HD patients, 65.0% in PD patients and 53.7% in patients with a functioning kidney transplant. Intriguingly, unlike in the general population where coronary atherosclerosis disease is the dominant cause of CV mortality, patients with CKD also exhibited a large proportion of left ventricular hypertrophy (LVH) driven by progressive VC as the most apparent cardiovascular abnormality [117,153–156]. Many therapeutic strategies targeting VC have therefore been explored and evaluated, including controlling athero- and arteriosclerosis and managing CKD-MBD. So far, the results are inconclusive and it remains unclear whether they are efficient in mitigating VC progression [4]. Prior to these problem-solving yet off-target attempts, it is possible that we may have missed out the full picture of VC in the context of CKD. The studies presented in this thesis further explore the prognostic value, predictive markers as well as treatment consequence of VC in uremic milieus. A better understanding of the clinical relevance of VC could advance the development of efficient preventive and therapeutic strategies, and ultimately exert a beneficial effect on the kidney health-related social and economic burden.

For instance, in *Study I*, in contrast to what observed in general population, we found an inverse J-shaped pattern between CAC density and mortality, which suggests a more complex scenario

between CAC density, volume and risk prediction in the uremic milieu. In *Study II*, we found a strong predictive value of AVC for mortality over traditional risk factors and inflammation, suggesting that AVC might be potentially included in the standard risk evaluation. From a scientific point of view, these findings provide a new perspective of the prognostic value of CAC and AVC, which can guide long-term prospective studies to evaluate the joint impact of evolution of CAC components, AVC, concomitant media calcification and other potential risk factors on clinical outcome. Additionally, from an economic point of view, our findings reflect potential large cost-effectiveness gains by a wider use of cardiovascular imaging in screening and risk stratification in advanced CKD. Clearly, more solid evidence is required to determine the clinical value of cardiovascular imaging, taking into account the current technique challenge of stratifying calcium density and volume, differentiating intima/media VC and identifying micro- and macrocalcification. It can be speculated that while overuse may generate a harmless yet redundant information, it will add extra costs and burden the health care system; underuse, on the other hand, may lead to a failure to collect critical information needed to diagnose and implement therapeutic strategies. Misuse, in this sense, without knowing the true role of calcium score in risk prediction, could drive false and misleading conclusions, both at a scientific level and in clinical practice, with similar unfavorable consequences as overuse and underuse.

In *Study III*, we identified 17 features including traditional risk factors and novel biomarkers associated with histologically verified media VC using the RLS method. Given the high prevalence of media VC and its critical impact on the development of arterial stiffening and LVH, predictions based on early and accurate diagnosis of media VC is vital in improving cardiovascular outcomes in CKD. Techniques quantifying media VC in clinical practice are however not available. Hence, alternative new generation data analysis methodology and machine-learning algorithms that are able to integrate biomarkers with mechanistic and imaging data to predict and quantitate the presence and extent of media VC - and more importantly - to discover novel therapeutic targets, are urgently needed. Echoing this, we applied the advanced mathematic modelling of the RLS method and provided a holistic as well as a less biased view of potential risk factors that concomitantly associate with presence of VC. Although the validation of phenotypic features associated with biopsy-verified vascular media VC requires replication in other cohorts, our findings may facilitate future investigations on media VC without taking arterial biopsies. This non-invasive machine-learning exploration exemplifies a potential cost-effective strategy with possible maximum benefits to patients, clinicians, policymakers and health care system as a whole.

In *Study IV*, we found an association between sevelamer use and disturbed microbial metabolism, suggesting clinical trade-offs of sevelamer therapy beyond its phosphate control benefits. This delivers an important message to clinicians and researchers of potential drug-bug interaction. In fact, a recent survey revealed that up to 24% of drugs may affect the gut microbiome [51]. Thus, it is conceivable that a better knowledge of drug-bug interactions may open new paths for side effect control.

In *Study V*, we found that dp-ucMGP did not correlate with VC but significantly associated with the study endpoint, i.e., all-cause mortality. Whereas the "non-positive" finding of non-apparent association between dp-ucMGP and VC might be attributed to study design, the association of dp-ucMGP with mortality may be more important when assessing the impact of this research. In scientific explorations, the obtained results are not always equivalent to expected ones. Taking account into the study-specific conditions (e.g., study design, methodology and limitations), our finding may encourage and add value to future research about the role of vitamin K deficiency in cardiovascular health, as well as its less explored function in mediating non-cardiovascular causes of poor outcomes in the context of CKD.

Taken together, we performed observational studies to expand the knowledge of risk factors and prognostic value of VC in the context of CKD. We observed several novel and intriguing associations that, as a next step, may guide the design of longitudinal explorations, interventional and mechanistic studies, aiming at establishing causal relationships and possibly leading to improved diagnostic, preventive and therapeutic strategies for this severe complication of CKD. Also, it is worth to note that in the pandemic of coronavirus disease-2019 (COVID-19), several reports have indicated that VC detected by chest CT, such as the presence and extent of CAC and the volume of aortic wall calcification, is a predictor of severe COVID-19 and associated with worse prognosis in hospitalized patients with COVID-19 [157,158]. Therefore, aside from the focus on CKD, the role of VC in risk stratification and prognosis in other disease scenarios is to be explored and highlighted.

The studies presented in the thesis are available to the public in peer-reviewed journals and the results have been largely disseminated and shared with scientific community through posters and oral presentations at several international conferences, symposiums, and other meetings. Aside from scientific aspects, findings reported in this thesis may bring several of the above-mentioned reflections to researchers, medical community, policymakers and health care managers above the boom and bust of cardiovascular imaging, the potentials of mathematical modeling in cardiovascular research, the interactions between drugs and microbes in sustainable treatment, and the hidden myths of vitamin K in human health.

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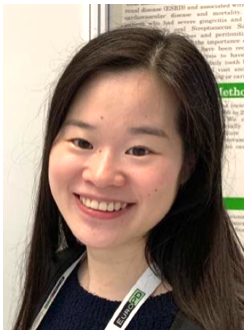
10 CURRICULUM VITAE

About the author



Lu Dai was born on December 21, 1989 in Shaoxing, China. After completing primary and secondary education in Shaoxing, she was enrolled in a seven-year medicine program (2007-2014) at Tianjin University of Traditional Chinese Medicine, Tianjin, China, during which she received Medical Doctor License (2013) and performed Master's research (2012-2014). Her master's thesis "Thrombin mediated renal interstitial fibrosis in obstructive nephropathy rat" was supervised by Prof. Hongtao Yang, at the Department of Nephrology at First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Tianjin, China. After obtaining a Master of Medicine Degree in 2014, she continued research activities and clinical training under the supervision of Prof. Hongtao Yang at the Department of Nephrology (First Teaching Hospital of Tianjin University of Traditional Chinese Medicine). In 2016, she received a scholarship from China Scholarship Council (CSC) and was accepted as a visiting researcher at Division of Baxter Novum and Renal Medicine, Department of Clinical Science, Intervention and Technology, Karolinska Institutet, Stockholm, Sweden, supervised by Prof. Bengt Lindholm. Her research was focused on associations between malnutrition, inflammation, and clinical outcome in patients with chronic kidney disease. In 2017, she was accepted for PhD studies at Karolinska Institutet as part of the EU-funded *International Network for Training on Risks of Vascular Intimal Calcification and roads to Regression of Cardiovascular Disease* (INTRICARE) Marie-Curie program. Her PhD research has been focused on the comprehensive investigation on risk factors and prognostic values of premature vascular calcification in chronic kidney disease using bio-banking and clinical cohorts. The PhD project was performed under the supervision of Prof. Peter Stenvinkel, Prof. Bengt Lindholm, Dr. Abdul Rashid Qureshi and Dr. Anna Witasp at Karolinska Institutet and Prof. Leon Schurgers at Maastricht University. In 2019, she also performed a five-month secondment at the Department of Biochemistry, Cardiovascular Research School Maastricht (CARIM), Maastricht University, Maastricht, the Netherlands. Her research work conducted with joint scientific output in INTRICARE consortium is described in this thesis entitled "Chronic kidney disease - a clinical model of premature vascular aging".

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Other Manuscripts

Dai L, Schurgers LJ, Shiels PG, Stenvinkel P. Can studies of nature help us understand the mechanisms of ageing in burden of life style diseases? *Submitted*

International Scientific Congress

Oral presentation as first author of accepted abstracts

Aortic valve calcium associates with all-cause mortality in patients with end-stage kidney disease, European Cooperation in Science and Technology (COST) meeting: The Complex Pathophysiology of Cardiovascular Calcification, Antwerp, Belgium, Dec 2-3, 2019

Sevelamer associates with vitamin K and microbial metabolism in ESKD, Annual Meeting of the American Society of Nephrology, ASN Kidney Week 2019, Washington D.C. USA, Nov 5-10, 2019

Oxidative DNA damage associates with clinical outcome in prevalent PD patients, 14th European Peritoneal Dialysis Meeting (EuroPD) 2019, Ljubljana, Slovenia, May 3-5, 2019

Serum osteoprotegerin associates with vascular calcification in ESKD, XIX International Congress on Nutrition and Metabolism in Renal Disease (ICRNM 2018), Genova, Italy, June 26-30, 2018

Inflammation modifies the mortality predictive capacity of oxidative DNA damage in CKD patients, 54th European Renal Association-European Dialysis and Transplant Association (ERA-EDTA) Congress, Madrid, Spain, June 3-6, 2017

Poster presentation as first author of accepted abstracts

Matrix Gla protein and premature vascular calcification in patients with end-stage renal disease, 57th ERA-EDTA Fully Virtual Congress, June 6-9, 2020

Predictors of vascular calcification in ESKD, 56th ERA-EDTA Congress, Budapest, Hungary, June 13-16, 2019

Osteoprotegerin associates with microbiota-derived phenylacetylglutamine in CKD, ASN Kidney Week 2018, San Diego, CA, USA, Oct 23-28, 2018

Subjective global assessment of nutritional status associates with all-cause mortality in CKD patients, World Congress of Nephrology (WCN) 2017, Mexico City, Mexico, April 21-25, 2017