Chasing the perfect storm

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Vascular calcification in chronic renocardiac syndrome

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Layout & design by Nikolas Rapp
Printed by Proefschriftspecialist

ISBN: 978-94-6469-258-7

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Dissertation

To obtain the degree of Doctor at Maastricht University

on the authority of the Rector Magnificus Prof. dr. Pamela Habibović

in accordance with the decision of the Board of Deans

to be defended in public on Tuesday, 16th of May 2023 at 13:00 hours

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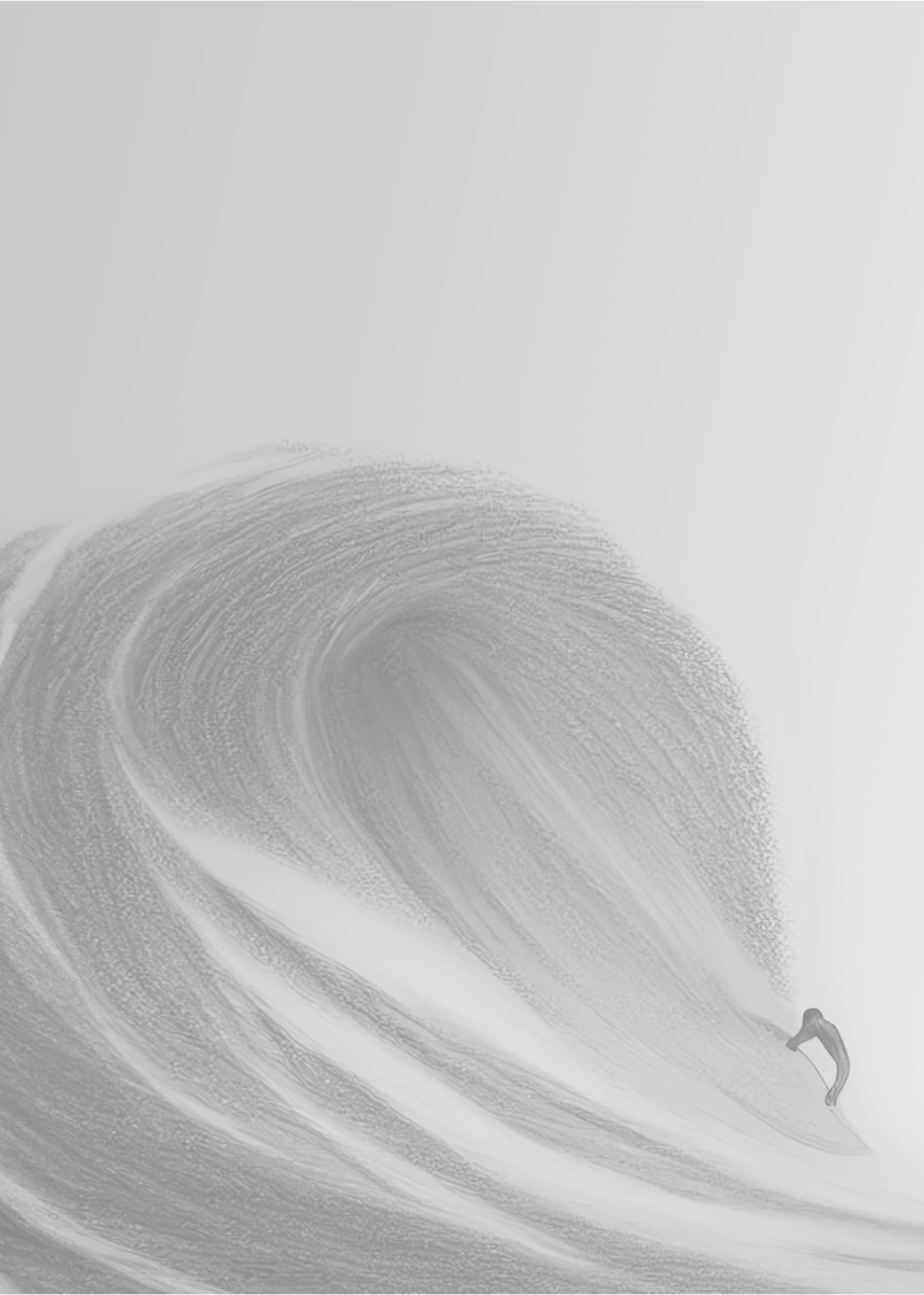
The research presented in this dissertation was funded with a grant from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant CaReSyAn (agreement No. 764474).

Content



Chapter 1

General introduction

Cardiorenal syndrome

Cardiovascular disease (CVD) is an umbrella term describing a multifaceted spectrum of disorders affecting heart and vessels. Globally, CVD is the most common cause of premature mortality and key contributor to rising healthcare cost[1]. Several factors are linked to CVD causally, including body mass index, diabetes mellitus (DM), high blood pressure and impaired kidney function[2]. The association between kidney function and CVD is reciprocal, strongly tied together and termed cardio-renal syndrome (CRS). The CRS can be subdivided in categories depending on whether acute or chronic cardiovascular (CV) or kidney disease are involved and whether kidneys or CV system are impaired initially (**Table 1**). Category 4, the chronic renocardiac syndrome, describes chronic kidney disease (CKD) resulting in chronic CVD[3]. CKD has been defined as "decreased kidney function shown by estimated glomerular filtration rate (eGFR) of less than 60 mL/min per 1,73 m^2, or markers of kidney damage, or both, of at least 3 months duration, regardless of the underlying cause"[4]. People with CKD are up to ten times more likely to die prematurely than that they progress to end-stage kidney disease. This increased mortality rises exponentially as kidney function declines and is largely attributable to mortality from CVD[5]. Vascular calcification (VC) has been identified as key contributor to excess morbidity and mortality in CRS[6].

Table 1: Classification of CRS

Phenotype	Nomenclature	Description
Type 1 CRS	Acute cardiorenal syndrome	HF resulting in AKI
Type 2 CRS	Chronic cardiorenal syndrome	Chronic HF resulting in CKD
Type 3 CRS	Acute renocardiac syndrome	AKI resulting in AHF
Type 4 CRS	Chronic renocardiac syndrome	CKD resulting in chronic HF
Type 5 CRS	Secondary cardiorenal syndrome	Systemic processes resulting in HF and kidney failure

Adapted from Rangaswami *et al*[3]*;* HF, heart failure; AKI, acute kidney injury

Ectopic calcification

Ectopic calcification (EC) is defined as "inappropriate biomineralization occurring in soft tissues, typically composed of hydroxyapatite (HA)"[7]. EC has received attention as pathological event in diseases of various aetiologies. Detection of mammary microcalcifications can be used in breast cancer screening, and brain calcifications are associated with a variety of neurological disorders, including dementia[8,9]. While also pulmonary, hepatic, renal, skin and tendon calcifications are recognized, calcifications of the CV system receive most attention among the EC affected organ systems[7,10,11]. Cardiac calcification may affect the heart valves, and to a lesser extend the myocardium[12]. Aortic valve calcification is among the most common heart diseases of the elderly, leading to stenosis, contributing to heart failure, and presenting a frequent cause of heart valve replacement[13,14].

In arteries, calcification may occur within the tunica intima or the tunica media. While different mechanisms may underly intimal and medial calcification, vascular smooth muscle cells (VSMC) play a central role in both[15]. VSMC are the most abundant

cell type in the medial layer and retain a remarkable phenotypic plasticity[16]. In response to microenvironmental changes, VSMC may lose key characteristics such as quiescence and contractile proteins, dedifferentiate, and become "synthetic", a physiological process contributing to vessel repair[17]. However, dedifferentiation of VSMC may occur in a pathological setting where it contributes to detrimental vessel remodelling and VC. Additionally, VSMC also hold the capacity to differentiate into a plethora of other phenotypes, including macrophage-like and osteochondrogenic VSMC, both of which are strongly associated with VC[16,18].

Intimal calcification relates to calcium deposition within atherosclerotic lesions, which lead to lumen narrowing and ultimately may result in myocardial infarction or stroke[19]. Size dependent, calcifications may be referred to as micro (<50 μm) or macro (>50μm) calcifications. Micro and macro calcifications may have opposing effects on atherosclerotic plaque stability with micro calcification being detrimental, and macro calcifications stabilizing[20]. Medial calcification, also known as "Mönckeberg sclerosis", named after the physician first describing the disease in 1903[21], can be found all over the vascular tree. Small vessels may suffer from calcium depositions, mainly in skin linked diseases, like calciphylaxis,[22] while calcification of the mid-sized and larger arteries strongly associates with aging, type 2 DM and CKD[23]. Medial calcification can lead to vascular stiffening, exacerbate hypertension, contribute to heart failure and cause peripheral artery disease independent of atherosclerosis[24,25].

Vascular calcification – clinical implications

In the asymptomatic population, by the age of 70, ≥ 90% of men and ≥70% of women show some degree of VC and widespread diseases like DM and CKD greatly accelerate development of VC[26]. Numerous studies support an association of VC with poor patient outcome, which may partially explain the excess morbidity and mortality in diseases with pronounced pro-calcific profile[27]. VC present at any vascular bed increases the risk for CV events by some 3.4-fold overall, and by 6.2-fold for patients with renal insufficiency. Additionally any present VC increases the risk for CV mortality 3.5-fold[27].

Various techniques to detect VC have been employed. Histological analysis of vascular specimen gained from autopsy or intervention greatly benefit research, but are not feasible as routine and repeated procedures[28]. At present clinical examination mainly depends on non-invasive imaging methods to detect and quantify calcium depositions e.g., plain X-ray radiography, ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI)[28]. CT scans are currently considered as gold standard for the detection of VC, however they fail to identify micro calcifications (<50 μm) and cannot distinguish between medial and intimal calcification[29]. Fused ^{18}F-fluoride positron emission tomography CT scan (PET-CT) is the only available technique at present, to detect micro calcification and ongoing (active) calcification. This technique however, is not widely available, requires the injection of radioactive material and suffers from low specificity and high cost[28,30].

Serum biomarkers, e.g. MGP[31], fetuin-A[32], pyrophosphate,[33] markers of inflammation[34,35] and oxidative stress[36] have been proposed to aid in VC risk assessment. They offer potential prognostic value while being minimally invasive and cost effective. However, no single marker has yet proven to be specific enough to provide reliable

information for clinical assessment[28]. Assays measuring serum calcification propensity, subsuming many factors into their outcome may prove to be more specific in reflecting VC. Indeed, the T_{50} assay, a cell free assay measuring calciprotein particle (CPP) development, can indicate CAC severity as well as progression among patients with CKD[37]. Like the vascular microenvironment, the T_{50} assay reacts to changes in the balance between calcification inhibitors and promoters.

Vascular calcification promoters and inhibitors

Under physiological conditions, calcification inhibitors suppress natural pro-calcific effectors, causing homeostasis for bone mineralisation and vascular health. Physiologically, human serum is supersaturated with calcium and phosphate, easing the formation of amorphous calcium phosphate products and HA crystals, creating by default a pro-calcific environment[38]. Thus, there is a need for endogenous calcification inhibitors, e.g., inorganic pyrophosphate (PP_i)[33], matrix gla protein (MGP)[39], fetuin-A[40] and Klotho[41], for homeostasis maintenance. PP_i, MGP and fetuin-A may hinder HA formation, by directly binding calcium or calcium phosphate products. Klotho acts as co-receptor for FGF23 and regulates VC by controlling serum-calcium and phosphate equilibrium via vitamin D metabolism and calcium / phosphate reabsorption[42]. MGP needs to undergo posttranslational activation by phosphorylation and vitamin K dependent carboxylation before being fully active[39]. Consequently, dephosphorylated and undercarboxylated MGP (dp-ucMGP) fails to fully exert its anti-calcific function. Dp-ucMGP independently predicts VC in CKD patients[31,43] and associates with CV events and CV mortality[44,45]. Additionally, dp-ucMGP serves as surrogate marker for vascular vitamin K deficiency[46].

Conversely, mutations in endogenous inhibitors lead to increased VC, e.g. impaired PP_i metabolism in pseudoxanthoma elasticum (PXE)[47]. Besides genetic factors, traditional CV risk factors associate with increased VC. Dyslipidaemia[48], smoking (nicotine)[49,50], DM[51] and age[52] are strongly associated with VC. Additionally, inflammation[53,54], oxidative stress[55,56] and disbalance in serum levels of calcium and phosphate[57] are linked to calcium deposition within the vascular wall. The latter being linked to mineral and bone disorders (MBD), defined as "a systemic disorder of mineral and bone metabolism manifested by either 1 or a combination of the following: abnormalities of calcium, phosphorus, PTH, or vitamin D metabolism; abnormalities in bone … and vascular or soft tissue calcification."[58] The contradictory association of abnormal bone metabolism in relation to VC has also been termed "calcification paradox"[59]. MBD has special relevance in CKD, which presents an exceptional case in pro-calcific diseases. CKD comes with an additional and unique set of risk factors for CVD and VC, termed uremic retention molecules (URM).

Uremic retention molecules

One of the functions of the kidney is removal of excess and waste products from the bloodstream. Kidney impairment leads to undesired retention of these molecules, called uremic toxins or URM[60]. These molecules may only be roughly classified according to their physicochemical properties as free water-soluble low molecular weight molecules, middle molecules, or protein-bound uremic toxins[61]. URM can be

endogenous e.g., interleukins and hormones, or exogenous like food derived gut metabolites[62,63]. Many are notoriously difficult to remove by dialysis, due to their size and/or attachment to proteins[64]. The increased risk of morbidity and mortality in CKD cannot solely be explained by traditional risk factors, highlighting an important contributing of URM[65]. Indeed, several URM are directly associated with renal function and negative patient outcome. Small water soluble URM Trimethylamine-N-oxide associates with all-cause mortality, atherosclerosis and CV events[66–68]. Disbalance of FGF23 links to MBD and the calcification paradox and associates with all-cause and CV mortality.[69] Prototype protein bound URM indoxyl sulfate (IS) and para-cresyl sulfate (pCS), directly associate with all-cause mortality in CKD and pCS associates with an increased risk of CV events[70]. While IS has also been linked to increased VC, most known URM have not been investigated in relation to VC[71]. The cardiotoxic nature of many URM, together with the imbalance in VC inhibitors and promoters, paired with MBD, drug side effects, hypercholesterolemia, hypertension and other factors in CKD create a milieu, sometimes referred to as "perfect storm" for VC (**Figure 1**)[72–74].

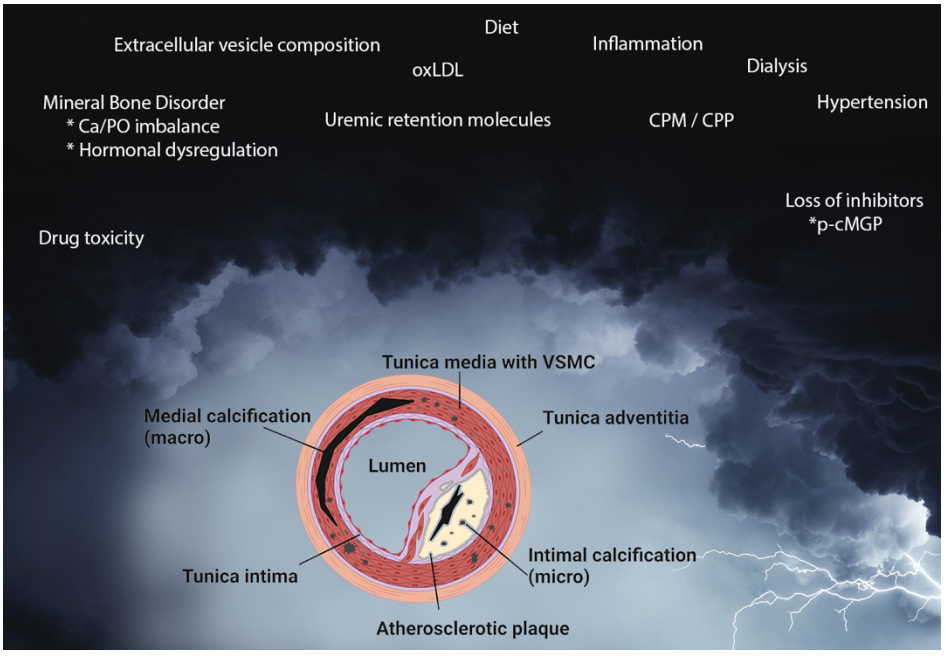


Figure 1 A myriad of factors contribute to the increases risk of VC in CKD, sometimes referred to as "the perfect storm". Abbreviations: oxLDL, oxidised low-density lipoprotein; CPM, calciprotein monomer; CPP, calciprotein particle; p-cMGP, phosphorylated and carboxylated MGP; Ca, calcium; PO, phosphate.

Therapeutic strategies and vascular calcification

There are currently no approved treatments to slow the progression or regress VC. However, several approaches are in pre-clinical and clinical development. Therapeutic options under investigation include agents interfering with HA crystal growth such as sodium thiosulfate and SNF472, a myo-inositol hexaphosphate formulation. Both are intravenously administered and have shown promising initial results in slowing progression of VC in end-stage CKD patients[75,76]. Furthermore, normalizing serum phosphate and calcium levels is an attractive approach of pharmacological targeting of VC. While the calcimimetic cinacalcet slowed progression of VC in CKD patients, the results for the phosphate binder sevelamer are conflicting[77–79]. Additionally, phosphate binders like sevelamer have adverse effects, including gastrointestinal disbalance, dysbiosis and malabsorption of micronutrients and vitamins, e.g. vitamin D and vitamin K[80].

Vitamin K constitutes a promising therapeutic option. Vitamin K is essential in activating vitamin K dependent proteins involved in coagulation, and bone and vascular health[81]. MGP has received attention for its beneficial role in vascular health for many years, especially its inhibitory role in VC. The precise mode of action has not been fully unravelled, but may include direct binding to calcium crystals, as well as scavenging bone morphogenic proteins[82]. Additionally, proteins in the coagulation cascade rely on vitamin K dependent activation e.g., prothrombin, which in its uncarboxylated form is called protein induced by vitamin K absence II (PIVKA II). Thus, clinical vitamin K deficiency impairs the functionality of the coagulation cascade and results in reduced blot clotting. This connection has been explored therapeutically using vitamin K antagonists (VKA) to deplete the vitamin K storage by inhibiting recycling[83]. However, VKA use comes with side effects such as increased VC and sometimes severe complications like calciphylaxis[84]. CKD patients commonly receive VKA therapy, further exacerbating the pro-calcific environment. But even without VKA use, CKD patients exhibit functional vitamin K deficiency, which may be due to dietary restriction recommendations[85]. A novel class of non-vitamin K dependent direct oral anti-coagulants (DOAC), e.g., Apixaban, has entered clinical practice to overcome VKA shortcomings. DOACs can also be combined with vitamin K supplementation, which is not possible with VKA therapy. However only one DOAC has been approved for use in CKD stage 4 and none for the use in end-stage renal disease patients with eGFR $\leq$ 15ml/min or on HD[86,87].

Outline of this thesis

This thesis aims to increase knowledge related to CRS, VC and its detection via blood biomarker as well as its relation to vitamin K status, with special focus on CKD related risk factors.

C*hapter 2* describes a novel cell-based approach for detection of serum calcification propensity. The assay, termed BioHybrid, is compared to current standards and applied to sera of a spectrum of different diseases. *Chapter 3* focuses on the advancement of the BioHybrid assay. We aim to optimise the BioHybrid using iPSC derived vascular cells as biosensors to create a more robust system and assess inter- and intra-assay precision. Furthermore, we investigate the effect of vitamin K and VKA use with the BioHybrid assay and explore its predictive power for VC. *Chapter 4* comprises an extensive review, summarizing the current knowledge on involvement of URM in VC. In *chapter 5,* we investigate the role of circulating particles and soluble serum components in relation to the calcification propensity of control and CKD serum. Moreover, we utilize the information of *chapter 4* to identify novel URM with potentially pro-calcific effects and aim to unravel their mechanism of action. *Chapter 6* comprises a study assessing vitamin K status in patients with high cardiovascular risk, using the two vitamin K dependent surrogate markers MGP and PIVKA II. Finally, in *chapter 7*, main conclusions of this thesis will be discussed considering broader context and future perspectives are provided.

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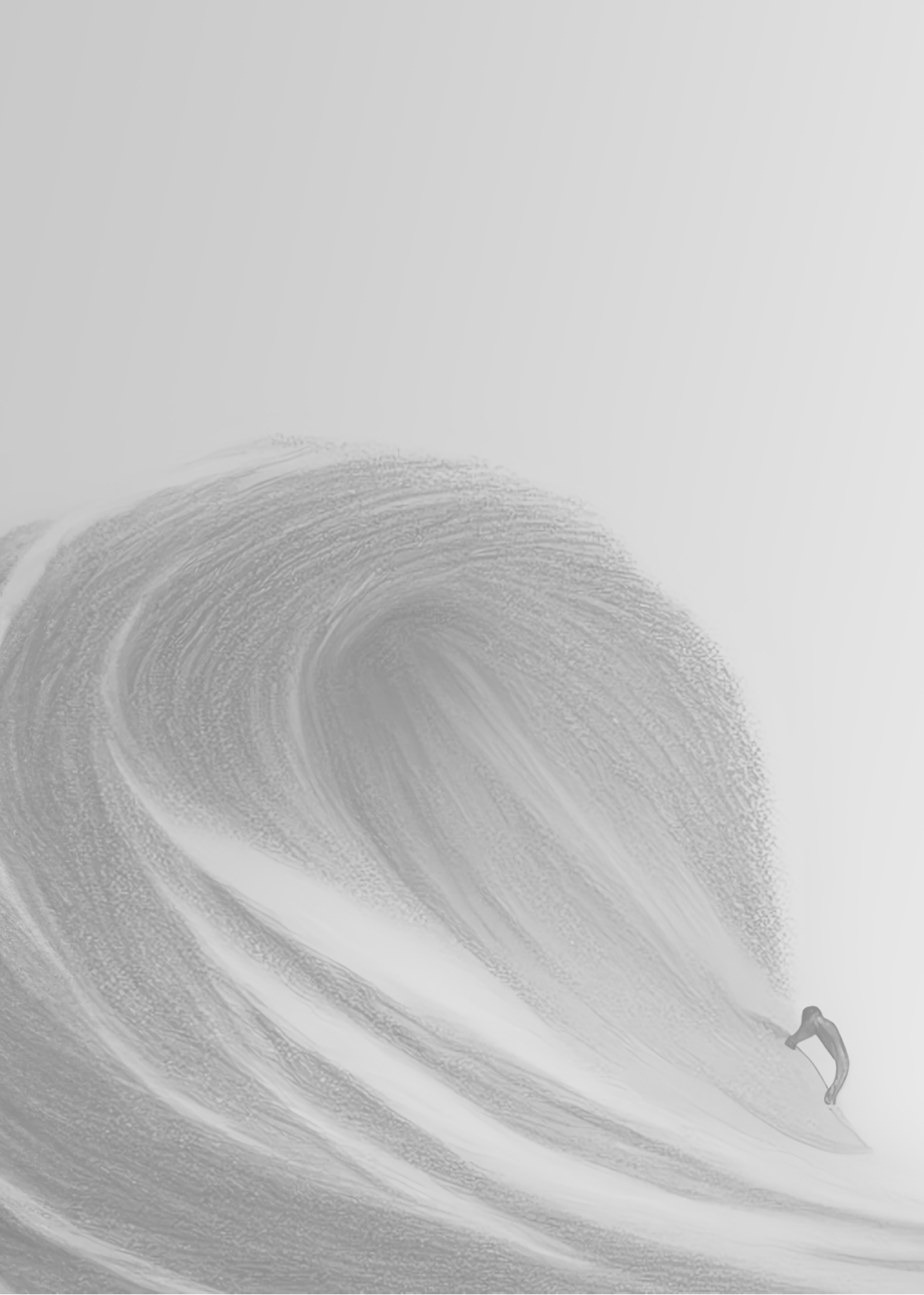

Chapter 2

Development of the BioHybrid Assay: Combining Primary Human Vascular Smooth Muscle Cells and Blood to Measure Vascular Calcification Propensity

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Cells 2021, *10*(8), 2097

Abstract

Background: Vascular calcification is an active process that increases cardiovascular disease (CVD) risk. There is still no consensus on an appropriate biomarker for vascular calcification. We reasoned that the biomarker for vascular calcification is the collection of all blood components that can be sensed and integrated into a calcification response by human vascular smooth muscle cells (hVSMC).

Methods: We developed a new cell-based high-content assay, the BioHybrid assay, to measure in vitro calcification. The BioHybrid assay was compared with the o-Cresolphthalein assay and the T50 assay. Serum and plasma were derived from different cohort studies including chronic kidney disease (CKD) stages III, IV, V and VD (on dialysis), pseudoxanthoma elasticum (PXE) and other cardiovascular diseases including serum from participants with mild and extensive coronary artery calcification (CAC). hVSMC were exposed to serum and plasma samples, and in vitro calcification was measured using AlexaFluor®-546 tagged fetuin-A as calcification sensor.

Results: The BioHybrid assay measured the kinetics of calcification in contrast to the endpoint o-Cresolphthalein assay. The BioHybrid assay was more sensitive to pick up differences in calcification propensity than the T50 assay as determined by measuring control as well as pre- and post-dialysis serum samples of CKD patients. The BioHybrid response increased with CKD severity. Further, the BioHybrid assay discriminated between calcification propensity of individuals with a high CAC index and individuals with a low CAC index. Patients with PXE had an increased calcification response in the BioHybrid assay as compared to both spouse and control plasma samples. Finally, vitamin K1 supplementation showed lower in vitro calcification, reflecting changes in delta Agatston scores. Lower progression within the BioHybrid and on Agatston scores was accompanied by lower dephosphorylated-uncarboxylated matrix Gla protein levels.

Conclusion: The BioHybrid assay is a novel approach to determine the vascular calcification propensity of an individual and thus may add to personalised risk assessment for CVD.

Introduction

Vascular calcification is an active process that occurs within the vessel wall and increases the risk of cardiovascular disease (CVD)[1–3]. Moreover, coronary artery calcification (CAC) is considered a marker for cardiovascular burden. High abundance of calcification present at any vascular site relates to approximately a 3.5-fold increase for CVD events[4]. Additionally, CAC progression is associated with a 17-fold increased risk of myocardial infarction[5]. Therefore, the means to measure vascular calcification amount or progression rate have the potential to accurately predict the risk for cardiovascular events.

The current methods to detect and assess calcification in vivo aim to visualise precipitated calcium phosphate deposition in the vasculature. Circulating biomarkers that reflect the propensity to develop and aggravate vascular calcification have not been identified so far. In vitro, diagnostics by the T50 assay may infer calcification propensity in serum[6]. Given this, the T50 assay is based on a chemical reaction and is largely affected in samples wherein a mineral imbalance or decreased circulating fetuin-A or albumin is present, thus lacking biological cause or consequence. In this paper, we reason that the circulating biomarker for vascular calcification is not a single component but the collection of all components in circulation.

As readout of this composite biomarker, we selected in vitro calcification mediated by human vascular smooth muscle cells (hVSMC), considered by many as the protagonists in vascular calcification[3]. This paper describes the BioHybrid assay, a cell-based assay for measuring the composite biomarker of vascular calcification. We measured the BioHybrid response that we consider to be the in vitro development of calcification. This was performed using either serum or plasma samples from several cohort studies. Cohorts included were that of variant stages of CKD wherein vascular calcification is synonymous with disease progression as well as PXE, a genetic disorder wherein ectopic mineralisation of soft tissue is present. In addition, we studied two cohort studies wherein enrolment was based on CVD status: one high vs. low CAC (determined by Agatston scoring), and the other was aortic valve calcification (AVC) with CAC. From both CVD cohorts, further information on Agatston scoring data was available.

In the following, we demonstrate that there is a strong and significant correlation between the Agatston score and the BioHybrid calcification readout. Additionally, serum and plasma samples from the various cohorts responded with increased in vitro calcification in the BioHybrid assay compared to respective controls. Serum or plasma samples from participants with extensive calcification responded with a faster development of in vitro hVSMC calcification than those with milder calcification. The BioHybrid calcification assay presented in this paper is an informative strategy to determine an individual's vascular calcification propensity.

Materials and Methods

hVSMC Culturing and Characterisation

hVSMC were isolated from non-atherosclerotic abdominal aortas of surgical biopsies in accordance with MUMC+ research and diagnostic procedure. Collection,

storage and use of tissue and patient data were performed in agreement with the Dutch Code for Proper Secondary Use of Human Tissue (https://www.federa.org/codes-conduct). This study complies with the Declaration of Helsinki.

Briefly, human vascular tissue samples were washed in phosphate buffered saline (PBS) before intima, fat and connective tissue was removed. Tissue was then cut into small fragments (approx. 2–5 mm in diameter) and left in M199 medium (Gibco, Bleiswijk, the Netherlands) containing 20% fetal bovine serum (FBS), 1% penicillin/streptomycin (PS, Gibco, Bleiswijk, the Netherlands) and 1% Amphotericin B on laminin coated plates (#L2020, Sigma, Saint Louis, MO, USA). When outgrowing cells reached confluency, they were passaged 1:2 on laminin-coated plates for two further passages. hVSMC were routinely cultured in M199 medium with 20% FBS and 1% PS. For experiments, cells were used between passages 5–10. All samples were mycoplasma tested.

For characterisation (**Supplemental Figure S2**), hVSMC were fixed in 4% paraformaldehyde before blocking in 2% BSA, 0.1% triton in PBS for one hour. Primary antibodies were incubated overnight at 4 °C: alpha-Smooth Muscle Actin (αSMA) 1:200 (DAKO, Glostrup, Glostrup, Denmark, M0851), phosphorylated-Myosin Light Chain (pMLC) 1:200 (Cell Signaling, Danvers, MA, USA, 3675S), Calponin (CNN1) 1:200 (Abcam, Cambridge, UK, ab46794), Smooth Muscle protein 22-alpha (SM22a) 1:200 (Abcam, Cambridge, UK ab14106) and S100 calcium-binding protein A4 (S100A4) 1:200 (DAKO, Glostrup, Glostrup, Denmark, A5114). Cells were incubated with secondary antibodies for one hour at room temperature: anti-mouse FITC 1:250 (Hycult Biotech, Uden, The Netherlands, HP2001) or anti-rabbit FITC 1:250 (DAKO, Glostrup, Glostrup, Denmark, F0205). Before imaging, cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI) for one minute. Imaging was performed on the Cytation 3 system on 20x magnification (BioSPX, Abcoude, the Netherlands).

BioHybrid Assay

For calcification experiments, hVSMC were seeded in culture well plates at a density of 10–15 × 103 cells/cm2. After 24 h, hVSMC were cultured in calcification medium (M199, 5% human serum or plasma, 1% PS and 3.6 mM Ca2+) for up to 14 days. Plasma samples were further supplemented with 143 µM Hirudin to prevent coagulation upon re-calcifying plasma. As positive control, a 0.5% serum/plasma condition was used due to the higher magnitude of calcification. For the BioHybrid assay, fetuin-A-AlexaFluor®-546 (1–3 µg/mL; prepared in-house) and Hoechst 33,342 (1 µg/mL, Invitrogen, Waltham, MA, USA) were supplemented at the start of the experiment. At various time points after calcification induction, red fluorescent protein (RFP) and DAPI channel (cell count) were imaged up to 14 days. After calcification was detected, cells were imaged regularly to follow calcification progression. Imaging was done with the Cytation 3 system (BioSPX, Abcoude, the Netherlands) and analysed using Gen5 software v.2.9 (BioTek, Abcoude, the Netherlands). As readout RFP confluence per well was normalised against cell count.

o-Cresolphthalein Assay

The o-Cresolphthalein assay (Randox, London, UK) was carried out according to manufacturer's instructions. In brief, cells were washed twice in PBS and mineral

deposits were solubilised in 0.1 M HCl for 2 h. After mineral deposits were solubilised, o-Cresolphthalein was added, which forms a violet complex with the calcium in the supernatant. A calcium standard was prepared with a range 0–2.54 mmol/L and concentration was determined by measuring absorbance at 570 nm.

T50 Assay

The T50 assay was performed as previously described[6]. In brief, sera of patients were centrifuged at 10,000 g and the supernatant was mixed with high concentrations of calcium and phosphate solutions to induce calciprotein particle (CPP) formation. Pipetting was performed with a high precision pipetting device (Liquidator, Mettler Toledo, Greifensee, Switzerland) and formation of CPP was monitored in a time-resolving manner using a standard nephelometer (Nephelostar, BMG Labtech, Ortenberg, Germany). The results were used to calculate the one-half maximal transition time, hence T50.

Serum and Plasma Preparation

Serum and EDTA plasma samples were obtained from several cohort studies. Enrolment to the cohorts was based on clinical diagnosis of the following parameters: CKD5D, CKD 3 to 5, PXE, high and low CAC score and AVC. In the AVC trial, patients received either vitamin K1 supplementation or placebo[7]. Serum and plasma of healthy individuals were used as a negative control. All patients and healthy volunteers had given written consent. This research and sample collection were approved by the Ethics Committee of the RWTH Aachen University Hospital, Aachen and Sidney Kimmel Medical College at Thomas Jefferson University, Philadelphia, Pennsylvania and conducted in accordance with the Declaration of Helsinki.

Dephosphorylated-Uncarboxylated Matrix Gla Protein (dp-ucMGP) Measurement

Plasma dp-ucMGP levels were determined using the commercially available IVD CE-marked chemiluminescent InaKtif MGP assay on the IDS-iSYS system (IDS, Boldon, UK). In brief, 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 and washed to remove unbound analyte. Trigger reagents were added; the resulting light emitted by the acridinium label is directly proportional to the level of dp-ucMGP in the sample. The within-run and total variations of this assay were 0.8–6.2% and 3.0–8.2%, respectively. The assay measuring range was between 300 and 12,000 pmol / L and was linear up to 11,651 pmol / L[8]. All assays were performed by Coagulation Profile laboratories, Maastricht, the Netherlands.

Data Analysis and Statistics

Real-time calcification development over time was analysed using Gen5 version 2.9 (BioTek, Abcoude, the Netherlands). Confluence of Alexa 546 signal was determined and normalised against cell count. Data are presented as mean ± standard deviation (SD). For over-time measurements, data is presented as mean. Non-parametric Mann–Whitney U test was performed for comparison between two groups. For more than two groups, significance was determined using one-way analysis of variance (ANOVA; Kruskal–Wallis) with comparison between groups by Dunn's multiple comparison test. For correlation determination, a linear regression model and R2 was determined. Statistical significance was defined as $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) and $P \leq 0.0001$ (****).

Results

Development of the BioHybrid Calcification Assay

Thus far, measurement of in vitro hVSMC calcification is only possible via end-point assays using either Alizarin Red or o-Cresolphthalein quantification. We developed a novel in vitro hVSMC calcification assay in which calcification development can be followed in real-time using fluorescently labelled fetuin-A-AlexaFluor®-546. To test the robustness of our live calcification assay, we compared the BioHybrid assay with the o-Cresolphthalein method, which measures calcification as calcium/protein ratio. We found a strong correlation ($R^2 = 0.72$) between fetuin-A-AlexaFluor®-546 fluorescence per cell (% RFP/cell count) and o-Cresolphthalein (µg Ca^{2+}/µg protein; **Figure 1A**; representative images **Figure 1C**; curve fit **Figure S1C and S1D**). Moreover, the BioHybrid assay has a dynamic range and is significantly more sensitive in both the earlier and lower detection of in vitro calcification compared to the o-Cresolphthalein method (**Figure 1B**).

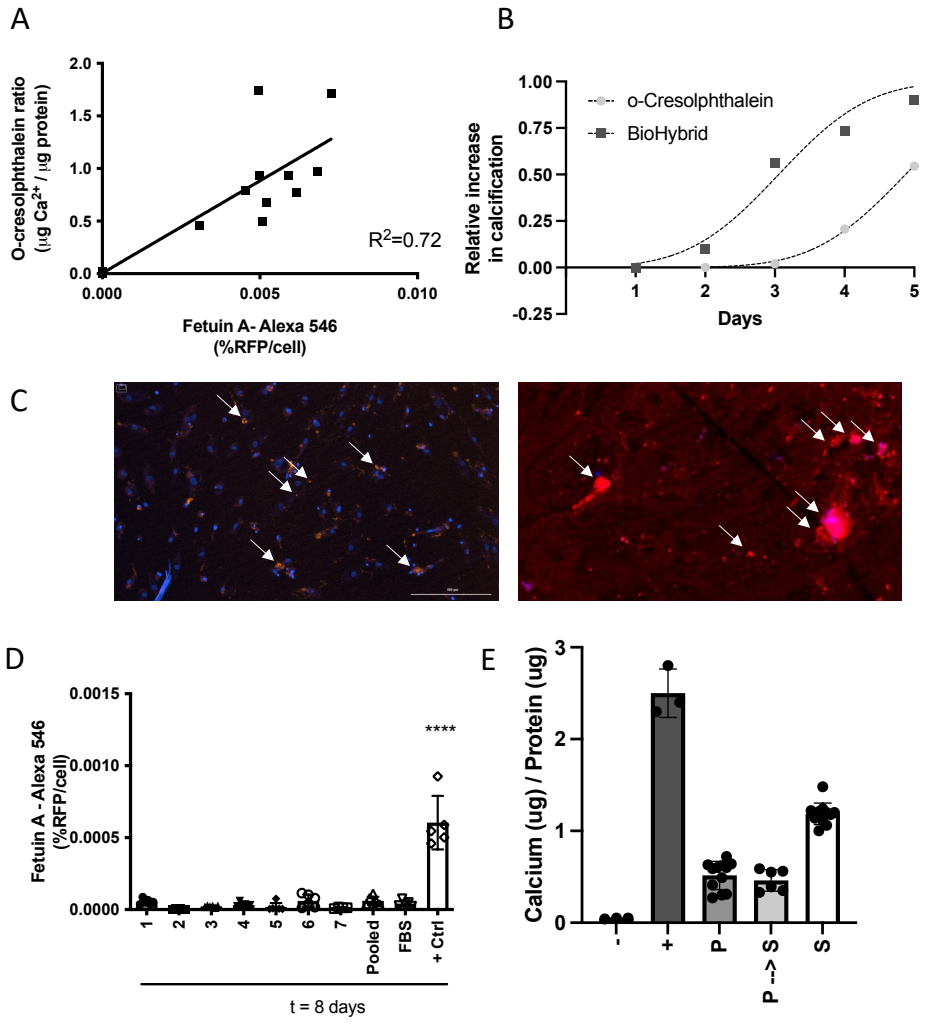

Figure 1. BioHybrid calcification assay. (**A**) Live calcification assay compared to the o-Cresolphthalein method provides a good correlation, $R^2 = 0.72$. (**B**) Relative increase in calcification of both BioHybrid and o-Cresolphthalein methods. (**C**) Representative images (4x magnification) of the BioHybrid calcification assay for minimal and severe calcification. (**D**) Calcification assay comparing FBS to serum of healthy individuals (nr. 1–7) and control serum from healthy donors (pooled; $n = 7$). I Calcification of control plasma vs. serum (P = plasma ($n = 4$); P → S = recalcified plasma ($n = 4$); S = Serum ($n = 4$)). Positive controls are always under 0.5% serum conditions. **** $p < 0.0001$.

A great advantage of using fetuin-A-AlexaFluor®-546 is that it can be used in real-time, measuring in vitro hVSMC calcification progression over time. This provides sequential, quantifiable measurements of in vitro hVSMC calcification in one single assay. We tested a variety of both serum and calcium conditions to establish the optimal parameters for our assay. We found that the use of 5% serum or plasma was optimal for a robust increase in in vitro hVSMC calcification (**Figure S1B**). Under 0.5% FBS condition, development of calcification was too rapid (**Figures S1A,B**) and when using 10% FBS calcification formation was low, as shown by us previously[9]. Another parameter that is often not uniform in calcification assays is the final concentration of calcium to trigger calcification development. All our assays were performed with 3.6 mM of calcium chloride ($CaCl_2$). Higher concentrations of $CaCl_2$ (4.5 mM and 5.4 mM total calcium) led to a much faster development of calcification, comparable to the use of lower amounts of serum (**Figure S1B**). Hence, we used 3.6 mM $CaCl_2$ and 5% serum or plasma for our BioHybrid assay.

Since we developed the BioHybrid assay to test clinical samples, we investigated whether human serum or plasma behave similarly with respect to calcification development. Comparing FBS with either a pool of healthy control serum or plasma or the individual serum or plasma from each of our healthy controls, we did not find differences in hVSMC calcification as assessed using both the fetuin-A-AlexaFluor®-546 and the o-Cresolphthalein method (**Figure 1D–E**). This indicates that serum as well as plasma samples can be used to measure calcification with our assay and that the fetuin-A- AlexaFluor®-546 probe gives comparable results to that of the o-Cresolphthalein method.

Calcification Propensity of CKD5D Serum

First, we used the T50 assay to measure hVSMC calcification propensity in a variety of samples pre- and post-dialysis as well as control serum. Pre-dialysis serum produced a significantly quicker T50 value compared to a corresponding serum sample post-dialysis. The T50 value post-dialysis was similar to that of control samples (**Figure 2A**). When screening serum from the same samples in our BioHybrid assay, we confirmed a significant difference between pre- and post-dialysis serum after three days in the BioHybrid assay (**Figure 2B**). Additionally, we were able to note a significant difference between post-dialysis serum and control samples in the BioHybrid assay (**Figure 2B**).

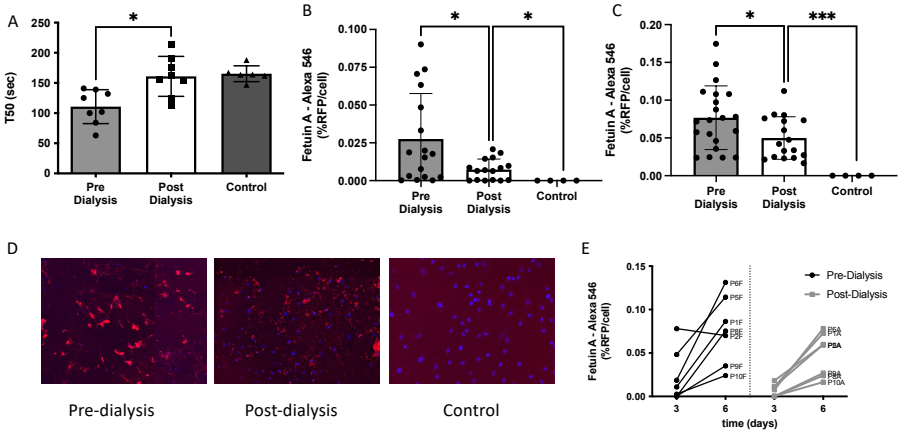

Figure 2. hVSMC calcification of CKD5D serum. (**A**) Serum from pre- (n = 8) and post-dialysis (n = 8) as well as control samples (n = 6) were tested in the T50 assay; a significant difference was found between dialysis, but no difference between post-dialysis and control serum. (**B**) Effect of dialysis on hVSMC calcification at day 3. A significant increase in calcification was found in serum of pre-dialysis (n = 7) vs. post-dialysis (n = 7). (**C**) Effect of dialysis on hVSMC calcification at day 6. A significant increase in calcification was found in serum pre-dialysis (n = 7) vs. post-dialysis (n = 7). (**D**) Representative images (4x magnification) of the calcification assay, control vs. pre- and post-dialysis CKD5D serum. (**E**) The effect of dialysis on the in vitro calcification rate. Serum of pre-dialysis has a significant higher calcification rate as compared to the same individual post-dialysis. * $p < 0.05$; *** $p < 0.001$.

After 6 days in the BioHybrid assay, pre- and post-dialysis serum continued to have progression of in vitro hVSMC calcification, whereas control samples remained low (**Figure 2C**). Moreover, serum from five out of the seven participants induced significantly less calcification post dialysis (**Figure 2E**). Serum from one individual had no significant difference and intriguingly, one showed a significant increase in calcification post-dialysis. This further suggests a sensitive and specific response that triggers in vitro hVSMC calcification and is only detectable by measuring real-time calcification development with the BioHybrid.

Calcification Propensity in Cohorts of Chronic Kidney Disease Patients, Pseudoxanthoma Elasticum Patients and Patients with Coronary Artery Calcification as Compared to Control

Next, we compared hVSMC calcification induction by serum of a cohort of participants with CKD stages 3, 4 or 5 against a pool of healthy donors. As shown in **Figure 3A**, healthy controls had the lowest rate of calcification development per hour. Serum from the CKD5 cohort participants showed the highest rate of hVSMC calcification followed by CKD4 and CKD3, respectively (trend; p = 0.0708). We observed a positive association of in vitro calcification rate from serum with increasing CKD severity (**Figures 3B**; p = 0.0859).

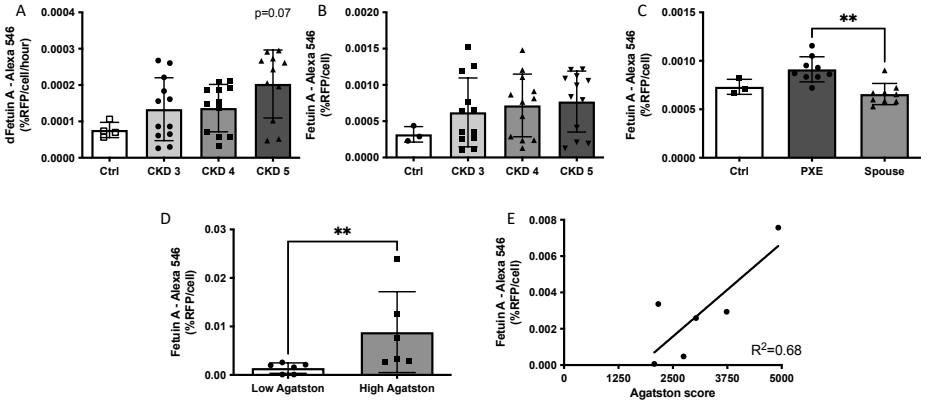

Figure 3. hVSMC calcification propensity of serum or plasma from cohorts of CKD stages 3–5, PXE and high vs. low Agatston score. (**A**) Calcification rate in the BioHybrid calcification assay. A trend was found in serum from CKD patients (n = 4) compared to control. (**B**) Calcification signal after 7 days in BioHybrid assay showing an increase in calcification when challenged with CKD serum (n = 4). (**C**) Calcification of PXE plasma (n = 3) compared to their spouses (n = 3) and healthy controls. A significant increase in in vitro calcification was found in PXE plasma compared to their spouses (p = 0.0012). (**D**) hVSMC calcification of serum from low (n = 6) vs. high (n = 6) Agatston score groups. A significant increase in calcification was found from serum of high Agatston scoring group compared to the low Agatston score group (p = 0.0022). (**E**) Correlation of Agatston score vs. in vitro calcification. A significant correlation was found in the high Agatston score group (R^2 = 0.68; p = 0.0436) but not in the low Agatston group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

We also compared hVSMC calcification induced by plasma samples from a PXE cohort against plasma from spouse controls and plasma from healthy controls. We found that plasma from the PXE cohort induced in vitro calcification faster compared to both control and spouse plasma (**Figure 3C**). This difference was significant between the PXE group and spouse controls (**Figure 3C**; p = 0.0012).

Next, we assessed how the BioHybrid system responded to serum from individuals known to have either mild or extensive CAC as determined by Agatston scores. For this, we tested serum from individuals with either a high (>1500) or low (<50) Agatston score. In vitro calcification was significantly higher from serum of the high Agatston score group compared to the low Agatston score group (**Figure 3D**; p = 0.0022). High Agatston score patient samples displayed a significant linear regression with in vitro calcification (**Figure 3E**; R^2 = 0.68) whereas no such correlation was observed in the low Agatston group (data not shown).

Calcification Propensity of Serum from Aortic Valve Calcification Patients with Vitamin K1 Treatment

Lastly, we tested serum samples from a cohort of individuals with AVC and CAC who participated in a proof-of-concept study with either vitamin K1 (phylloquinone) or placebo treatment for one year[7]. When comparing serum from the start of the trial with serum after 12 months of vitamin K1 supplementation, we found a reduction of in vitro hVSMC calcification (vitamin K1 −21.4%; **Figure 4A**). Conversely, serum from the placebo group did not have the same response, whereas in fact an increase

in in vitro hVSMC calcification was found (placebo +73.2%; **Figure 4A**). There was a trend in hVSMC calcification between 12 months of vitamin K1 and placebo supplementation (**Figure 4A**; $p = 0.06$).

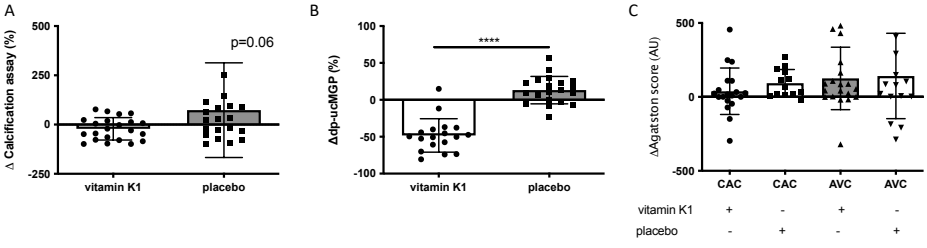

Figure 4. hVSMC calcification induction from serum of AVC and CAC patients. (**A**) In vitro calcification after 1 year vitamin K1 supplementation ($n = 22$) or placebo ($n = 20$). Supplementation of vitamin K1 showed a decreased response to in vitro calcification development comparing baseline serum to 12 months. Conversely, the placebo group exhibited increased in vitro calcification development in our assay ($p = 0.06$). (**B**) Dp-ucMGP change after 1 year. Dp-ucMGP levels significantly decreased following 1 year of vitamin K1 supplementation ($n = 18$) compared to the placebo ($n = 20$) group ($p < 0.0001$). (**C**) Development of Agatston score. No differences were found in either CAC or AVC score in both the vitamin K1 (CAC $n = 18$; AVK $n = 19$) and placebo (CAC $n = 13$; AVK $n = 16$) group. **** $p < 0.0001$.

Next, we assessed dp-ucMGP levels between the two groups, as dp-ucMGP is both a vitamin K-dependent protein and a circulating marker associated with the development of vascular calcification (**Figure 4B**). Dp-ucMGP plasma levels were significantly decreased in the serum of individuals who received vitamin K1 supplementation ($p < 0.0001$). Comparing the delta in Agatston scores (change in calcification between pre- vs. post-supplementation), no differences between vitamin K1 or placebo could be observed (**Figure 4C**).

Discussion

Vascular calcification independently predicts the risk of cardiovascular disease[4]. Vascular calcification can be measured clinically by imaging techniques such as computed tomography, intravascular ultrasound and magnetic resonance imaging. These techniques are expensive and place burdens on patients. Measurement of circulating biomarkers can be a more cost-effective and less burdensome alternative for diagnosis of vascular calcification. However, to date, no such biomarkers have been identified that adequately reflect the calcification burden.

This paper describes the BioHybrid assay for determining the vascular calcification propensity of an individual by measuring the calcification response of cultured hVSMC brought into contact with serum or plasma of that individual. The BioHybrid assay is built on the idea that the circulating biomarker of vascular calcification is not a single biomarker but a collection of circulating components. These can be sensed, integrated, and converted into a measurable calcification response by cultured hVSMC. We describe the setup of the BioHybrid assay and its first validation.

Two methods are widely used for measurement of precipitated calcium salts in cell culture in vitro. These are Alizarin Red staining and o-Cresolphthalein

quantification, and both are endpoint measurements. In order to be able to retrieve kinetics of calcification in the presence of hVSMC, the BioHybrid assay employs fluorescently labelled fetuin-A in combination with live cell imaging. Fetuin-A is a protein abundantly present in the blood with the ability to bind to minerals with high affinity[10]. The amount of calcification measured with fetuin-A-AlexaFluor®-546 significantly correlated with the results obtained with the o-Cresolphthalein method (R^2 = 0.72). During the early phase of in vitro hVSMC calcification, the fetuin-A-AlexaFluor®-546 probe is vastly superior with a greater degree of sensitivity than o-Cresolphthalein. Additionally, end-point assays require multiple replicates to record calcification development over time, which also increases chances of variability. With the BioHybrid assay, we exploit live-cell imaging, enabling us to follow the calcification development of a single condition and its replicates over time. Trialling the BioHybrid assay with a variety of both human serum and plasma samples grants further efficacy to the use of the BioHybrid assay as well as confidence in its robustness.

To date, most measurements are based on single protein biomarkers. The T50 assay was the first attempt to more comprehensively analyse the calcification propensity test by measuring the transformation of CPPs[6,11]. The T50 determines poor cardiovascular prognosis and has been shown to be associated with CVD, all-cause mortality and aortic stiffening in renal disease[12–14] based on serum factors and time to form CPPs. However, this assay is solely based on a chemical reaction without considering the vascular influence.

After optimizing calcification conditions, we tested whether T50 results could be replicated in the BioHybrid, comparing serum samples from pre- and post-dialysis. We found that the BioHybrid assay could measure differences between pre- and post-dialysis serum. Additionally, the BioHybrid could also discriminate between post-dialysis and control serum, i.e., a discrimination that the T50 assay was unable to make. The comparison between pre- and post-dialysis was performed since dialysis removes circulating components involved in calcification. The T50 is based on systemic proteins (pro-/anti-calcification) and minerals (i.e., phosphate, calcium and magnesium). However, factors impacting vascular cells that are involved in the calcification process (i.e., hVSMC) are not measured in the T50. In contrast to the T50, these factors (i.e., toxins, cytokines) are also measured with the BioHybrid assay. Thus, the BioHybrid is measuring the sum of all factors, including vascular response, contributing to vascular calcification. Following the samples in real-time, six days in culture revealed a greater difference between pre- and post-dialysis as well as between post-dialysis and control. In addition to this, the ability to follow the same sample live and its replicates in real-time allows us to find specific responses as to the rate of calcification development. The differential response is possibly representative of the varying profiles exhibited by individuals on dialysis and requires further investigation. We suggest that as a next step, a more clinically coupled application of the BioHybrid in dialysis could serve as a warning or signal to non-response to treatments.

Given our application of the BioHybrid assay to the serum of CKD5D patients, we next checked how serum or plasma samples from a variety of cohorts would respond. This was done with serum or plasma samples from cohort participants with CKD stages 3–5, PXE and high vs. low Agatston scores.

PXE is a monogenetic liver disorder, and reduced plasma anti-mineralisation capacity is observed along with extensive ectopic calcification. As PXE is a metabolic disorder, assessing whether the blood-metabolites produced by the liver cause ectopic mineralisation makes it ideal for our BioHybrid screening[15,16]. We found that PXE plasma samples had a significantly increased effect on in vitro hVSMC calcification when compared to plasma from spouse controls. This suggests a circulating factor causing increased in vitro ectopic calcification. Further research is required to define the factors that are involved in PXE mediated vascular calcification, but our research indicates that the BioHybrid platform can distinguish factors present in the blood that affect calcification. Further, the BioHybrid could serve as a screening platform of drugs that target PXE induced mineralisation.

Severe renal artery and aortic calcification, CAC and AVC, are often symptomatic in CKD[17]. Additionally, up to 93% of CKD individuals on dialysis display imageable vascular calcification[18,19]. The Agatston score is evaluated as the gold-standard clinical predictor for the accumulation and progression of vascular calcification over time[5,20]. Further, CAC score is used to identify non-CKD high-risk individuals that need immediate medical attention or intervention[21]. In the BioHybrid assay, we found that all cohort patients (CKD stages 3–5, PXE and high Agatston scoring) showed increased in vitro calcification compared to respective controls. In vitro calcification progression with CKD5 serum was faster and greater than that of serum from CKD 3 and 4 patients and non-CKD controls. Serum samples from CKD 4 had the second greatest induction, followed by CKD3 serum and lastly control serum. This is somewhat representative of the clinical situation wherein an increase of severity of CKD is associated with an increase in vascular calcification[22–24].

Next, we tested whether serum from high and low Agatston scored individuals would have an influence in the BioHybrid assay. We found that hVSMC calcification with serum from the high Agatston score group developed more calcification compared to the low Agatston score group. We further observed a positive correlation of calcification development within the high Agatston score group which was non-existing in patients with low Agatston score. Lastly, we tested serum samples from a cohort of participants with CVD diagnosis who had received either vitamin K1 supplementation or placebo for 12 months. Interestingly, serum from the vitamin K1 supplementation group had a reduced effect on in vitro hVSMC calcification compared to serum from the placebo group following 12 months of supplementation. The effect was most likely caused by changes in serum composition induced by vitamin K1, which is known to act as cofactor to activate vitamin K-dependent proteins (VKDPs) involved in the inhibition of calcification. This was not a direct effect of vitamin K serum levels as exogenous addition of vitamin K1 to serum had no effect on hVSMC calcification. We postulate that the protective effect of vitamin K1 in our BioHybrid assay is based on post-translational modifications of VKDP.

Although in the vitamin K1 study, all groups' cardiovascular status continued to decline, the placebo group had on average a 25% increase in Agatston scoring during the 12-month period, whereas the vitamin K1 group had only a 12% increase[7]. Combined, these results indicate that the BioHybrid assay might have the potential to

predict in vivo vascular calcification development and has potential sensitivity to distinguish amongst highly susceptible at-risk individuals.

Serum levels of the vitamin K-dependent protein and circulatory biomarker dp-ucMGP positively associates with vascular calcification[4,25]. Vitamin K supplementation decreased dp-ucMGP levels in all samples, yet this was not reflected in the clinical outcome for development of vascular calcification. We reason that dp-ucMGP is only reflective as a single biomarker involved in vascular calcification, a multifactorial biological process. Whereas dp-ucMGP reflects the status of a singular protein in circulation, the BioHybrid assay employs the whole blood compartment. We hypothesise that the BioHybrid platform can become an assay for clinical risk assessment of vascular calcification progression.

Conclusions

In conclusion, we present a novel BioHybrid assay that can determine personal vascular calcification propensity. Using fetuin-A-AlexaFluor®-546, we developed a real-time in vitro calcification assay that can give a quantifiable readout of in vitro hVSMC calcification development over time. We showed that vascular calcification is the consequence of the collection of all blood components that can be sensed and integrated into a calcification response by hVSMC. Further, the sensitivity of this assay has been demonstrated in response to dialysis, vitamin K treatment, as well as both metabolic and non-metabolic disorders that directly affect cardiovascular status. We propose wide scale application of this assay in larger cohorts to further validate the potential application of the BioHybrid assay as a non-invasive cardiovascular diagnostic tool, which may ultimately add to personalised risk assessment for CVD (**Figure 5**).

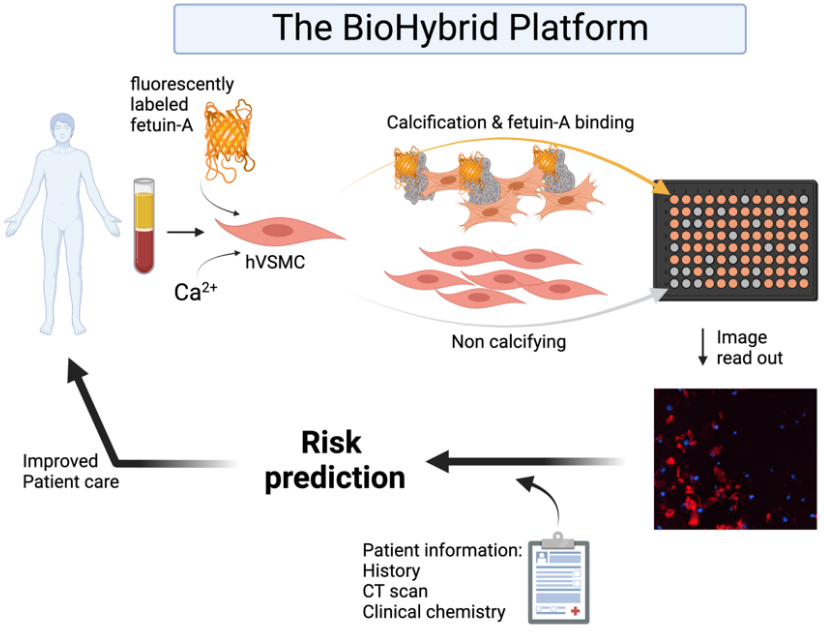

Figure 5. The BioHybrid platform. hVSMC mediated calcification is measured by fluorescent imaging using fetuin-A-AlexaFluor®-546. Created with BioRender.com.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Validation of the BioHybrid assay, Figure S2: Characterisation of hVSMC.

Funding: This research was funded by the European Union's Horizon 2020 research and innovation programmes under the Marie Skłodowska-Curie grant agreement No 722609 and 764474, NWO ZonMw (MKMD 40-42600-98-13007) and by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation)—TRR 219—Project-ID 322900939.

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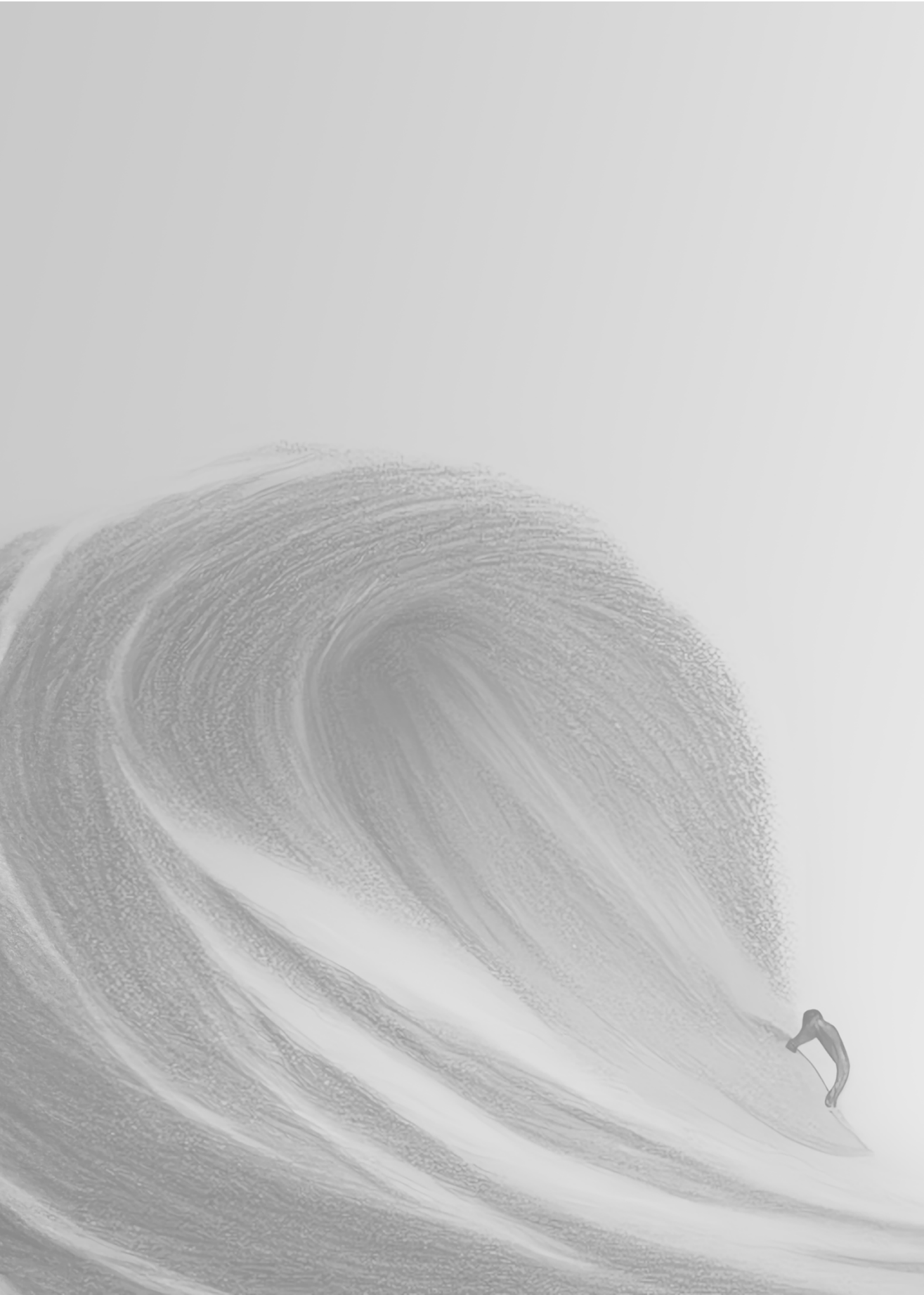
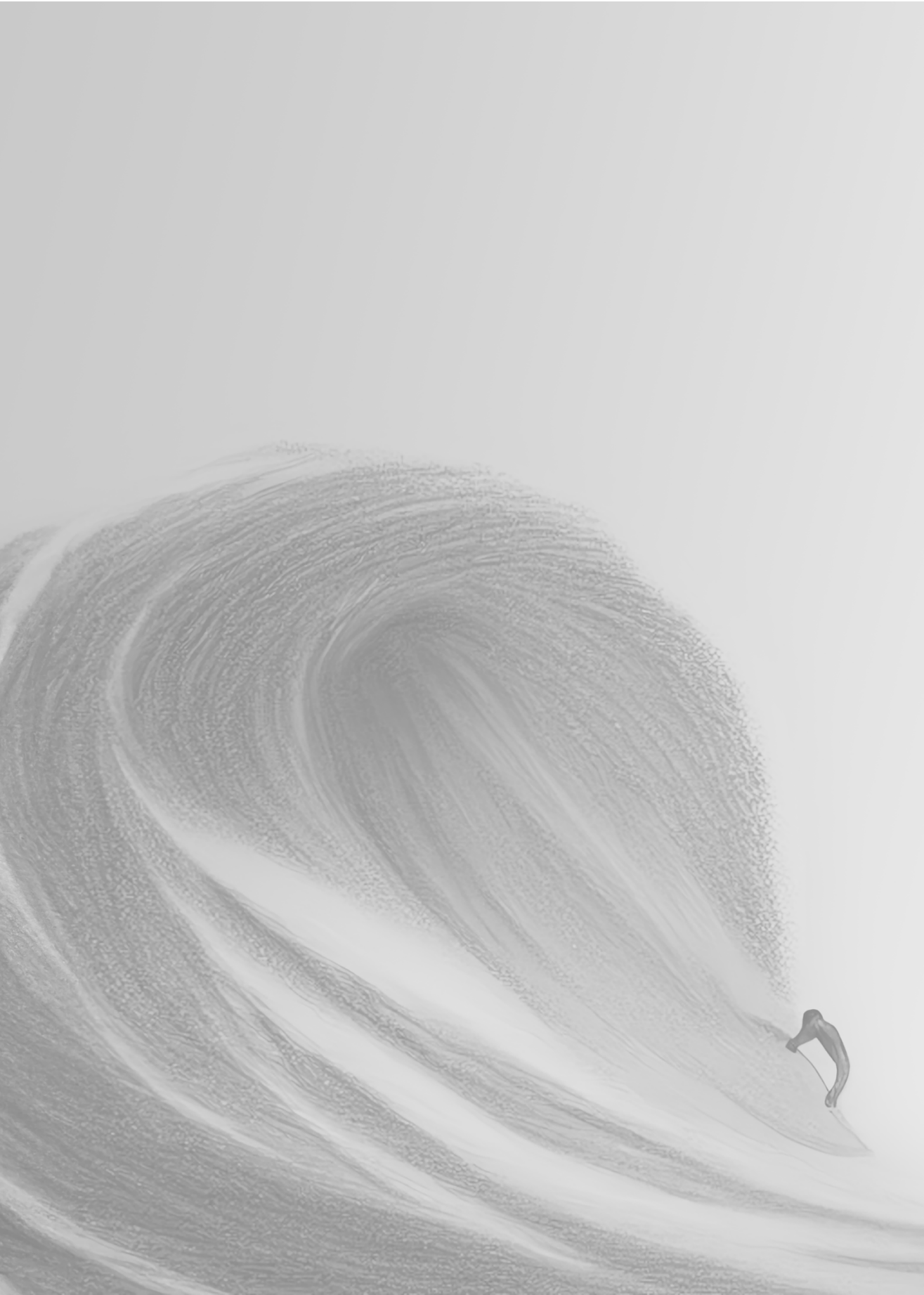


Chapter 3

Combining iPSC derived human vascular cells and serum to study and predict vascular calcification – the BioHybrid 2.0

Rapp, N., Akbulut, C., Jaminon, A.M.G., Furmanik, M., Kramann, R., Jahnen-Dechent W., R. Stöhr, V. Brandenburg, Reutlingsperger, C., Schurgers, L.J.



Chapter 4

Uremic toxins and vascular calcification – missing the forest for all the trees

Rapp N, Evenepoel P, Stenvinkel P, Schurgers LJ

Toxins 2020, *12*(10), 624

Abstract:

The cardiorenal syndrome relates to the detrimental interplay between the vascular system and the kidney. The uremic milieu induced by reduced kidney function alters the phenotype of vascular smooth muscle cells (VSMC) and promotes vascular calcification, a condition which is strongly linked to cardiovascular morbidity and mortality. Biological mechanisms involved include generation of reactive oxygen species, inflammation and accelerated senescence. A better understanding of the vasotoxic effects of uremic retention molecules may reveal novel avenues to reduce vascular calcification in CKD. The present review aims to present a state of the art on the role of uremic toxins in pathogenesis of vascular calcification. Evidence, so far, is fragmentary and limited with only a few uremic toxins being investigated, often by a single group of investigators. Experimental heterogeneity furthermore hampers comparison. There is a clear need for a concerted action harmonizing and standardizing experimental protocols and combining efforts of basic and clinical researchers to solve the complex puzzle of uremic vascular calcification.

Relevance and objective

Chronic kidney disease (CKD) is recognized as a major non-communicable disease of growing epidemic dimension worldwide. The prevalence of CKD has increased by 19.6% between 2005 and 2015[1] and reaches an overall global level of 11-13% [2]. Up to 70% of the healthcare cost related to CKD are due to hospitalization[3], the majority of which is accounted for by cardiovascular events[4]. The risk for cardiovascular disease (CVD) related hospitalization or death has been shown to increase with progression of CKD[5]. Dialysis increases cardiovascular mortality by up to 20-fold[6]. The reciprocal detrimental effects of the cardiovascular (CV) system and the kidneys on each other has been termed cardiorenal syndrome (CRS). CRS can be classified in five types according to duration and aetiology. CRS type 4, the chronic renocardiac syndrome, describes CKD leading to CVD[7]. While traditional Framingham risk factors like dyslipidaemia and diabetes contribute to CVD, they cannot fully explain the excessive mortality observed in patients with CRS type 4. Non-traditional risk factors including inflammation, oxidative stress or abnormal calcium-phosphate metabolism, have been found to account, at least partly, for the excessively high cardiovascular morbidity and mortality in these patients[8]. Non-traditional risk factors become more important along the progression of renal failure. Importantly, CKD patients are likely to die of CVD before even reaching end stage renal disease (ESRD)[9]. Prevalent CV phenotypes in CKD include atherosclerosis, cardiac arrythmia and vascular calcification (VC)[10]. VC affects up to 60% of CKD patients and is even more prevalent in dialysis patients[11]. Furthermore, it has been independently associated with CV morbidity and mortality[11,12]. Impaired renal function leads to retention of numerous compounds (referred to as uremic retention molecules, URMs). Information on the impact of URMs on vascular (patho)biology, in general, is limited and fragmentary, and pathophysiological mechanisms remain largely obscure. In this review, we present a state of the art on the effects of uremic retention molecules on vascular smooth muscle cells (VSMC) with relevance for early vascular ageing (EVA) and VC related CVD. Early vascular ageing - a modifiable, not passive entropic process[13] - is an evolving construct that has been growing around accumulating evidence surrounding arterial stiffness as an intermediate end-point and independent predictor of CVD. CKD-associated EVA is characterized by a loss of plasticity and/or resilience to adaptions against the changing internal uremic environment, which results in a marked discrepancy between chronological and biological vascular age.[14] In depth analysis of translational cohort studies investigating extreme EVA, such as CKD, provides valuable insights in factors that drive vascular ageing, and which may be also present in the older general population but take considerable longer time to evolve.

Vascular calcification

VC as consequence of EVA can occur at two distinct sites: in the intimal layer of vessels where it is associated with atherosclerosis and in the medial layer, where it is also referred to as Mönckeberg sclerosis. Medial calcification is linked to non-traditional risk factors and is a common feature in CKD, which often co-exist with intimal calcification[15,16]. Arterial calcium depositions have long been perceived as a passive process strongly associated with aging. This common opinion shifted as more recent research showed that VC is an actively regulated process involving a delicate balance between calcification activators and inhibitors, present both in the vessel wall and in the circulation[17]. The medial layer of larger arteries is predominantly composed of VSMC, a differentiated cell type expressing a set of characteristic proteins (e.g., smooth muscle myosin heavy chain, sm22 alpha and myocardin). Contractile VSMC are significantly involved in maintaining vascular tone and structural integrity of the vessel wall. However, VSMC are not terminally differentiated and retain a high degree of phenotypic plasticity. Mechanical and/ or chemical stress promote a phenotypic transition of the VSMC into a synthetic state, characterized by an increased ability to synthesize extracellular matrix components and accompanied by increased capacity for migration and proliferation[18]. Dedifferentiated VSMC have a variety of cell-fates, including macrophage-like, myofibroblast-like and osteo-chondrogenic-like phenotype[19]. Osteo-chondrogenic VSMC are characterized by a loss of contractility marker and an increase in osteo-chondrogenic marker proteins (e.g., Runt-related transcription factor 2 [Runx2], alkaline phosphatase [ALP], SRY-Box Transcription Factor 9 [Sox9], bone morphogenetic protein 2 [BMP2]). It has been reported that VSMC calcification is related to cellular senescence.[14] Furthermore the change in phenotype is accompanied by an increase in release of calcifying extracellular vesicles, elastin degradation and creation of a calcification-prone matrix[20]. Osteo-chondrogenic VSMC support the growth of hydroxyapatite (HA) crystals in the vessel wall, which defines calcification. Osteo-chondrogenesis and VC are driven by stressors including high phosphate, high calcium, oxidative stress, inflammation, senescence, apoptosis and alkalinization[21,22], all (except alkalinization) common manifestations of uraemia. Thus, non-surprisingly, uremic serum accelerates VC in VSMC *in vitro*[23–25].

Uremic toxins and their effects on VSMC

Uraemia (in Greek) literally means “urine in the blood”. A key future of the uraemic syndrome is the accumulation of URMs. URMs comprise an extremely heterogenous group of molecules of different origins, molecular weight (MW), biological functions and physico-chemical properties[26,27]. Their classification into middle molecules (MM, MW >500 Da), protein bound uremic retention molecules (PBURM) and low molecular weight solutes (LMWS, MW <500 Da) has been widely accepted [28].

The present literature review aims to update current evidence of the role of URMs in VC[29]. A total of 151 URMs were included of which 46 were classified as MM, 32 as PBURM, and 73 as LMWS (**Table 1**). For only 17.2 % of these URMs, the impact on VC has been investigated in an *in vitro* setting. *In vivo* and clinical data are even more limited (**Figure 1).** Thus, huge research gaps persist on whether and how URMs influence VC development or modify its progression

Table 1 151 described URM where selected to be part of this review on basis of the database maintained by the European Uremic Toxin work group, complemented by two further publications. Table 1 summarizes the basic information of the included URM, showing the number of molecules per group (#) and the respective percentage from all molecules (%), as well as the characteristic size range and a typical example molecule.

Middle molecules	46 (30,5)	# (%)	MW >500 Da	e.g. TNF
Protein bound	32 (21,2)	# (%)	-	e.g. Indoxyl sulfate
Water soluble	73 (48,3)	# (%)	MW <500 Da	e.g. Urea
Total	151 (100)	#(%)		

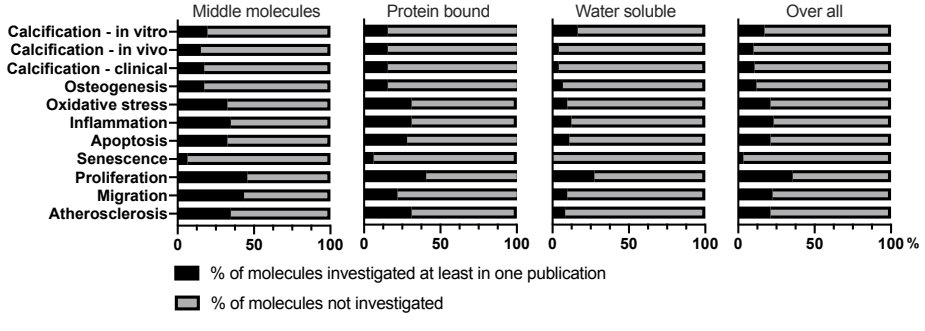


Figure 1 : Literature was screened for effects of URM on VSMC, with relevance for VC. Relevant review terms have been defined as the three levels of calcification research – in vitro, in vivo and clinical, and were completed by pertinent phrases. The extent to which molecules of each group have (black bar) or have not (grey bar) been investigated with respect to a given topic, displayed as % per group, is presented.

MM are predominantly proteins and peptides generated endogenously as a response to uraemia, such as cytokines and peptide hormones. Dialysis has limited efficacy in controlling MM, partly related to size restriction of the pores of dialyzers[26]. Of the 46 MM included in this review, 9 (19,6%) have been investigated with respect to effects on VC**.** Of these 46, all belong to the subgroups of inflammatory cytokines and peptide hormones. A summary of the effects of MM on VSMC calcification is given in **Table 2** (**Supplemental Table S1**).

Table 2 Repository of studies investigating MM towards their effect on VC, as well as their effects on VC related processes in VSMC

	Calcification - in vitro	Calcification - in vivo	Calcification - Clinical studies	Osteogenesis	Oxidative stress	Inflammation	Apoptosis	Senescence	Proliferation	Migration	Atherosclerosis
APN	↓[30–32] —[33]	↓[34,35]	↑[36–38] ↓[39,40] —[41–43]	↓[30,35]		↓[44]	↓[32,34]		↓[45,46]	↓[47,48]	↓[48,49]
ADM	↓[50,51]	↓[50,52]		↓[52]	↓[53,54]				↑[55,56] ↓[57,58]	↓[59,60]	
ET	↑[61]	↑[61–63]	↑[64–66]		↑[67–69]	↑[68,70]	↑[71] ↓[72]		↑[73,74]	↑[75,76]	↑[77,78]
IL-8	↑[79]		↑[80] —[81]			↓[82]			↑[83,84]	↑[83,84]	
IL-18	↑[85,86]		↑[87–89]	↑[85,86]	↑[90]	↑[91,92]			↑[93,94]	↑[95,96]	↑[97,98]
IL-1β	↑[99–101] ~[102]	↑[100,103,104]	—[100,101,105]	↑[99,101] ~[106]	—[107]	↑[108,109]	↑[110,111]	↑[101]	↑[112,113]	↑[113,114]	↑[100,115]
IL-6	↑[116–121]	↑[117,118,122]	↑[40,105,123–128]	↑[117,119,121,129]	↑[130]	↑↓[129]	↑[131,132]	↑[120]	↑[133,134]	↑[134,135]	↑[117,119]
PTH	↑[136,137]	↑[136,138,139] ↓[140,141]	↑[128,142] —[143]	↑[136]	↓[140]				—[144]		
TNF	↑[106,145–151]	↑[148]	↑[125,128] —[43,127]	↑[106,145,148,152]	↑[153,154]	↑[155,156]	↑[157,158]		↑[159,160]	↑[159,161]	↑[162,163]

↑ = increase, ↓ = decrease, — = tested, but no effect, ~ = unclear effect
Abbreviations: Adrenomedullin = ADM, Endothelin = ET, Interleukin = IL, Parathyroid hormone = PTH, Tumor necrosis factor alpha = TNF-α, Adiponectin = APN

PBURM are a heterogenous group of solutes, characterized by reversible protein binding capacity. PBURM are considered a major threat to ESRD patients, partly because of their limited removal by conventional dialysis[164]. Of the 32 PBURM included in this review 5 (15,4 %) have been investigated with respect to effects on VC. A summary of their effects on VSMC calcification is given in (**Table 3** and **Supplemental Table 2**).

Table 3 Repository of studies investigating PBURM towards their effect on VC, as well as their effects on VC related processes in VSMC

	Calcification - in vitro	Calcification - in vivo	Calcification - Clinical studies	Osteogenesis	Oxidative stress	Inflammation	Apoptosis	Senescence	Proliferation	Migration	Atherosclerosis
Hcy	↑[165–169]	↑[165,168–171]	↑[170,172–177] —[105,127,178–181]	↑[165,166,168,169]	↑[182,183]	↑[184,185]	↑[186,187] —[188]		↑[189,190]	↑[191,192]	↑[193,194]
IS	↑[29,79,195–197]	↑[195,197,198]	↑[199,200] —[201]	↑[79,196,197,202]	↑[202,203]	↑[204,205]	↑[29]	↑[29,29]	↑[206,207]	↑[208,209]	↑[29,210]
Leptin	↑[211–213]	↑[211,214,215]	↑[39,216–219] —[40,43]	↑[211,211,213,214,220]	↑[221,222]	↑[221,223]	↑[222] ↓[224]		↑[223,225] ↓[226]	↑[227,228]	↑[212,229]
CML	↑[230]	↑[230]	↑[231]	↑[230]		↑[232]	↓[233]		↑[233]		↑[230]
pCS		↑[198]	↑[234]	↑[235]	↑[235,236]	↑[198,235]			↑[237]	↑[237]	↑[237,238]
SM	↓[239]				↑[240]		↑[241,242]		↑[243,244] ↓[245]		

↑ = increase, ↓ = decrease, — = tested, but no effect, ~ = unclear effect
Abbreviations: Homocystein = Hcy, Indoxyl sulfate = IS, N(6)-Carboxymethyllysine = CML, p cresyl sulfate = pCS, Spermine = SM

The group of LMWS comprises compounds with molecular weight < 500 kDa and minimal protein binding. Reduction rates of LMWS during dialysis are overall high, but show substantial variability[26]. Twelve out of the 73 LMWS (16,4 %) have been investigated with respect to potential effects on VC (**Figure 1, Table 4 and Supplemental Table S3).** LMWS have been neglected for a long time but interest in this class of URMS has increased in recent years, as it has become clear that they considerably affect patient well-being and may contribute substantially to vascular pathobiology[26]. Thus, LMWS might hold significant potential as therapeutic target, also considering that it is the largest and most understudied class with respect to VC. URM of the class MM as well as PBURM are approximately 2.5 times more often investigated compared to LMWS. That might partially be due to the fact that MM contain many non-

uremia specific molecules, e.g. inflammatory cytokines and peptide hormones also relevant for many other diseases[28]. This leads to a seemingly higher level of clinical evidence for these molecules, while the level of evidence generally remains low.

Table 4 Repository of studies investigating LMWS towards their effect on VC, as well as their effects on VC related processes in VSMC.

	Calcification - in vitro	Calcification - in vivo	Calcification - Clinical studies	Osteogenesis	Oxidative stress	Inflammation	Apoptosis	Senescence	Proliferation	Migration	Atherosclerosis
ADMA	↓[246]		↑[247–249,248,250,251] —[252]		↑[253]	↑[254]	↑[253]		↑[254,255] —[246]	↓[256,257]	
G	—[246]								—[246]		
GAA	—[246]								—[246]		
GSA	↓[246]								—[246]		
MG	—[246]								—[246]		
MMA	↓[258]			↓[258]			↑[259]				
NA	↑[260]	↑[260]		↑[260]					↑[261,262]	↑[263,264]	
SDMA	↓[246]								—[246]		
TMAO	↑[265]	↑[265]	—[266]	↑[265]		↑[265,267]					↑[267]
UA	↑[268,269]	↑[269]	↑[270–273] —[274]	↑[268,269]	↑[275–278]	↑[276,279]			↑[280,281]	↑[282]	↑[276]
GPA	—[246]								—[246]		
GBA	—[246]								—[246]		

↑ = increase, ↓ = decrease, — = tested, but no effect, ~ = unclear effect
Abbrevations: γ-guanidinobutyric Acid = GBA, β-Guanidinopropionic Acid = GPA, Uric acid = UA, Trimethylamine-N-oxide = TMAO, Symmetric Dimethylarginine = SDMA, Asymmetric Dimethylarginine = ADMA, Noradrenalin = NA, Monomethylamine = MMA, Methylguanidine = MG, Guanidino succinic acid = GSA, Guanidino acetic acid = GAA, Guanidine = G

Overall, information on the impact of uremic toxins on the process of vascular calcification, in all its aspects, is fragmentary (**Figure 1**) and by large incomplete; e.g., for none of the URM, information was available on the impact of either release or composition of extracellular vesicles released by VSMC, which recently gained attention as modifiers of the VC process[283].

Challenges in uremic toxin – VC research

Experimental heterogeneity hampers comparison of data. Primary human, bovine, mouse and rat VSMCs as well as cell lines have been used by different investigators. Interspecies differences may account at least partly for inconsistent findings. VSMC derived from different topographic locations of the vascular tree may differ in their physiology and pathophysiology[284]. In addition, experimental conditions diverge substantially and may introduce substantial heterogeneity with major impact on the outcome. As an example, URM are tested as standalone or in combination with either Ca^{2+} or Pi. Also, for Ca^{2+} or Pi, concentration, donor (e.g., β-Glycerophosphate vs. Na_2HPO_4), exposure time and the use of foetal bovine serum (FBS) are subject to differences. A proposal for standardization has recently been put forward, however consensus has not been reached yet[285]. Additionally, the concentration of the URMs to which the VSMC are exposed matters and should ideally reflect/mimic the clinical situation. The European uremic toxin work group (EUTox) published recommendations on handling and use of URM which serves as guideline. In this respect, especially the protein bound uremic toxins deserve attention since guidelines recommend to add human serum albumin at the average uremic concentration of 35 g/l to any test system not containing protein[286]. This recommendation assumes that only the free fraction of uremic toxins exerts an effect. However, none of the publications included in this review was performed in a protein free system but used FBS in varying concentrations containing an untested concentration of protein. Using FBS free conditions in a calcification experiment remains challenging but might be desirable to overcome this limitation. FBS also contains unknown substances and factors known to influence calcification, like Fetuin-A[287,288]. In lieu of FBS free conditions, reporting of medium protein concentrations could be an alternative and to assess the effect of the free URM fraction and foster reproducibility.

As discussed earlier, URM may affect the process of calcification also by indirectly targeting VSMC. For instance, urea and thiocyanate serve as substrates for carbamylation of LDL and proteins[289,290], an event that has recently been identified as a key driver of VC in CKD[291]. Furthermore, the multifaceted effects of especially cytokines and hormones might lead to effects relevant for VC which cannot be assessed by looking at VSMC alone e.g., PTH (**Table 2**). The pleiotropic endocrine nature of hormones may not allow a credible conclusion by examining VSMC alone. Therefore, even though a direct impact of certain URM on VSMC calcification has not been established, it might not render them obsolete. Reviewing the indirect effects of URM on VSMC calcification was beyond the scope of this review. However, the above underlines the need for more research in advanced models, comprised of *in vitro* co-cultures and *in vivo* experiments.

Literature also inheres a considerable inconsistency in effects of URM on VC between experimental and clinical studies. Biomarker assays only reflect a snapshot in time and neglect the slow progressive nature of CKD[292] and its metabolic complications, including VC. Furthermore, the relevance of systemic URM levels for the vascular microenvironment remains a concern.

The conflicting results obtained in clinical and *in vitro* studies preclude strong conclusions. Furthermore, consistency of the findings is often limited. Indeed, 15 out of

26 URM have been tested only once *in vitro* and thus lack independent confirmation. Additionally, we found inconsistencies between clinical studies investigating the same URM. Both case-mix and variable residual confounding may account for these inconsistencies. Furthermore, clinical studies can only indicate correlation and not prove causation. In this context also correction for confounding factors might be challenging giving the multifaceted nature of CKD and the huge body of yet explored influences of the URM. Moreover, the Bradford Hill criteria have not been fulfilled by any of the solutes, which underscores that current evidence is insufficient to establish a reliable cause-effect relationship, between any single URM and VC. Thus, future recommendations should be based on *in vitro* and *in vivo* preclinical and clinical research to provide impactful decisions for the advancement of quality of life for CRS patients.

Uremic serum and Pi induces phenotypic modulation and calcification of VSMC differently, thereby underscoring the importance of the uremic toxins as contributors to VC[23]. However, the complexity of the composition of uremic serum and the fact that 26 different molecules have been linked to VC already, with many more having yet unclear impact (**Figure 1**), make it unlikely that one single substance can be identified as the "holy grail". It is more probable that the effects leading to increased VC are attributable to an orchestra of factors questioning the effects found by testing individual molecules as being representative for the situation *in situ* (uremic toxin storm). It is more likely that different toxins have synergistic effects, however, might also partially antagonize each other or prime the vasculature for other toxins to be effective, without exerting an effect on its own. However, these possible ties between uremic toxins remain yet unstudied.

These findings call for a more standardized and systematic approach in order to ensure relevance of results for the therapeutic setting and to facilitate comparability between studies.

Treatment strategies

Although there is currently no approved therapy to stop, attenuate or regress VC there are several appealing approaches[293,294]. These may be categorized as: 1) direct pharmacological inhibition of hydroxyapatite (HA) growth, 2) increasing level or activity of anti-calcifying factors, 3) reducing pro-calcifying factors. Multiple compounds for direct pharmacological inhibition of HA crystal growth are currently in development e.g., sodium thiosulfate, myo-inositol hexaphosphate or bisphosphonates. SNF472, a myo-inositol hexaphosphate formulation, was recently reported to significantly reduce progression of VC in HD-patients in a phase 2b trial[295]. Supplementation with vitamin K is under investigation to increase activity of anti-calcifying vitamin K dependent proteins. Vitamin K is an unequivocal cofactor for the gamma-glutamyl carboxylation of protein bound glutamate residues. This carboxylation is pivotal to activate the endogenous VC inhibitor matrix Gla-protein (MGP)[296]. Several clinical trials are currently underway to examine the potential of vitamin K supplementation to hold or reduce VC[293]. Additionally, also certain URM can be protective against VC (**Table 2-4**), yet this has not been studied in detail. The newly emerging field of senolytics as medication might also be a promising new treatment

strategy for reducing VC[297]. Renal replacement therapy as a strategy to reduce pro-calcifying factors has its limitations, e.g., the removal of PBURM by conventional haemodialysis is poor. As many vasotoxic compounds originate from gut microbial metabolism, the large intestine is increasingly recognized as a promising target of therapy.

Future perspectives

The composition of the uremic serum differs vastly among CDK patients. Thus, although CKD patients suffer from VC, the underlying cause for VC development may differ. In order to progress towards more personalized CKD and CRS treatment strategies a better understanding of the individual uremic toxin profile is needed. This might pave the way for the early identification of patients with a higher risk for VC development and accelerated progression. Thus far, comprehensive URM profiles of individual patients are not available and information stem from measurements of a limited number of URM. Moreover, since the list of URM is constantly updated, it is difficult to define all molecules relevant in the context of VC[26]. A comprehensive understanding of the effect of individual URM is needed to identify and reduce the burden of pro-calcifying factors caused by the entire pool of URM. Since testing each URM in depth is nearly impossible, evaluating URM by classifying and clustering them to determine potential relevance will pave the way for targeted treatment strategies. We strongly put forward a structured, systematic, and unbiased approach in assessing effects of URM on VSMC being most effective in revealing detrimental or beneficial effects on VC (**Figure 2**). Clustering of known URM based on their chemical similarity combined with proven effects might be an attractive possibility. As such, systematic screening by testing representative URM of each cluster will provide knowledge on similar URM in that cluster. Moreover, it could be worthwhile identifying common signalling pathways activated by multiple URM. This would open new avenues for counteracting the effects of URM, with very different origins and challenges for their removal. Alternatively, segregation of the overwhelming uremic toxin "storm" into manageable units might present an idea worth exploring. Such units may include the CKD-mineral bone disorder or rebalancing the inflammatory profile. A newly emerging link between intestinal microbiota and VC could also present an accessible unit[298]. A considerable fraction of the uremic toxins have been found to be gut derived[26] some of which have already been linked to VC including TMAO, IS and pCS (**Table 3 & 4**). TMAO has further been found to be indicative of intestinal dysbiosis[299], further supporting the idea of a intestine – vascular axis in CRS pathology. Therefore, a more detailed understanding of the origin of each URM might identify novel intervention sites. Moreover, senescence as feature of EVA, which has been linked to VC, should receive more attention during the design of future experiments. Extracellular vesicles are recognized as contributor to VC development, and increased vesicle secretion is associated with VSMC phenotypic switching[300,301]. Furthermore, circulating extracellular vesicles isolated from CDK patients increase VSMC osteogenic-phenotypic switching and VC[302]. Therefore, we strongly advocate for acknowledging extracellular vesicle release as process indirectly linked VC, which should receive attention also in CRS centred research.

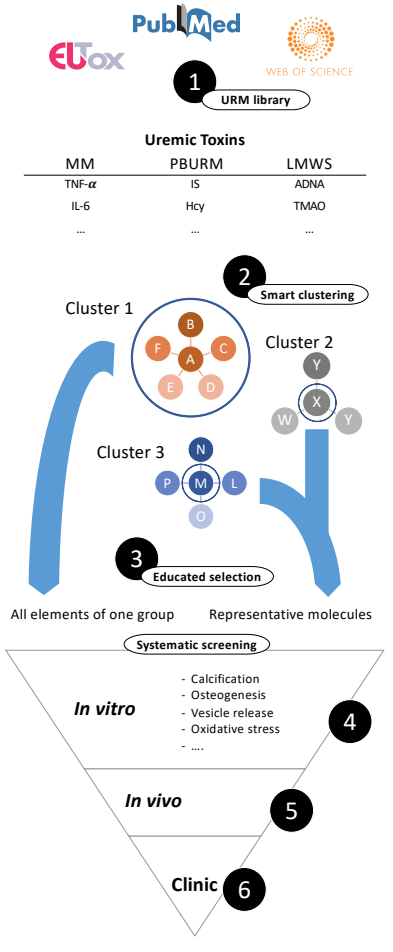


Figure 2 Outline of a systematic screening approach for URM. 1. URM need to be collected and organized in a library, possibly starting with the EuTox database, extended by recent discoveries. 2. URM need to be logically sorted into smaller, accessible groups. Clustering strategies could be based on chemical similarity, origin, or others. 3. A selection for actual testing needs to be applied which could be by, educated guessing based on known effects, testing the representative molecule from each cluster to identify relevant subgroups or by testing cluster wise. 4. Selected molecules are tested systematically in on one or more cell types for relevant effects like calcification, inflammation, apoptosis, etc. in vitro, 5. if successful in vivo and 6. on a clinical level.

Summary

In this review, we included literature investigating URM and VSMC calcification *in vitro*, *in vivo* and on a clinical level. A clear definition of URM being detrimental, harmless or beneficial with respect to vascular calcification is currently lacking. Furthermore, we identify many gaps in URM research related to VSMC. A large fraction of URM has not been investigated with respect to their effects on VSMC calcification. However, some are linked to processes indirectly associated with VSMC calcification, such as ROS and inflammation. We also include potential novel avenues of VSMC driven calcification, such as extracellular vesicles, senescence and the influence of the microbiome. Effects of URM are often reported once in publications, and experimental conditions often lack an adequate degree of standardization and are not designed in relation to CRS. The composition of uremic serum is divers and may differ

vastly between patients. Moreover, the influence of many uremic retention molecules on VSMC and VC, remain largely obscure and the underlying effects poorly understood. Better understanding of the effects of URM on the vasculature could be the first step towards reducing VC and a personalized CKD treatment strategy. This underscores the need for deep patient serum profiling to understand the underlying cause and consequence relationship between URM and clinical manifestations. In conclusion, our narrative overview puts forward the hypothesis to test effects of URM on VSMC in relation to VC more systematically and advocates for more CRS centred research.

Search strategy

The European uremic toxin work group has created an extensive database[303] containing many relevant uremic retention molecules, which served as the basis for the literature research and has been supplemented by compounds from two further publications[28,304]. The search was performed in the Medline library accessed via Pubmed using the search term " -molecule- AND (smooth muscle cell OR vascular smooth muscle cell OR VSMC OR SMC) AND (calcif* OR inflam* OR oxidative stress OR vesicle OR proliferation OR migration OR apoptosis OR necrosis OR senescence OR osteo* OR chondro* OR monocyte OR macrophage OR athero*)." If no article could be found with this strategy, at least one alternative name for this molecule has been tried in the same search term. If more than 160 articles have been found for a single molecule, the articles were sorted by "best match", and the first 160 hits, as well as reviews from the past 10 years were included. Furthermore, if publications concerning *in vitro* and/or *in vivo* results on calcification could be found, the search was extended for only these molecules to identify clinical cohort studies that relate the respective molecule to vascular calcification in a clinical setting by using the search term " -molecule- AND calcification AND cohort" as well as " -molecule- AND calcification".

Supplementary Materials:

The following are available online at www.mdpi.com/xxx/s1, Table S1: Full table of the included MM and their influence on calcification, as well as their effects on VC related processes in VSMC. Table S2: Full table of the included PURM and their influence on calcification, as well as their effects on VC related processes in VSMC. Table S3: Full table of the included LMWS and their influence on calcification, as well as their effects on VC related processes in VSMC

Funding:

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 764474.

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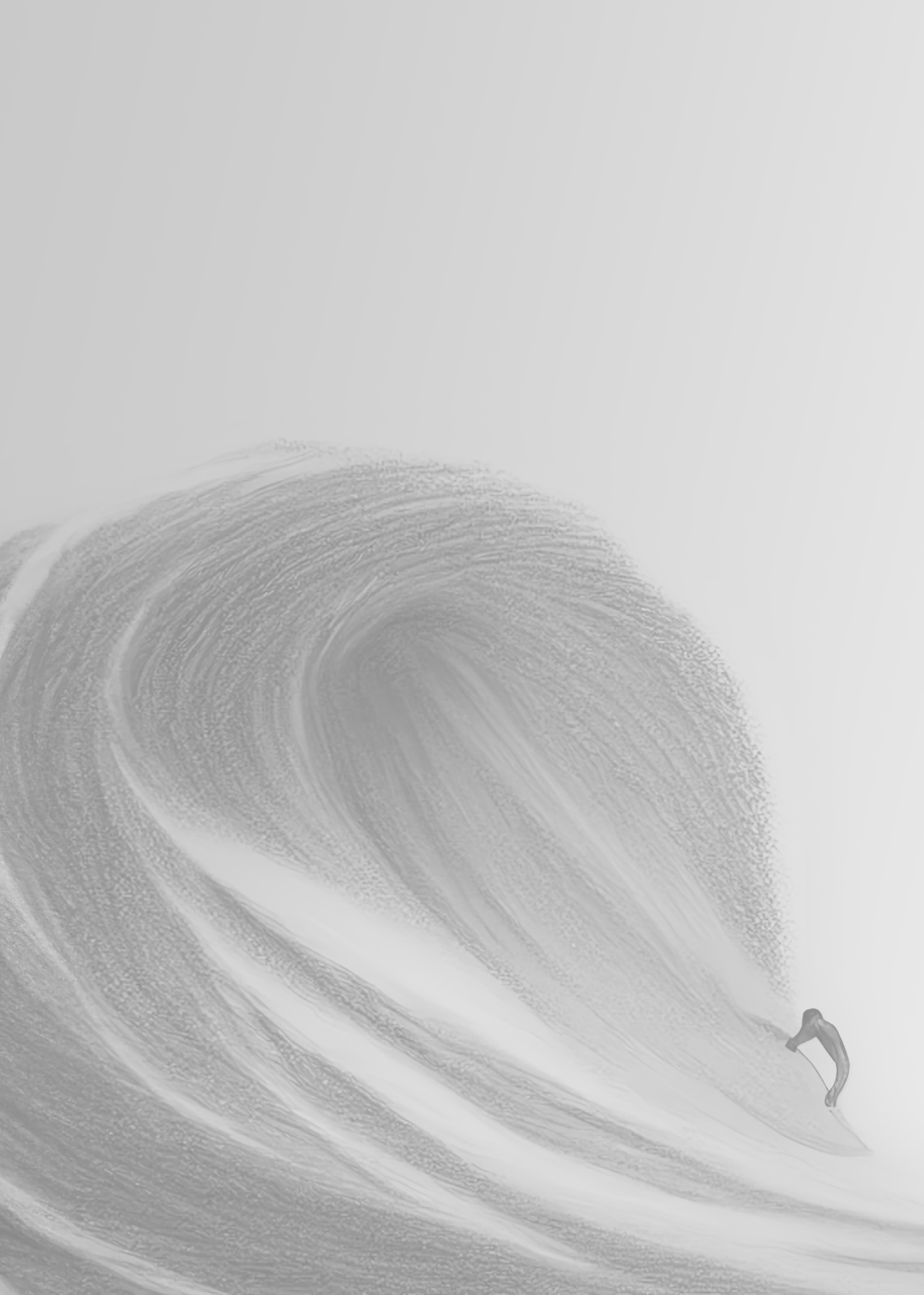
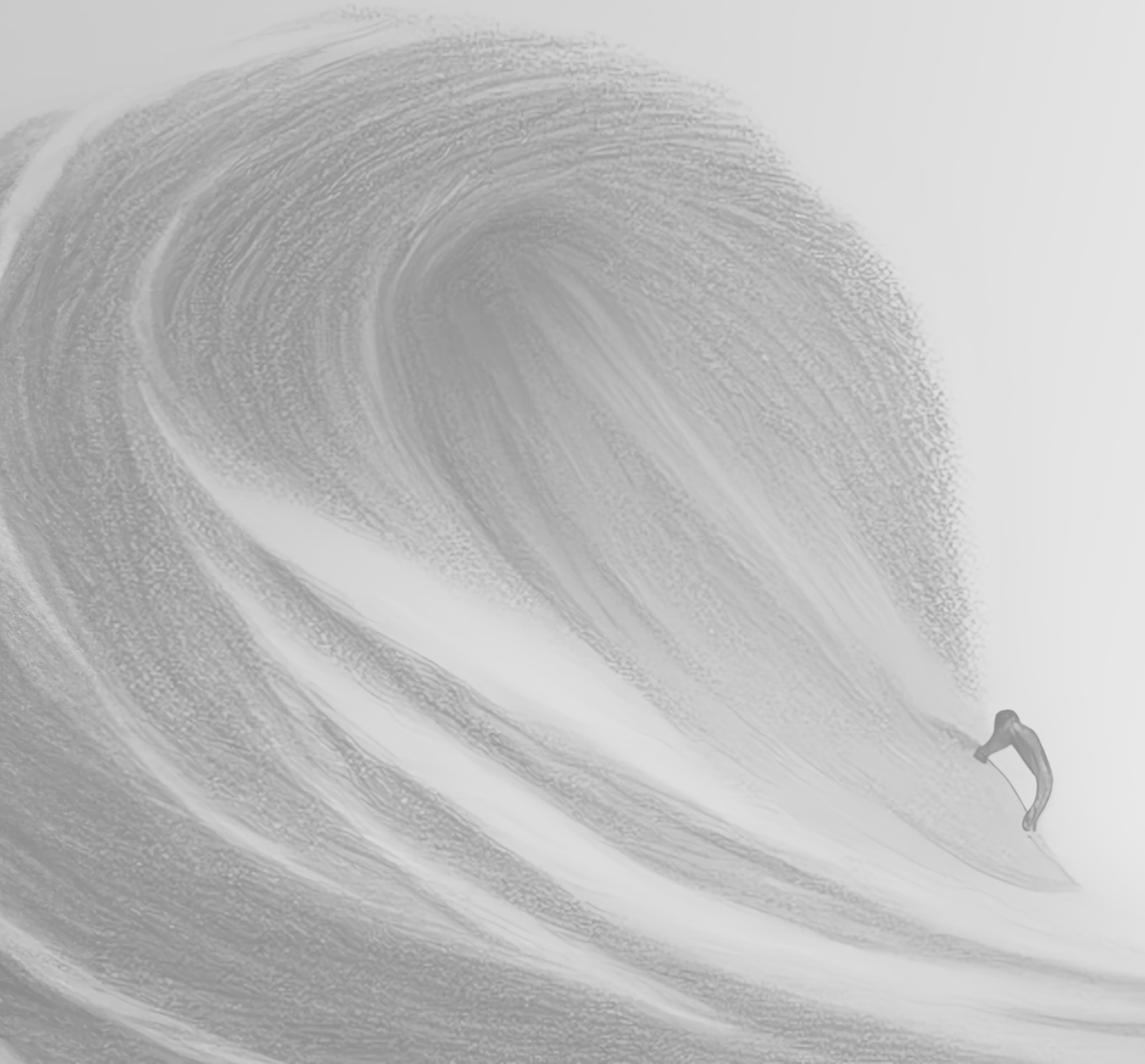


Chapter 5

Utilizing iPSC derived human vascular smooth muscle cells to study uremia related vascular calcification

Nikolas Rapp, Cengiz Akbulut, Malgorzata Furmanik, Rafael Kramann, Armand Jaminon, Leon J. Schurgers



Chapter 6

Hepatic and Vascular Vitamin K Status in Patients with High Cardiovascular Risk

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Nutrients 2021, *13*(10), 3490

Abstract

Vitamin K dependent proteins (VKDP), such as hepatic coagulation factors and vascular matrix Gla-protein (MGP), play key roles in maintaining physiological functions. Vitamin K deficiency results in inactive VKDP and is strongly linked to vascular calcification (VC), one of the major risk factors for cardiovascular morbidity and mortality. In this study we investigated how two vitamin K surrogate markers, dephosphorylated-undercarboxylated MGP (dp-ucMGP) and protein induced by vitamin K absence II (PIVKA-II) reflect vitamin K status in patients on hemodialysis or with calcific uremic arteriolopathy (CUA) and patients with atrial fibrillation or aortic valve stenosis. By inter- and intra-cohort comparisons we assessed the influence of vitamin K antagonist (VKA) use, vitamin K supplementation and disease aetiology on vitamin K status, as well as the correlation between both markers. Overall, VKA therapy was associated with 8.5-fold higher PIVKA-II (0.25 to 2.03 AU/ml) and 3-fold higher dp-ucMGP (843 to 2642 pM) levels. In the absence of VKA use, non-renal patients with established VC have dp-ucMGP levels similar to controls (460 vs. 380 pM), while in HD and CUA patients' levels were strongly elevated (977 pM). Vitamin K supplementation significantly reduced dp-ucMGP levels within 12 month (440 to 221 pM). Overall, PIVKA-II and dp-ucMGP showed only weak correlation ($r^2 \leq 0.26$) and distinct distribution pattern in renal and non-renal patients. In conclusion, VKA use exacerbated vitamin K deficiency across all aetiologies, while vitamin K supplementation resulted in a vascular VKDP status better than in the general population. Weak correlation of vitamin K biomarkers calls for thoughtful selection lead by the research question. Vitamin K status in non-renal deficient patients was not anomalous and may question the role of vitamin K deficiency in the pathogenesis of VC in these patients.

Introduction

The unequivocal role of vitamin K is to mediate the posttranslational gamma-glutamyl-carboxylation of specific, protein bound glutamate (Glu) residues into gamma-carboxyglutamate (Gla) residues. Hence, proteins carrying these residues are called vitamin K-dependent proteins (VKDP). VKDP play a well-known major role in the coagulation system (factors II, VII, IX, X). Another prototypic VKDP is MGP. MGP represents a major, local anti-calcification factor in the vascular wall and thus, protects against vascular calcification (VC)[1]. VKDP rely upon sufficient vitamin K for full biological activity[2–4]. The undercarboxylated fraction of VKDP can be specifically measured in the blood, which as a marker reflects functional vitamin K status more robustly than direct vitamin K measurements[5]. However, measuring a vitamin K surrogate marker cannot determine the status of different vitamin K species, such as phylloquinone and menaquinones, because both subtypes can be used as cofactor and are bioactive and because after absorption vitamin K can be converted into menaquinone-4[6]. While menaquinones are the prevalent active form in extra hepatic tissue, and hence the vessel wall[7], there is currently no data available if any of the surrogate markers shows correlation with a specific vitamin K subtype. Thus, using vitamin K-dependent proteins as surrogate marker reflects all vitamin K forms. Synthesized in different cell types within the body, the undercarboxylated fractions of some VKPD have been linked to the vitamin K status of certain tissues. dp-ucMGP has been described as a parameter to assess vascular vitamin K status, while (PIVKA-II) reflects hepatic vitamin K status[5]. Increasing the supplementation with vitamin K substantially increases the carboxylation status of these marker proteins,[8,9] while application of vitamin K antagonists (VKA) or oral anticoagulant therapy (OAT) interferes with the carboxylation process of Gla-proteins[10]. Thus dp-ucMGP and PIVKA-II can be used to assess the efficacy of vitamin K supplementation or the impact of VKA therapy[4,11].

We investigated plasma samples from patients with four different disease backgrounds. Chronic kidney disease (CKD) patients on hemodialysis (HD), CKD patients undergoing HD with calcific uremic arteriolopathy (CUA), patients with atrial fibrillation (AF) and patients with aortic valve calcification (AVC). All groups have either established or are at increased risk for cardiovascular (CV) disease and additionally, have confirmed or are at high risk for VC. Considering the increasingly acknowledged role of vitamin K in CV health the aim of the present study was threefold. Firstly, we sought to determine whether vitamin K levels differ between patients representing a wide range of CV disease burden and CKD by the means of dp-ucMGP and PIVKA-II, and how each group compares to the general population. Secondly, we investigated if both surrogate markers reflect the vitamin K status in a similar manner. Thirdly, we studied how VKA treatment or vitamin K supplementation affected vitamin K status in our cohorts.

Materials and Methods

Design and Patients

We performed a post-hoc analysis on data collected in the frame of 4 different cohort studies including: HD patients scheduled for kidney transplantation

(NCT01886950, Leuven, Belgium); HD patients with CUA (German calciphylaxis registry, Aachen[12]); patients with aortic valve calcification enrolled in a randomized controlled trial to placebo versus vitamin K1 supplementation ("Vitamin K Supplementation for Inhibition of the Progress in Aortic Valve Calcification", NCT00785109[8]). And patients with AF randomized to rivaroxaban versus phenprocoumon ("Rivaroxaban Compared to Vitamin K Antagonist Upon Development of Cardiovascular Calcification", NCT02066662, Aachen[4]).

Hemodialysis patients (HD)

40 HD patients, of whom 20 were treated with a VKA, were recruited from an ongoing, prospective observational study investigating outcomes after kidney transplantation. The study has been registered as clinical trial with the unique identifier NCT01886950. All patients eligible for renal transplantation were included, excluding only bisphosphonate users. Blood samples were collected at the time of the call for transplantation (random, non-fasting) and stored at −80˚C until use. Demographic characteristics and data on immunosuppressive and mineral metabolism therapy were retrieved from electronic files

Hemodialysis patients with calcific uremic arteriolopathy (CUA)

Thirty-two CUA patients were randomly selected from the German calciphylaxis registry. Detailed results of the entire registry cohort were previously published[12]. In brief, the German calciphylaxis registry is a prospective, nation-wide, internet-based registry in which treating physicians can notify cases of CUA. Confirmation of the diagnosis is based on plausibility check of the incoming data about medical history, clinical aspects and picture documentation when available. Plausibility check in cases of doubt includes verification discussion between both parties. The registry team at the Aachen University Hospital requested full blood, plasma, and serum sampling of the patients according to standard procedures and asked for immediate freezing at the peripheral study site. Long-term storage was done at −80°C immediately after arrival at the central biobank at the RWTH Aachen University Hospital. We analysed stored blood samples of patients on long-term HD prior to the diagnosis of CUA. Patients were divided into two groups according to VKA treatment longer than 6 months prior to the diagnosis (n = 14) or no VKA prior to the diagnosis of CUA (n = 18).

Patients with aortic valve calcification (AVC)

We analysed stored blood samples of a previously published randomized controlled open-label study about the influence of 2 mg vitamin K1 (daily, orally administered phytomenadion) application per day over 12 months compared to placebo in AVC patients. Data of an intention-to-treat cohort were previously published and the study has been registered as clinical trial with the unique identifier NCT00785109[8]. Exclusion criteria for this mono-centre, open, controlled, randomized phase I study were, amongst others, chronic kidney disease, recent additional vitamin K intake, oral anticoagulation with vitamin K antagonists, venous thrombosis, or embolization of lung arteria. Prescence of aortic valve calcification was confirmed via echocardiography. For the present analysis we included all patients for whom stored blood samples at baseline

and at 12 months were available. These patients had no or only mild chronic kidney disease (**Table 1**).

Patients with atrial fibrillation (AF)

We selected randomly 30 patients from a randomized, prospective interventional study in which the influence of VKA upon coronary artery calcification in patients with AF or deep vein thrombosis / pulmonary embolism is evaluated compared to rivaroxaban, registered as clinical trial with the unique identifier NCT02066662. Main inclusion criteria were existent coronary or valvular calcification, or both and Agatston score > 50 in at least one location and need for long term anti-coagulant therapy. Relevant exclusion criteria were Liver disease with coagulopathy or other bleeding disorders including cirrhotic patients with Child Pugh B and C, clinically significant active bleeding, acute gastrointestinal disease. Coronary artery calcification and AVC were assessed by multi-slice spiral computed tomography scanning. Blood samples of patients after 12 months of randomized treatment were selected. These patients had no or only mild chronic kidney disease (**Table 1**).

Individuals from the general population (control)

The present study included individuals of the LifeLines-MINUTHE (MIcroNUTrients and Health disparities in Elderly) sub cohort of the LifeLines Cohort Study. This sub cohort consists of 1605 individuals aged between 60 and 75 years, with available plasma, serum, and 24-h urine samples from the biobank of the LifeLines cohort. Fasting blood samples are processed on the day of collection and stored at −80 °C. The 1605 individuals comprised 400 men and 403 women with low socioeconomic status (SES) and 402 men and 400 women with high SES. Since education is more differentiating than income in the Dutch population, the classification of SES was based on educational status. Low SES was defined as never been to school or elementary school only, or completed lower vocational or secondary schooling; high SES was defined as completed higher vocational schooling or education.[13–15] Of this subpopulation 65 individuals, age matched to disease cohorts, were randomly selected for the present study.

Biochemistry

Blood samples were collected in either potassium-ethylenediaminetetraacetic acid (EDTA) or serum vacutainers, immediately centrifuged and stored in aliquots at -80°C. Circulating dp-ucMGP levels were determined in EDTA plasma using the commercially available IVD CE marked chemiluminescent InaKif MGP assay on the IDS-iSYS system (IDS, Boldon, UK). 50 µL of patient sample or calibrators are incubated with magnetic particles coated with murine monoclonal dpMGP antibody, an acridinium labelled murine monoclonal ucMGP antibody and assay buffer. The magnetic particles are captured using a magnet and a wash step performed to remove any unbound analyte. Trigger reagents are added, the resulting light emitted by the acridinium label is directly proportional to the concentration of dp-ucMGP in the sample. The within-run and total precision of this assay were 0.8 – 6.2% and 3.0 – 8.2%, respectively. The assay measuring range is between 300 – 12,000 pmol/L and was found to be linear up to 11,651 pmol/L. dp-ucMGP values below 300 pmol/L are considered to be in the normal healthy

range. Assays were performed in a single run by Coagulation Profile BV, Maastricht, the Netherlands.

Circulating PIVKA-II (ucFII) levels were measured using a conformation-specific monoclonal antibody in an ELISA-based assay[16]. Results are expressed as arbitrary units per litre (AU/l) because in states of vitamin K deficiency circulating ucFII may comprise multiple forms of partially carboxylated FII and neither their relative abundance in serum nor their relative affinity for the antibody is known. Using electrophoretic techniques 1 AU is equivalent to 1 μg of purified ucFII[16]. The detection limit was 0.15 AU/ml ucFII in serum. Other routine mineral metabolism parameters were extracted from the (electronic) patient files. Parathyroid hormone levels are reported as times upper normal limit (UNL), to account for inter assay variability.

Statistical analyses

Prior to analysis data has been square root transformed. Plasma dp-ucMGP concentration as well as PIVKA-II plasma levels are compared using ordinary one-way ANOVA with Tukey post-hoc test for multiple comparisons (no VKA and VKA groups of the CUA, HD and AF cohorts and comparison between all diseased groups and the control group). The Wilcoxon matched pairs signed rank test has been used to compare the paired AVC study groups. The Kruskal Wallis test, followed by Dunn's post hoc correction for multiple comparisons was used to compare the AVC groups to the control group. Ordinary one-way ANOVA has been used to analyse differences in the clinical characteristics for continuous variables and the Chi Square test for proportional data. To assess the correlation between dp-ucMGP and PIVKA-II calculation has been performed as Pearson correlation. Significance levels are displayed as: Ns =$p > 0.05$; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$. In **Figure 2**, # also indicates a significance level of $p \leq 0.0001$ and $ indicates no significant difference.

Results

In total, 40 chronic HD patients (20 VKA / 20 no VKA), 32 CUA patients (14 VKA / 18 no VKA), 30 AF patients (14 VKA / 16 no VKA) and 66 AVC patients (34 no vitamin K / 32 vitamin K) were included in this study. The patients' clinical characteristics are shown in **Table 1**. There was a significant difference in age between the cohorts ($p \leq 0.0001$), with the HD patients being younger. Furthermore, PTH ($p \leq 0.0001$), serum calcium ($p \leq 0.01$) and serum phosphate ($p \leq 0.0001$) levels showed significant differences between the HD and CUA group (**Table 1**).

Table 1. Clinical characteristics.

	HD		CUA		AF		AVC		
	VKA No	VKA Yes	VKA No	VKA Yes	VKA No	VKA Yes	K1 No	K1 Yes	*p* value
7	57 ± 13	57 ± 13	69 ± 17	72 ± 14	71 ± 11	69 ± 9	69 ± 8	69 ± 10	≤ 0.0001
male %	85	70	69	63	65	67	73	79	≤ 0.01
PTH xUNL	5.57 ± 2,22	3.14 ± 2.22	2.40 ± 2.05	2.08 ± 3.23	nm	nm	nm	nm	≤ 0.0001
Calcium mmol/L	2.3 ± 0.1	2.3 ± 0.1	2.2 ± 0.1	2.2 ± 0.2	2.4 ± 0.1	2.3 ± 0.1	2.3 ± 0.2	2.4 ± 0.2	≤ 0.01
Phosphate mmol/L	1.52 ± 0.61	1.42 ± 0.52	0.5 ± 0,13	0.53 ± 0.14	nm	nm	nm	nm	≤ 0.0001
month on dialysis	43 ± 32	49 ± 46	46 ± 17	30 ± 21	not applicable	not applicable	not applicable	not applicable	0.39
hypertension %	75	60	91	89	74	78	78	71	≤ 0.0001
Diabetes mellitus %	20	35	56	44	23	25	29	36	≤ 0.0001
eGFR ml/min/1.73²	all dialysis	all dialysis	all dialysis	all dialysis	63 ± 12	71 ± 15	71 ± 14	69 ± 18	0.36

To gain an overview of the vitamin K status, all groups were separated by PIVKA-II and dp-ucMGP measurements and ranked by their respective median values. Over all groups, dp-ucMGP levels are higher in patients using VKA, with HD and CUA patients showing higher levels than AF patients. AVC patients supplemented with vitamin K1 showed the lowest dp-ucMGP plasma levels (**Figure 1A**). For PIVKA-II the rank was differently. AF - VKA patients showed higher PIVKA-II levels. CUA patients have lower PIVKA-II plasma levels than AF and HD patients. AVC patients supplemented with vitamin K1 showed the lowest PIVKA-II levels. (**Figure 1B**).

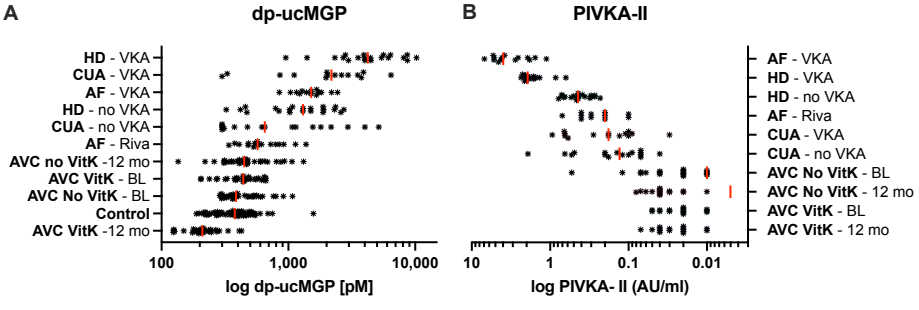

Figure 1. dp-ucMGP and PIVKA-II measurements of all cohorts sorted by median values from high (top) to low (bottom). Red line indicates the median. A: VKA treated patients showed higher dp-ucMGP compared to untreated patients of the same cohort. AVC dp-ucMGP levels were lowest with Vitamin K supplemented patients below the control group. B: PIVKA-II ranks differ from the dp-ucMGP ranks. AF and HD patients showed highest PIVKA-II levels, followed by CUA and AVC.

Compared to the control group, HD and CUA groups show substantially higher dp-ucMGP levels, regardless of VKA use, indicating a strong functional vitamin K deficiency in the dialysis patients. AF patients exhibited values slightly higher than controls, but levels were much higher with VKA use. None of the AVC groups at baseline did show dp-ucMGP values significantly different from the control group. Hence, AF and AVC patients might not by default be characterized by insufficient vitamin K status (**Figure 2**, **Supplemental table 1**).

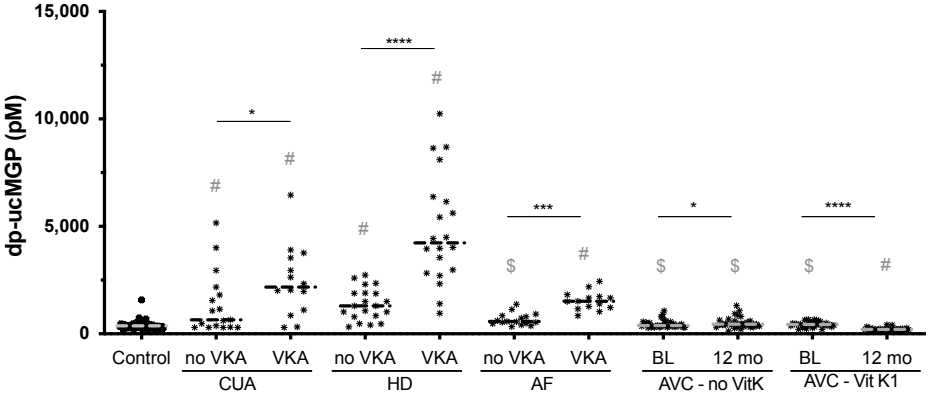

Figure 2. dp-ucMGP measurements of all disease cohorts and a control group. VKA use exacerbated dp-ucMGP status in all cohorts, while vitamin K supplementation ameliorated the dp-ucMGP status beyond healthy controls. Compared to controls, all HD and CUA groups had significantly increased dp-ucMGP levels, while in the AF cohort only the VKA group was increased. Dash indicates the median. Significance levels: * = $p \leq 0.05$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$. # and $ indicate the comparison between each disease group and the control group. # Indicates a significance level of $p \leq 0.0001$ and $ indicates no significant difference between the respective group and the control group.

Next, we compared VKA users against non-users of each disease. dp-ucMGP levels of VKA treated patients were significantly higher for the CUA ($p \leq 0.0001$, 1.8-fold higher), HD ($p \leq 0.0001$; 3.5-fold higher) and AF ($p \leq 0.001$, 2.3-fold higher) cohorts. PIVKA-II levels of VKA treated patients were significantly higher for the HD ($p \leq 0.0001$, 3.9-fold higher) and AF ($p \leq 0.0001$; 16.6-fold higher) cohorts only. In the AVC cohort, patients not receiving vitamin K1 supplements did show significantly higher dp-ucMGP levels after 12 month ($p \leq 0.05$), while in patients receiving the supplements levels were significantly lower ($p \leq 0.0001$). PIVKA-II levels, conversely, remained stable in either AVC group. (**Figure 2 & 3, Supplemental table 2**).

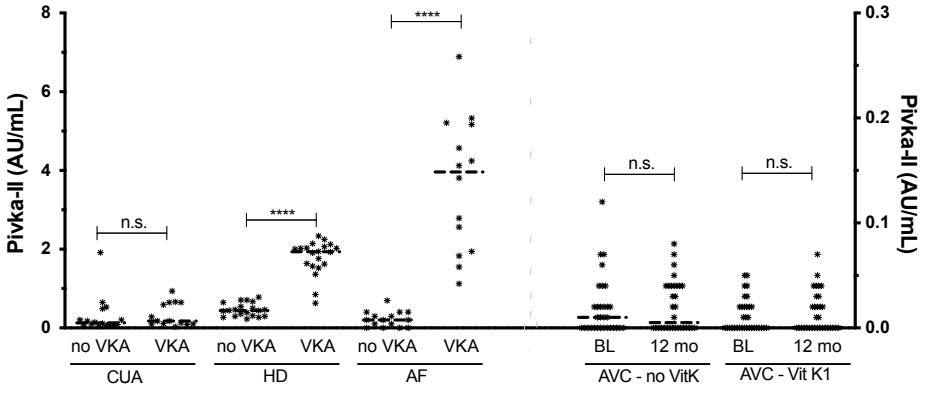

Figure 3. PIVKA-II measurements of all cohorts. HD and AF patients had higher PIVKA-II levels with VKA use. CUA and AVC cohorts did not change with VKA or vitamin K supplementation, respectively. CUA, HD and AF values correspond to the left Y-axis, AVC values correspond to the right Y-axis. The dash indicates the median. Significance levels: n.s. = $p > 0.05$; **** = $p \leq 0.0001$.

Furthermore, the vitamin K status of CUA and HD patients was compared. Overall dp-ucMGP levels were similar, with levels being modestly higher only in HD patients treated with VKA. HD patients also showed over all higher PIVKA-II concentrations, an observation that was not made in patients not receiving VKA (**Supplemental Figure 1**). Finally, we investigated the correlation between dp-ucMGP and PIVKA-II levels in the various cohorts. Correlation between both markers was generally very weak ($r^2 \leq 0.26$, **Figure 4A**). HD, CUA, and AF patients showed a distinct distribution pattern of PIVKA-II and dp-ucMGP. HD and CUA cohorts revealed a pattern skewed towards higher dp-ucMGP, while the AF cohort showed a pattern skewed towards higher PIKVA-II, especially in those treated with VKA (**Figure 4B**). Moreover, pooling the measurements from all patients of the AF, CUA and HD cohorts did not reveal a good correlation between dp-ucMGP and PIVKA-II neither on nor off VKA.

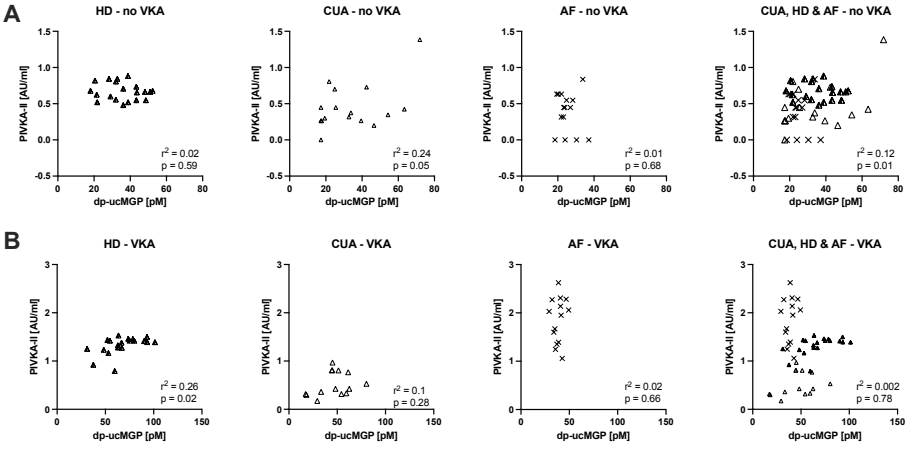

Figure 4. Weak correlations between PIVKA-II and dp-ucMGP of all patients of the CUA, HD and AF cohorts, separated into panel A) without VKA treatment ($r^2 \leq 0.24$) and panel B) with VKA treatment ($r^2 \leq 0.26$). Symbols in the right most graph correspond to the respective patient group.

Discussion

Our study compares for the first-time indicators of vitamin K status in various diseases known for their cardiovascular disease burden. Moreover, assessing the influence of either VKA treatment or vitamin K substitution upon markers of vitamin K status is a unique feature of the present study. Although a detailed quantification of the amount of cardiovascular calcification was not available in these patients (e.g., via autopsy or CT scanning) our data nevertheless add substantially to our understanding how vitamin K status, antagonist therapy and supplementation might associate with CV calcification in humans.

HD patients are known to have increased risk for vascular calcifications and cardiovascular mortality[17]. Furthermore, the plasma vitamin K status is known to deteriorate with CKD progression[18] and, reflected by dp-ucMGP, independently predicts CV calcification burden[19]. Vascular calcifications are a hallmark of CUA, but risk factors for the development are still ill defined. Vitamin K status and vitamin K antagonist treatment as anti-coagulation therapy have been suspected to increase the odds for the development of CUA, but study results have been inconsistent[20].

Somewhat surprising, the vitamin K status in dialysis patients presenting with CUA was not worse compared to counterparts free of the devastating complication. This observation conflicts with a study demonstrating a significantly reduced relative cMGP concentration and a higher prevalence of vitamin K deficiency reflected by PIVKA-II in CUA patients[21]. While in the present study vitamin K deficiency is not more severe in CUA cases than HD patients, our findings do not oppose a contribution of VKA use to the development of CUA per se. The exact mechanism leading to calcific and thrombotic events in subcutaneous arterioles is still unknown. Next to the effect of inhibiting MGP

carboxylation, VKAs have additional detrimental effects on human endothelial cells (hEC) and vascular smooth muscle cells (hVSMC) that could fuel CUA development. VKA treatment decreases the carboxylation and secretion of protein S, a coagulation inhibitor also synthesised by hVSMC and hEC[22,23]. Furthermore, VKAs induce hVSMC calcification via endoplasmic reticulum stress, a process independent of Gla-protein activation[24]. The precise role of how the coagulation system is involved in CUA development is to date unclear. However, occluding thrombus formation in the dermal arterioles with subsequent hypoxia and skin necrosis are events known to contribute to CUA pathology[25]. Patients with CUA show a high prevalence of thrombophilia, underscoring the potential importance of congenital and acquired thrombotic propensity, potentially contributing to the pathogenesis of this disease[26,27]. Therefore, an in-depth assessment of the coagulation system in patients with CUA, preferentially prior to initiation of the disease, could potentially yield new treatment strategies for this, to date, difficult to handle complication.

AF patients included in this study were previously diagnosed with either coronary or valvular calcification and were enrolled in an ongoing trial assessing the influence of VKA treatment on calcification progression (NCT02066662). Remarkably, AF patients with calcifications do not exhibit a strong vitamin K deficiency. However, VKA therapy was associated with considerably higher PIVKA-II levels while differences in dp-ucMGP were much lower. This response clearly opposes what was observed in dialysis patients, in whom VKA therapy predominantly results in higher dp-ucMGP levels. This observation fuels the hypothesis that especially vascular vitamin K stores are critically depleted in dialysis patients. The discrepancy might also be partially related to differences in the half-life of MGP (<15h)[28] and PIVKA-II (62h)[29] and the filtration rate of both proteins. MGP can be filtered by either the kidneys or a dialysis membrane, whereas PIVKA-II is mostly retained[30,31]. Thus, kidney function might be related to circulating plasma levels. However, in the vessel wall of CKD patients MGP is expressed 5-fold higher compared to non-CKD controls and since vitamin K supplementation decreases dp-ucMGP levels in CKD patients it indicates a filtration independent effect[32,33]. Recent studies found that vitamin K supplementation in CKD does improve the carboxylation status of VKDP, but without slowing VC progression[33,34]. Vascular calcification in CKD has multiple drivers, including vitamin K deficiency. But other factors like phosphate disbalance[35] and uremic toxins[36] also contribute, and thus mono therapy with vitamin K might not be sufficient to slow the progression of VC. The CKD uniqueness is supported by the contrasting results of the AVC cohort, as well as other non-CDK cohorts, where vitamin K supplementation was capable of lowering dp-ucMGP levels even below levels observed in healthy controls and consequently attenuated progression of AVC[8,37]. Overall, research coverage on the effect of vitamin K supplementation in VC remains unsatisfactory and several other studies are currently under way to build a stronger data foundation[38,39].

In the present study, the correlation between PIVKA-II and dp-ucMGP was weak. This might partially be explained by a limited assay sensitivity for PIVKA-II and the therefor reduced precision while measuring lower concentrations. However, other publications investigating CKD and non-CKD samples also reported very weak[40,41], or weak[42] to moderate[11] correlation, while one time a strong[43] correlation between both

markers was reported. Moreover, also the uncarboxylated fraction of other VKDP such as osteocalcin and Growth-arrest-specific protein-6 correlate poorly with PIVKA-II and dp-ucMGP[40,44]. This might potentially indicate, VKDP may be subject to relevant regulatory influences other than vitamin K status, and isolated interpretation of individual markers might be difficult.

The results of the present study should be interpreted considering its limitations. Data on indication, duration, dose, and target INR of VKA treated patients are not available. Information on phosphate binder treatment and residual renal function are equally lacking. There are however no reasons to assume a systematic bias by these (potential) confounders.

Conclusions

The observation of normal vitamin K status in non-renal patients with established VC (AVC and AF) questions the role of vitamin K in the pathogenesis of VC in these patients. PIVKA-II levels and dp-ucMGP show only weak correlations at best, supporting the thesis that these markers most probably represent different functional vitamin K stores, which moreover are poorly related. VKA therapy was strongly associated with higher dp-ucMGP levels in all cohorts, but without VKA elevated in HD patients only. Vascular vitamin K stores thus are especially compromised in dialysis, rendering these patients susceptible to accelerated VC. Finally, vitamin K status was not inferior in CUA patients compared to HD patients free of this complication. Yet, VKA use might still be a driver of CUA development by influencing coagulation and VC via vitamin K independent mechanisms. Given the design of our study and inherent limitations, our data should be considered hypothesis-generating, but call for additional studies.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki. All studies were approved by the appropriate Institutional Review Board or Ethics Committee. German calciphylaxis registry: approved by University Hospital RWTH Aachen ethical committee (No. 10/024), the AVC trial registered as NCT00785109 by RWTH Aachen Institutional Review Board (No. 165/08). AF trial registered as NCT02066662, approved by RWTH Aachen Institutional Review Board. And the HD trial, registered as NCT01886950, approved by the ethical committee of the University Hospital Leuven. The study collecting data from the general population was approved by the Medical Ethics Committee of the University Medical Center Groningen, the Netherlands.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

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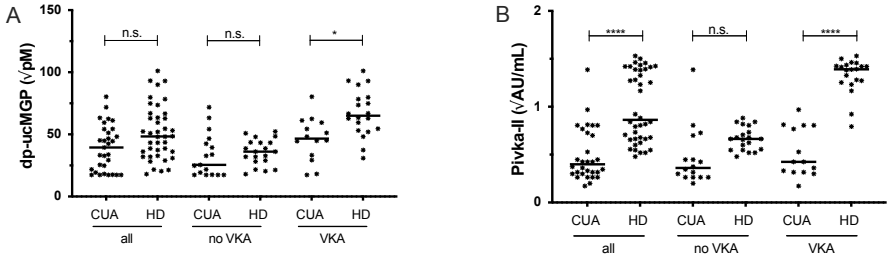
Supplementary Materials

Supplemental Table 1: dp-ucMGP measurements: patients vs. healthy controls,

Group	dp-ucMGP [pM]	p value: control - disease
Control	399 ± 190	Does not apply
CUA		
VKA	2443 ± 1649	<0.0001
no VKA	1384 ± 1446	<0.0001
HD		
VKA	4844 ± 2551	<0.0001
no VKA	1385 ± 7478	<0.0001
AF		
VKA	1535 ± 434	<0.0001
Rivaroxaban	671 ± 286	0.36
AVC		
No Vitamin K		
BL	463 ± 189	0.42
12 months	508 ± 251	0.09
Vitamin K		
BL	437 ± 221	0.2
12 months	221 ± 72	<0.0001

Data are presented as mean ± SD. Significance levels: n.s. =P > 0.05; * = P ≤ 0.05; ** = P ≤ 0.01; *** = P ≤ 0.001; **** = P ≤ 0.0001.

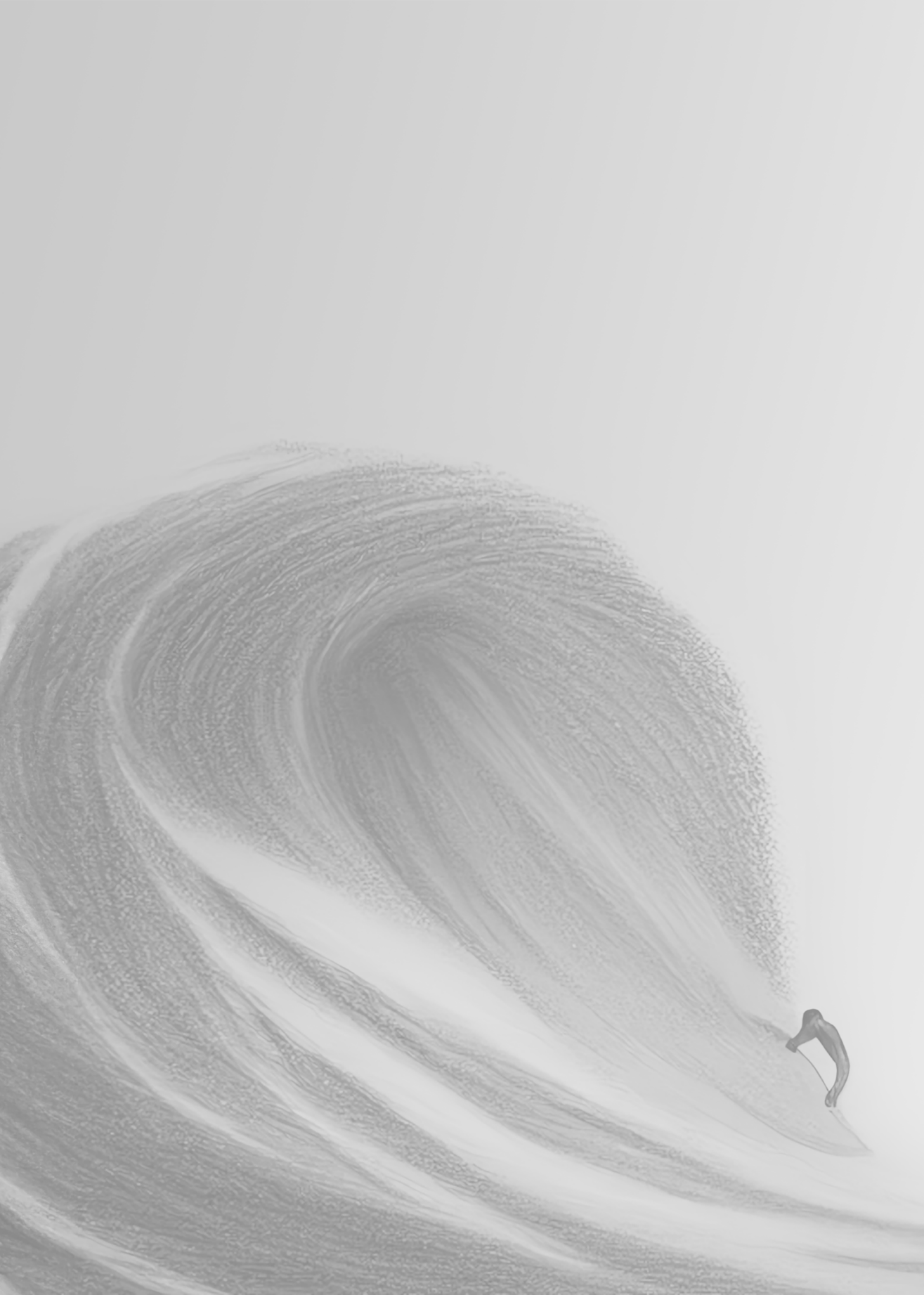


Supplemental Figure 1: dp-ucMGP (A) and PIVKA-II (B) levels of CUA and HD cohorts.

Supplemental Table 2: dp-ucMGP and PIVKA-II measurements for each cohort**,** and

Group	**Treatment**			
CUA	**no VKA**	**VKA**	**Foldchange**	**p value$^{\$ / \#}$**
dp-ucMGP [pM]	1384 ± 1446	2443 ± 1649	1.8	0.001
PIVKA-II [AU/ml]	0.2 ± 0.19	0.34 ± 0.3	1.7	0.99
HD	**no VKA**	**VKA**		
dp-ucMGP [pM]	1385 ± 7478	4844 ± 2551	3.5	<0.0001
PIVKA-II [AU/ml]	0.46 ± 0.17	1.79 ± 0.44	3.9	<0.0001
AF	**Rivaroxaban**	**VKA**		
dp-ucMGP [pM]	671 ± 286	1535 ± 434	2.3	0.002
PIVKA-II [AU/ml]	0.22 ± 0.2	3.65 ± 1,72	16.6	<0.0001
AVC				
No vitamin K	**Baseline**	**12 months**		
dp-ucMGP [pM]	463 ± 189	508 ± 251	1.1	0.02
PIVKA-II [AU/ml]	0.02 ± 0.03	0.02 ± 0.02	1	0.78
Vitamin K	**Baseline**	**12 months**		
dp-ucMGP [pM]	437 ± 221	221 ± 72	0.5	<0.0001
PIVKA-II [AU/ml]	0.01 ± 0.02	0.01 ± 0.02	1	0.68

Data are presented as mean ± SD. Significance levels: n.s. =P > 0.05; * = P ≤ 0.05; ** = P ≤ 0.01; *** = P ≤ 0.001; **** = P ≤ 0.0001.



Chapter 7

General discussion

Key findings of this thesis

Cardiovascular disease (CVD) is one of the major contributors to increased morbidity and mortality and is driven considerably by vascular calcification (VC). CVD and VC are more prevalent in chronic kidney disease (CKD) patients, where development of both is fuelled by a unique set of risk factors, called uremic retention molecules (URM). VC has long been regarded a passive process but is now recognized as actively regulated. VC regulation involves modifiers, both inhibitors and promoters, such as URM in CKD. VC modifiers can be utilized for the development of anti-calcification therapies as well as in patient risk profiling. Identifying, investigating, and capturing effects of VC modifiers is vital, since there is currently no approved therapy to prevent, hold or treat VC and methods for detection of VC are restricted by availability, radiation burden and only assess status quo.

Chapter 2 describes a novel screening assay termed the BioHybrid assay, a cell-based approach for detection of serum calcification propensity. Chapter 3 focuses on the advancement of the BioHybrid assay, using induced pluripotent stem cell derived vascular smooth muscle cells (iVSMC) as biosensors and we explore its improved variability, stability, and predictive power. In Chapter 4 we review current knowledge on the involvement of URM in VC. In Chapter 5 we investigate the role of nanoparticles and soluble serum components in VC and identify novel URM with pro-calcific potential. Chapter 6 comprises a study assessing vitamin K status in patients with high cardiovascular and VC risk.

In summary, this thesis adds to the growing body of evidence centred around VC, its detection and relation to vitamin K status, with a special focus on CKD related risk factors.

Key findings of this thesis are:

I. The BioHybrid is a novel VSMC-based system to assess serum calcification propensity to investigate CVD risk profiles (Chapter 2).
II. iVSMC are superior to primary VSMC in BioHybrid assay precision and the BioHybrid readout serves as important contributor to prediction of VC progression (Chapter 3).
III. The impact of URM on VSMC calcification *in vitro* and *in vivo* remain largely obscure and underlying effects are poorly understood. A better understanding of the effects of URM is key to develop strategies to reduce VC (Chapter 4).
IV. para-Cresyl Sulfate exacerbates VC *in vitro* and vitamin K counteracts its detrimental effects (Chapter 5).
V. Vitamin K status is normal in non-renal patients with VC, and vascular vitamin K stores are especially compromised in dialysis patients. Moreover, markers for vitamin K status PIVKA-II (liver) and dp-ucMGP (vessel wall) correlate poorly (Chapter 6).

VC diagnostics in perspective

The reversing demographic pyramid and an ever-older society are one of the major challenges for industrial countries in the near and developing countries in the mid distant future[1]. Socioeconomic burden of health care, including increasing prevalence of chronic diseases like CKD, diabetes mellitus and CVD, is expected to rise exponentially,

especially as multiple morbidities accumulate in one patient[2]. Personalized preventive, instead of reactive care, can reduce health care expenditure and improve health management[3–6]. Even though VC is considered a strong risk marker for cardiovascular morbidity and mortality, the diagnostic use of VC in routine clinical care, even for CKD patients, is not well established, and there is no consensus on its application[7]. This might be attributed to quality screening methods for VC requiring expensive equipment and highly skilled personal to conduct and interpret. Thus, VC imaging is limited in availability, especially in low-income countries[8]. Additionally, the lifetime excess cancer risk from a single CAC - CT examination has been estimated at 9 cancers per 100,000 men and 28 cancers per 100,000 women[9]. Nonetheless, VC testing is an effective and cost saving risk-stratification tool, and may enable physicians to better tailor preventive therapy to patients' CVD risk[10]. Moreover, it has been suggested that the use of CAC scoring can detect disease in asymptomatic patients who would be classified low risk when assessed by conventional risk factors[11].

A blood-based assay would provide a simple test, which could be integrated in routine measurements of other biomarkers which are analysed frequently, thereby reducing burden and increase compliance. A number of studies has investigated biomarkers as predictors for VC or CV risk, and while associations between individual markers and VC are present, none has proven to be suitable for clinical use[12–14]. This might be due to the complex interplay of all factors contributing to VC and CVD risk, which can hardly be captured by one individual marker. It may more likely be the entirety of blood constituents that better predict VC. The BioHybrid has shown its potential in reflecting VC propensity in a wide spectrum of disorders. However, studies with greater patient numbers may be warranted to clinically prove its power in prediction and reflection of VC and CVD risk. It may be used as standalone, or together with other features, to give an estimation of present VC status, but additionally offer benefits in predicting progression of VC, as well as general CVD risk. For routine future use, we foresee the need of a standardized benchmark, comparable to the international normalized ratio in coagulation, based on a standard serum pool which reflects "normality." A defined serum pool with criteria for donors e.g., age or sex but also medication use e.g., VKA, will allow for comparable results between sites of conduct, but also for temporal comparisons of the same patient.

Regarding the future of personalized medicine, we envision an individualized version of the BioHybrid. Personalized therapy and clinical trials based on iPSC derived products are already ongoing, also for CVD. Additionally, iPSC derived cardiomyocytes are used in diagnostic cardiology, which may extend to VC diagnostics in the future[15–18]. The cell-based nature of the BioHybrid will allow for full personalization. Combining patient specific iVSMC as biosensors with serum of the same patient, the BioHybrid will provide advanced risk assessment. It may even advance beyond diagnostics and aid therapy decision making by providing a basis for personalized drug screening. Unfortunately, no therapy has been approved yet for VC, and this is likely because the underlying mechanisms may differ fundamentally between individual patients making it difficult to apply standardized therapy. This applies to non-renal patients, but also to CKD patients where URM profiles and thus risk factors are highly individual.

The changing landscape of URM

Prevalence of chronic kidney impairment remains high on a global scale, with challenging, potentially life-threatening consequences for patients[19]. CKD is characterized by a unique set of risk factors for VC and CVD, comprised by a build-up of URM in circulation[20]. In our review we outlined the relationship between URM listed in the European uremic toxin work group (EUTox) database and VC. We found only a fraction of URM referenced in the EUTox database have been investigated with regards to impact on VC[21]. Additionally, it is suggested only a fraction of relevant pathological mediators in CKD are known at present, and more URM are being identified and described continuously, further expanding their quantity and diversity[22]. Expansion of the application of metabolomics and proteomics will not only extend the list of URM but also offer the possibility to generate personalized profiles and thus improved risk stratification models. This has been shown to be promising in predicting decline in kidney function[23] and cognitive impairment[24] and may prospectively apply to VC and CVD as well.

Traditionally, URM are classified according to their physicochemical properties into one of the three classes: small water soluble compounds, protein bound solutes and middle molecules[25]. A recent consensus publication proposed an advancement of the definition and classification of URM, linking it to clinical outcomes, quality of life measures, and toxicity[26]. While inorganic solutes like phosphate and water were intentionally excluded from the definition as URM, we noticed that the spectrum of macro molecules e.g., extracellular vesicles (EVs), miRNA, small & oxidised low-density lipoproteins (sLDL), calciprotein particles (CPP), and microplastic or nanoparticles seem not to be in the field of view of the uremic toxin research community. Even high cut off dialysis membranes only have a mean pore size of 10 nm[27], many fold smaller than the size of sLDL[28], CPPs[29] or EVs,[30] suggesting these larger molecules are not depleted using haemodialysis. EVs have been put forward as a possible link in the bone-vascular axis in the calcium paradox[31]. Moreover, oxidised sLDL and CPPs are identified as serum components with pro-calcific capacity in CKD[32,33]. Furthermore, miRNAs can show pro-calcific properties and are dysregulated in CKD[34,35]. Nanoparticles (~100 nm) are frequently used as food additive, but can exert adverse health effects, e.g., cytotoxicity of several organs including nephrotoxicity[36]. Nanoparticles are also know to interact with and alter the gut microbiome, which in turn is linked to VC[37,38]. Additionally a recent study reported the pollution of human blood with microplastic particles (>700nm), the health consequences of which are not fully investigated yet[39]. Although evidence associates these macromolecules with adverse health effects in CKD, they are frequently overlooked as uremic toxins. However, they might hold potential as biomarker or therapeutic target, but more research is needed to unravel the role of these macro sized mediators in the context of CKD, VC and CVD.

More knowledge of pathological mediators is the first step towards understanding disease mechanisms and developing treatments. In our review we put forward a systematic analysis of URM to identify relevant mediators. Screening all molecules on a continuously changing list is resource intensive, and therefore we propose to sort URM in clusters by similarity. Further, we propose to analyse only representative molecules and extrapolate this knowledge to clustered molecules. However, sorting

URM by chemical similarity proved ineffective, since their diversity did not allow for meaningful clustering (data not included). Instead, we employed a weighted scoring matrix based on existing knowledge of URM features to identify potential novel mediators of VC (Chapter 5). The EUtox workgroup recently suggested, to choose candidate biomarkers representing different types of uremic retention solutes and use these as proxies to study removal strategies, stratified per dialysis type. For future studies this panel can serve as representative to gain knowledge on VC, susceptibility to infection and other clinical parameters. Subsequently, the information can be utilized to adjust the dialysis strategy to match the outcome personalized to each patient[40].

In addition to personalized URM removal strategies, pathological effects of uremic solutes can be treated therapeutically. Many of the underlying mechanisms driving VC in CKD are still not well understood, but some common features e.g., inflammation or oxidative stress, have been identified and targeted. Pre-clinical research supports unspecific treatment of the targets such as oxidative stress and inflammation in VC inhibition[41–43]. However, they have not been successfully applied in clinical applications yet[44–46]. In contrast, statins which are often prescribed for their lipid lowering effect, also act anti-inflammatory, yet increase VC[47,48]. Therefore, unravelling detailed mechanisms of action of URM may prove a more successful road to targeted inhibition of VC.

Vitamin K in vascular health

Vitamin K plays a pivotal role in the activation of γ-carboxyglutamate (Gla) residues in so called vitamin K dependent proteins (VKDP), such as e.g., hepatic coagulation factors, vascular matrix Gla protein (MGP) and bone linked osteocalcin (OC). Vitamin K exists in two major isoforms: vitamin K1 (phylloquinone) and K2 (menaquinones). K1 and K2 isoforms have distinct absorption and bioavailability profiles, with K1 being more taken up by the liver, and K2 more associated with extra hepatic tissues[49]. Analysing vitamin K status of patients of 4 different cohorts we showed that different vitamin K surrogate markers (PIVKA-II; proteins induced by vitamin K absence or antagonism FII and dp-ucMGP; unphosphorylated and undercarboxylated MGP) correlate poorly. Other studies reported similar findings, aiding the idea of functionally distinct vitamin K tissue stores[50]. Vascular vitamin K deficiency reflected by increased dp-ucMGP is linked to increased VC and worse CVD outcome[51,52]. Up to 30% of the general population suffer from vitamin K insufficiency and vitamin K stores are even more compromised in CKD patients[53,54]. However, supplementation with vitamin K did not yield significantly improved outcomes of VC or CVD in CKD patients in most clinical trials[55–57]. In the context of progressive CKD, the question remains: “Is it too late for vitamin K treatment?”. Low bioavailability due to impaired uptake, distribution and recycling, which deteriorate with progressing CKD, and medication interfering with vitamin K metabolism e.g. antibiotics, phosphate binders or statins may hinder vitamin K supplementation in end stage CKD to be effective[56], while other patient groups might benefit[58]. Additionally, in some countries treatment of patients with K2 was hindered by the non-availability of a pharmaceutical grade product, and thus researchers only could only use K1[56]. Thus, a lack of bioavailability may be responsible for the inadequate action of vitamin K in treating VC in CKD, rather than a lack of

potency. It remains subject of further investigation to include measurements of absorption profiles in CKD patients, thereby establishing personalised vitamin K intake. This raises the question whether vitamin K can be administered differently in CKD groups, e.g., via the dialysis fluid or injections. Moreover, treatment before patients reach advanced CKD might circumvent some of the bioavailability issues and slow progression of VC, CVD and progression to end stage renal disease. Additionally, monotherapy may not be sufficient to improve VC outcomes in a multifaceted disorder like CKD. And indeed pre-clinical research shows combined phosphate binder and vitamin K therapy was effective, while neither reduced VC alone[59].

Vitamin K related health benefits historically revolved around its role as a cofactor for the enzyme gamma-glutamyl carboxylase. Up to 20 VKDP proteins have been identified thus far, with partially unknown functions. For some proteins conflicting reports have been published about the existence of a carboxylase binding site[60,61]. Moreover, the widespread expression of proteins of the vitamin K cycle in many tissues together with the difficult identification of Gla-residues suggests that more VKDP may exist which are net yet identified, indicating yet unknown contributions of vitamin K[60,61]. Complementary, we showed that vitamin K directly counteracts the pro-calcific effects of the cardiotoxic URM, para-cresyl sulfate, suggesting a non-canonical role for vitamin K in protecting VSMC from detrimental URM[20,62]. Next to its known oxygen radical scavenging capacity, which we also reported, vitamin K has recently been shown to protect cells efficiently from ferroptosis[63]. Ferroptosis associates with VC and other CVD, and may therefore be a novel route via which vitamin K exerts its cardioprotective effects[64,65]. Additionally vitamin K acts as a ligand of the nuclear steroid and xenobiotic receptor (SXR), thereby regulating cellular function on a transcriptional level[66]. SXR activation regulates vascular homeostasis, partially by regulating inflammation as well as lipid and glucose metabolism. Thus far the details of the interplay between SXR and vitamin K remain mostly obscure[67]. Vitamin K may be a key player in vascular health beyond VKDP activation, however more research is needed to understand and unleash the full potential of vitamin K as nutraceutical for CVD.

Concluding remarks

In this thesis VC, CVD, URM, and vitamin K are researched and discussed from both a basic research and clinical perspective. Today, treatment of CVD is based on resolving symptoms rather than preventing disease development. This is likely caused by the often-late diagnosis of CVD. We provide a novel BioHybrid platform which may provide a simple, low cost, and accessible tool for early detection of VC and CVD. URM are strong contributors to VC and CVD in CKD. The relation of VC with many URM is poorly understood and a fast-changing landscape calls for systematic and iterative evaluation. More knowledge allows for better risk stratification and potentially individualized treatment strategies. With a more personalized approach, including optimised dosage, timing, and co-treatments we foresee an important role for vitamin K in the treatment and prevention of VC and CVD, in the general population as well as in CKD patients. This would reduce the socioeconomic consequences of an aging population by reducing the development of CVD, bone loss, and potentially, other age-related diseases by means of a safe and cost-effective tool.

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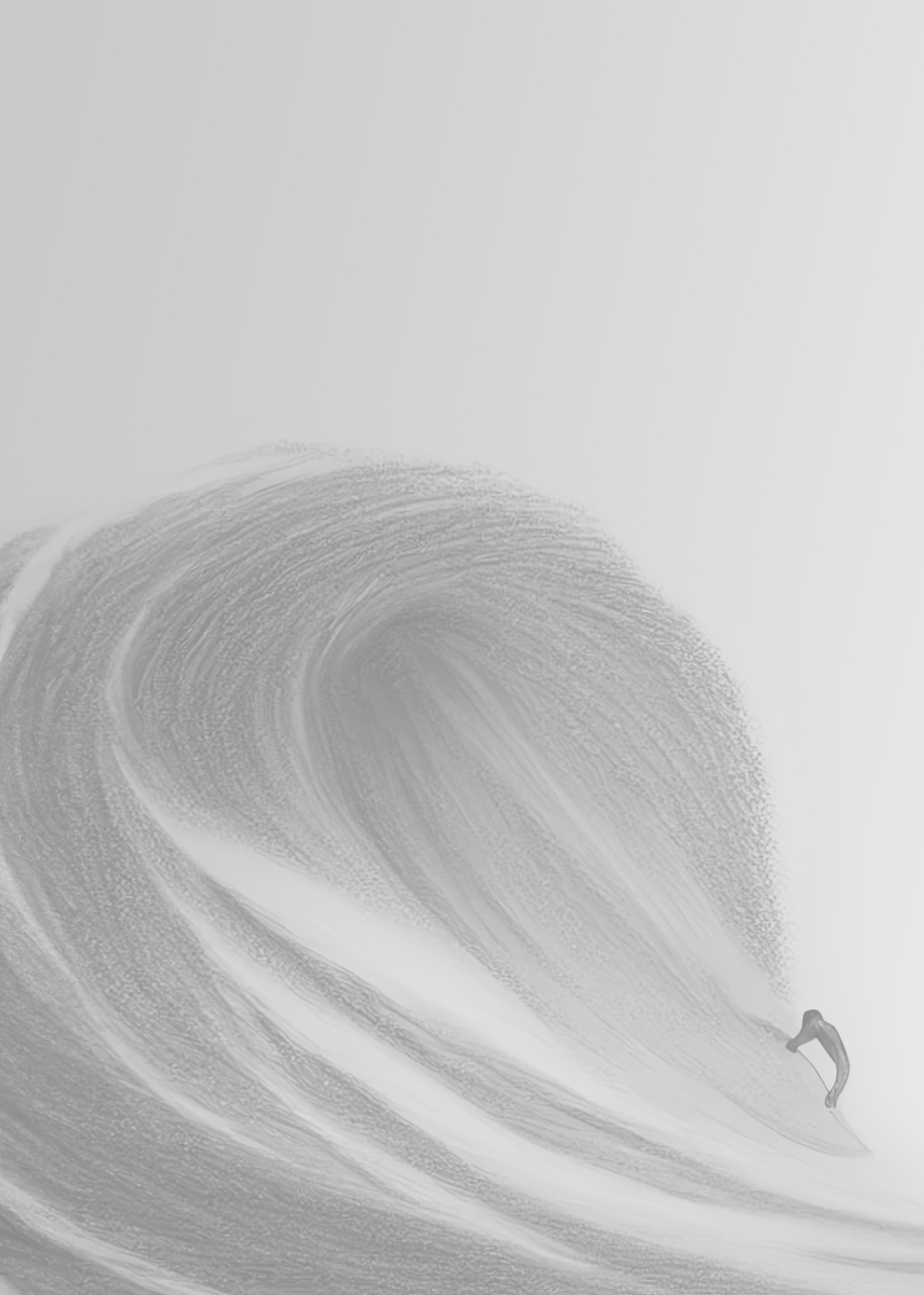


Appendix

Summary

This thesis adds to the growing body of knowledge around the impact of vascular calcification (VC) on cardiovascular disease (CVD), its detection and relation to vitamin K status, with a special focus on chronic kidney disease (CKD) and related risk factors.

As outlined in **Chapter 1**, CVD is one of the major contributors to mortality globally and significantly impacted by VC. CVD and VC are more prevalent in CKD patients, where development of both is fuelled by a unique set of risk factors sometimes referred to as ”the perfect storm”, and which include uremic retention molecules (URM). Despite VC being an actively regulated process, there is currently no approved treatment to halt or slow its progression. Vitamin K may be a promising therapy option. However, at present patients often receive vitamin K antagonist as coagulation inhibitor, inducing vitamin K deficiency and thereby contributing to VC progression. Moreover, detection of VC currently relies on insensitive and complicated methods with restricted availability.

In **Chapter 2**, we present a novel assay to determine a patient’s personal VC propensity, termed the BioHybrid. Using fetuin-A-AlexaFluor®-546, we developed a real-time calcification assay which provides a quantifiable readout of *in vitro* primary vascular smooth muscle cell (pVSMC) calcification development. We showed that VC is a consequence of all blood components which can be sensed and integrated into a calcification response by human pVSMC. Further, the sensitivity of this assay has been demonstrated in response to dialysis, vitamin K treatment, as well as both metabolic and non-metabolic disorders that directly affect cardiovascular status.

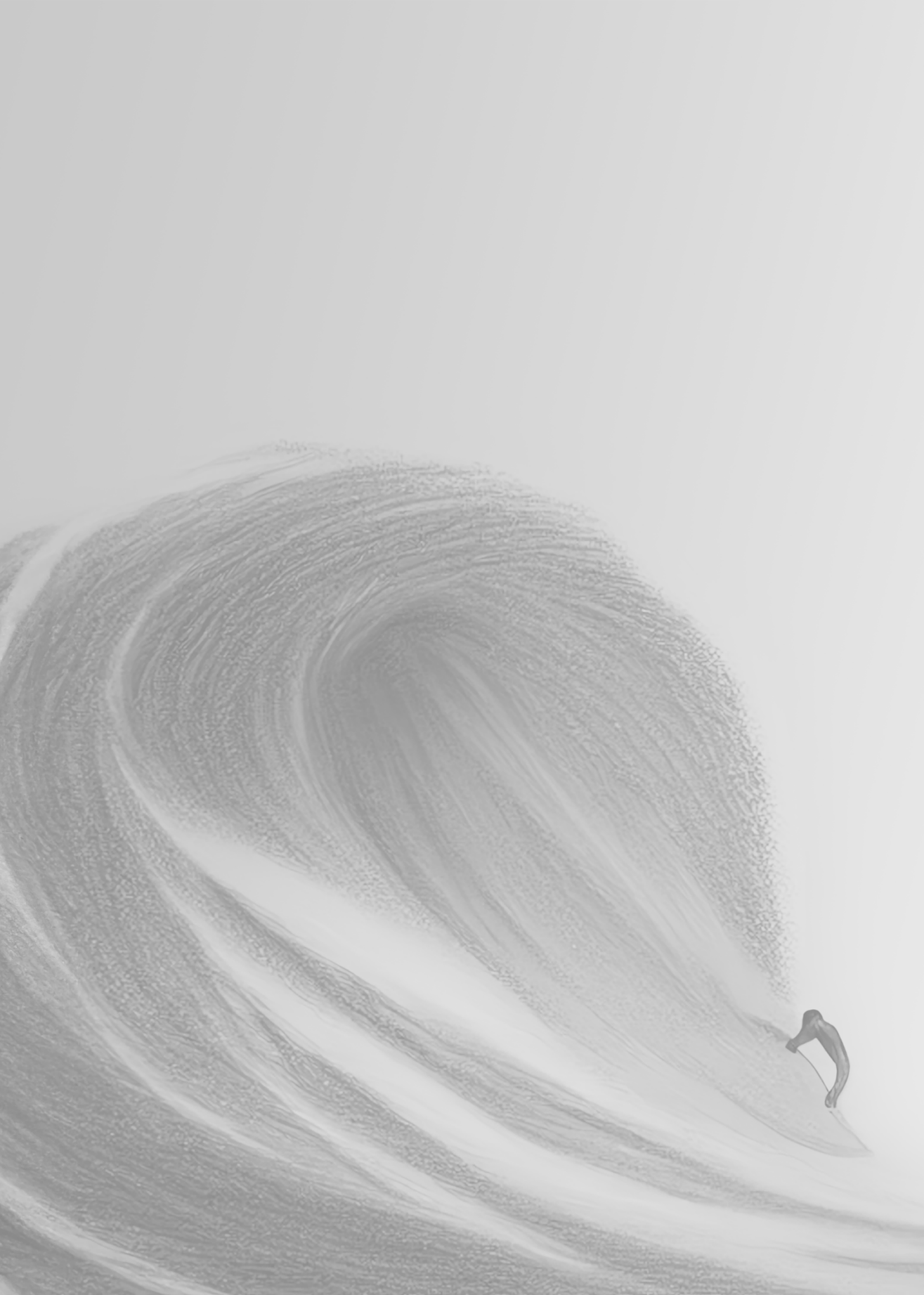
Chapter 3 focuses on the advancement of the BioHybrid assay. We optimised the BioHybrid using iPSC derived vascular smooth muscle cells (iVSMC) as biosensors as a more robust system with increased repeatability and lower variability. Primary and iPSC derived VSMC reflect CKD5D serum calcification propensity in a similar manner. Furthermore, inter-, and intra-assay precision of iVSMC is superior to pVSMC. The BioHybrid assay, using iVSMC, reflects VKA use by increased serum calcification propensity of two different patient populations. Moreover, we show the platform can be used for drug screening, holding potential for personalized medicine. Lastly, we showed that the BioHybrid readout of serum at baseline is an important contributing feature in a machine learning model predicting the progression of coronary artery calcification.

Chapter 4 is comprised of an extensive review, summarizing the current knowledge on the involvement of URM in VC. In this review, we included literature investigating URM and VSMC calcification *in vitro, in vivo* and on a clinical level. A clear definition of URM being detrimental, harmless, or beneficial with respect to VC is currently lacking. Furthermore, we identified gaps in URM research related to VSMC responses. A large fraction of URM has not been investigated with respect to their effects on VSMC calcification. However, some URM are linked to processes associated with VSMC calcification, such as oxidative stress and inflammation. We included potential novel avenues of VSMC driven calcification, such as extracellular vesicles and

senescence. However, effects of URM are often reported only once in literature, and experimental conditions often lack an adequate degree of standardization and are not designed in relation to chronic reno-cardiac syndrome. The composition of uremic serum is divers and may differ vastly between patients. Moreover, the influence of many URM on VSMC behaviour *in vitro* and VC *in vivo* remains largely obscure, and the underlying effects poorly understood. Better understanding of the effects of URM on the vasculature could be a first step towards reducing VC and a personalized CKD treatment strategy.

In **Chapter 5** we show iVSMC are a suitable platform to study CKD and URM related VC *in vitro*. Particles significantly contribute to calcification propensity of serum, while soluble factors also play an important role in CKD related VC. We developed a systematic scoring matrix to identify URM with potential pro-calcific effects. Acrolein and para-cresyl sulfate (pCS) increased VC in our iVSMC based *in vitro* model, while 4-OH-noneal and methyl glyoxal did not. Acrolein may act via increased cell death, while pCS likely exerts its action on iVSMC via RAGE, intracellular oxidative stress, alkaline phosphatase activation and increased extracellular vesicle release, with a potential role for disturbed energy metabolism. Lastly, we show that vitamin K supplementation may be a promising therapeutic approach to counteract URM induced vascular damage. More research is necessary to further substantiate the findings of this study.

Chapter 6 is comprised of a study assessing vitamin K status in patients with high CVD risk, using two different vitamin K dependent surrogate markers. The observation of normal vitamin K status in non-renal patients with established VC (aortic valve calcification and atrial fibrillation with VC) may question to what extend vitamin K is involved in the pathogenesis of VC in these patient groups. Indeed, more pathways are leading to VC than only vitamin K-deficiency driven VC. PIVKA-II levels and dp-ucMGP show only weak correlations, supporting the hypothesis that these markers most likely represent different functional vitamin K stores. VKA therapy was strongly associated with higher dp-ucMGP levels in all patient cohorts. However, without VKA treatment dp-ucMGP levels were only elevated in haemodialysis patients. Thus, vascular vitamin K stores are especially compromised in patients on dialysis, rendering these patients susceptible to accelerated VC. Finally, vitamin K status was not inferior in patients with calcific uremic arteriolopathy (CUA; calciphylaxis) compared to HD patients. Yet, VKA use might still be a driving factor of CUA development by influencing coagulation and VC via vitamin K independent mechanisms. Given the design of our study and inherent limitations, our data should be considered hypothesis-generating, but call for additional studies.



Appendix

Social and scientific impact

Age-related diseases, including cardiovascular diseases (CVD), chronic kidney disease (CKD) and vascular calcification (VC) are on the rise globally. VC is of particular interest because it is related to, as well as can cause multiple complications such as heart failure and high blood pressure. In CKD, kidneys fail to excrete waste products from the blood, thereby accumulating them in the blood. The waste products are then referred to as uremic retention molecules (URM). These URM strongly contribute to increased VC, CVD, and mortality in CKD patients. Ceasing health comes with a heavy burden on quality of life and rapidly increasing health care cost. Furthermore, multi-morbidity tends to accumulate within an individual with age, and with the global population aging, over-all disease burden is rising.

This thesis aims to identify risk factors contributing to increased CVD burden, with a focus on CKD related contributors, since CKD is considered a disease of accelerated ageing. Further we investigated methods to detect individuals which potentially are at high risk to develop CVD, and we investigate how to cost effectively provide save therapy for prevention and treatment of CVD.

In chapter 2 of this thesis, we describe the development of a novel test to detect the propensity of human serum to induce calcification by using cells from the vasculature and termed this assay BioHybrid. We showed that the BioHybrid detects the anticipated reaction to sera from patients, by showing more calcification with sera from people suffering from a range of VC related diseases. The advantage of the BioHybrid over previously available biomarker assays is, that it needs less serum to test the calcification reaction over time, and it is more reliable in its readout. Another perk of this test is, it considers all serum components and not just one single biomarker. In chapter 3 we describe how the test is further developed, using vascular cells derived from induced pluripotent stem cells (iPSCs). This allows to provide an endless source of similar vascular cells, allowing for more standardisation and distribution of the BioHybrid. Additionally, using iPSC derived vascular cells makes the BioHybrid more precise and increases repeatability. Furthermore, we applied the BioHybrid to serum samples of patients with established and proven coronary artery calcification. Next, we used these results as well as other clinical data to predict progression of VC. We showed that the BioHybrid contributes substantially to identification of high-risk patients. Another advantage of the BioHybrid lays in its simplicity. It can potentially be used in middle- and low-income countries, with common laboratory equipment, and it can strongly contribute to the identification of high-risk patients, enabling early treatment thereby preventing future health care cost and disease burden. The BioHybrid could be used commercially and may have a future in routine clinical practice, with specific applications in personalized medicine.

In chapter 4 we provide an extensive review and summary of the literature on URM and VC. Currently, only few URM are researched with respect to their relation to VC. We point out gaps in existing research, paving the way for a better understanding of this subject. We also provide a roadmap for systematic assessment of URM to effectively allow fact-based decision making. In the subsequent chapter, we utilize this information, and create a list, ranking URM by likelihood of being pro-calcific. We tested the top 4 candidates, yet the list may serve for informed future decision making. Of the tested 4 candidates, 2 are newly identified molecules being pro-calcific. Para-cresyl sulfate

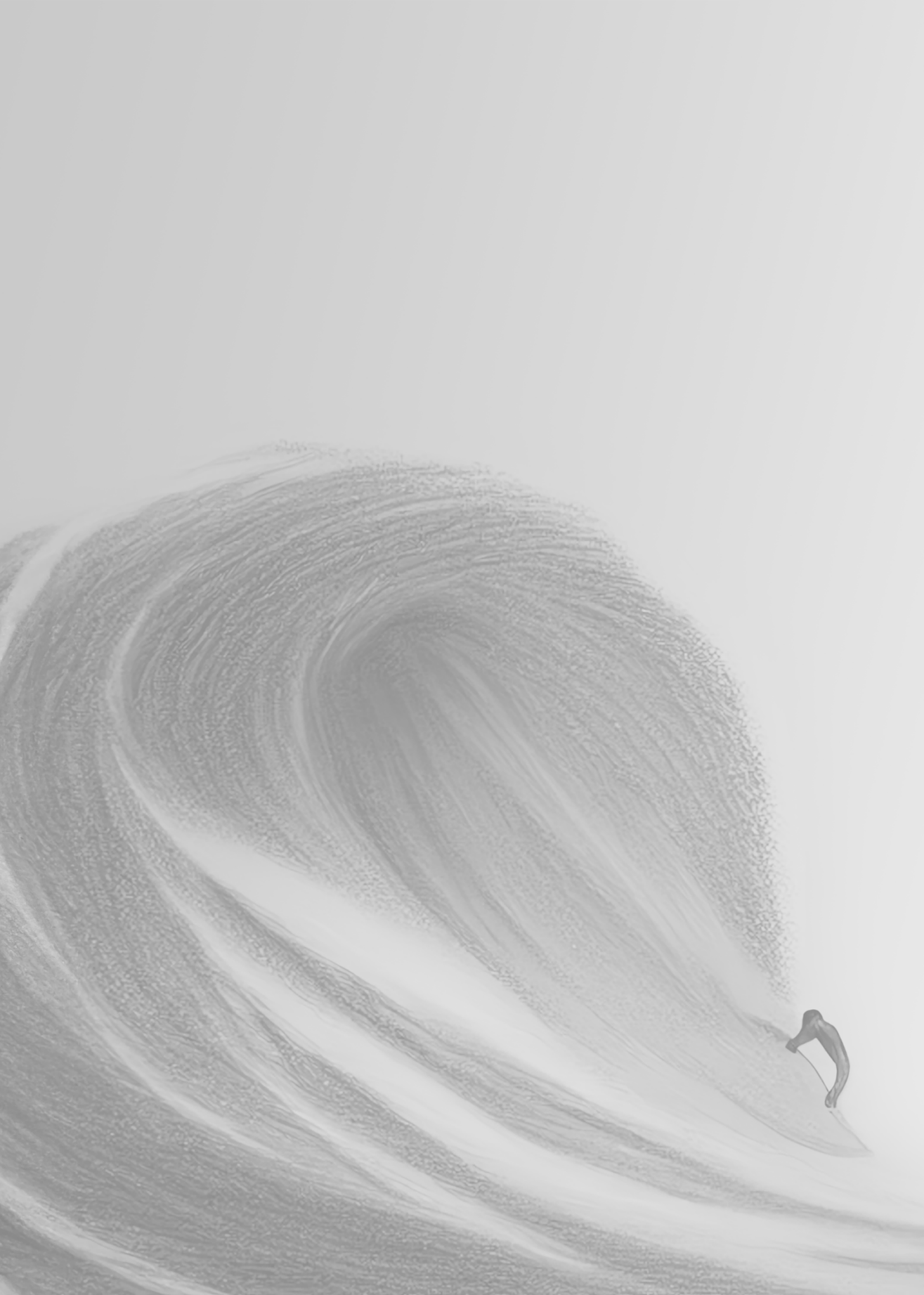
(pCS), being among the most recognized URM. We showed that vitamin K directly counteracts the detrimental effects of pCS and investigated the molecular mechanism behind it. Understanding how pCS and other URM contribute to VC, allows to better understand the process of VC in CKD providing the basis for development of targeted therapies.

In chapter 6, we provide an assessment of vitamin K status in patients with high cardiovascular risk, using two different vitamin K dependent surrogate markers. Four different patient groups were included: 2 dialysis dependent and 2 non-dialysis dependent patient groups. We observed normal vitamin K status in non-dialysis patients and especially compromised vascular vitamin K stores in dialysis, likely rendering these patients susceptible to accelerated VC. Both surrogate markers correlated poorly with each other, highlighting the importance of which marker is chosen. Our findings may aid physicians in their analytical and therapeutical decision making, and in finding optimal treatment strategies.

The results of this PhD project are published in various open-access scientific journals (complete list in subsequent chapter) and more research is in preparation for publication. All published articles combined have been read more than 9609 times and been cited over 28 times, underscoring the relevance of this research for the scientific community. We encourage scientists to use the BioHybrid, by publishing our detailed, video supported, protocols. Furthermore, the results of this work have been presented at international conferences, thereby exchanging knowledge, creating discussion, and sparking new ideas among researchers.

Importantly, and in light of the recent COVID-19 pandemic, the discussion around the impact of scientific evidence in public decision making intensified. Also, outreach and dissemination of results by researchers has received more attention and gained importance. Given the funding from public resources, research should benefit the public, but also reach the general population to increase understanding, acceptance, and support. To raise awareness, we shared the scientific background and novel findings of this thesis via various ways, including distribution on several social media platforms, development of flyers for patients distributed at the hospital, a podcast explaining the project in layman's terms and training of students.

In its essence, research conducted in this PhD project aims to reduce the disease burden coming with an ageing population, one of the major challenges for mankind in this century. We worked on a low cost, easy to implement, reproducible test to aid risk assessment for VC, a major contributor to CVD. Even in the COVID-19 pandemic, CVD is still the number one cause of premature death globally. We provided a framework for researchers to build on for URM research and started to fill-in knowledge gaps. Lastly, we contributed to the growing body of knowledge supporting vitamin K as a potent, cost effective and safe way of treating and preventing CVD.

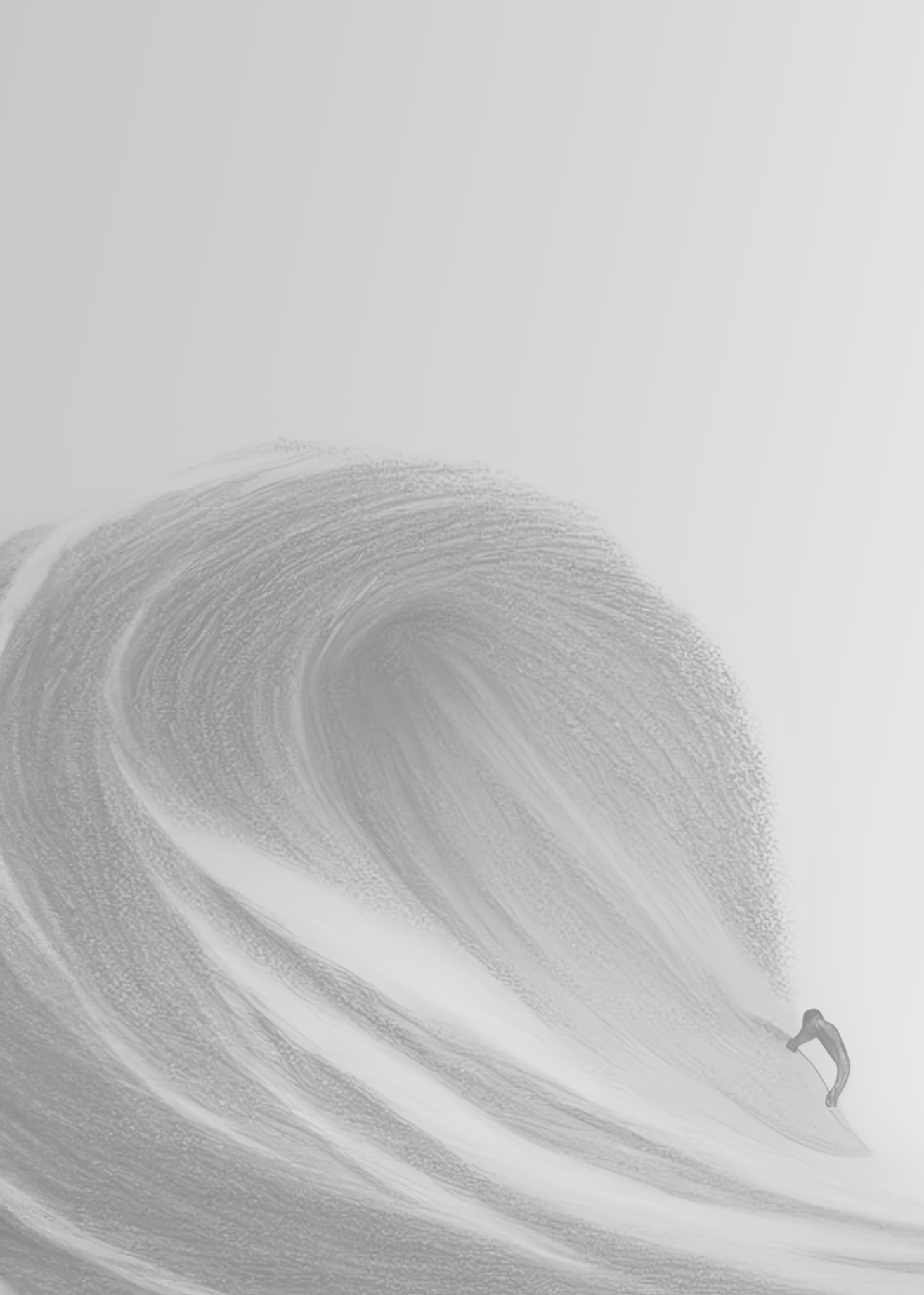


Appendix

Curriculum vitae

About the author

Nikolas Andreas Rapp was born on the 21st of July 1991 in Bruchsal, Germany, where he also visited high school. He graduated from the "Justus Knecht Gymnasium" in 2011, obtaining his university entrance qualification and started the Bachelor of Science program "Bio sciences" at the Goethe University in Frankfurt am Main, Germany, the same year. 2014 he received his bachelor's degree, specialized in molecular biology, and subsequently started the Master of Science program "Pharmaceutical biotechnology" at the university of Ulm and the university of applied sciences Biberach, Germany. During his master's program he accepted internships at Roche Diagnostics in Mannheim, Germany and Roche Pharma Research and Early Development, Basel, Switzerland. In 2017 he obtained his Master of Science Degree, with a focus on the production and development of biopharmaceuticals. In 2018 he became a Marie-Skłodowska-Curie fellow, within the European "Cardio-Renal-Syndrome-Analysis" (CaReSyAn) international training network and started his PhD position at the biochemistry department of Maastricht University, the Netherlands under supervision of Prof. Dr. Leon Schurgers. His PhD project revolved around vascular calcification, its detection and relation to vitamin K, with a special focus on chronic kidney disease related risk factors.



Appendix

List of publications

Publications

Rapp, N.; Jaminon, A. M. G., Akbulut; A. C., Dzhanaev, R.; Reutelingsperger, C. P.; Jahnen-Dechent, W.; Schurgers, L. J. A Semi-Automated and Reproducible Biological-Based Method to Quantify Calcium Deposition In Vitro. J. Vis. Exp. (184), e64029, (2022)

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Jaminon AMG; Akbulut AC; **Rapp N**; Kramann R; Biessen EAL; Temmerman L; Mees B; Brandenburg V; Dzhanaev R; Jahnen-Dechent W; Floege J; Uitto J; Reutelingsperger CP; Schurgers LJ. Development of the BioHybrid Assay: Combining Primary Human Vascular Smooth Muscle Cells and Blood to Measure Vascular Calcification Propensity. Cells. 10(8):2097, (2021)

Akbulut, A. C.; Wasilewski G. B.; **Rapp N**.; Forin F.; Singer H.; Czogalla-Nitsche K.; Schurgers L. J. Menaquinone-7 Supplementation Improves Osteogenesis in Pluripotent Stem Cell Derived Mesenchymal Stem Cells. Front. Cell Dev. Biol. 8, (2021)

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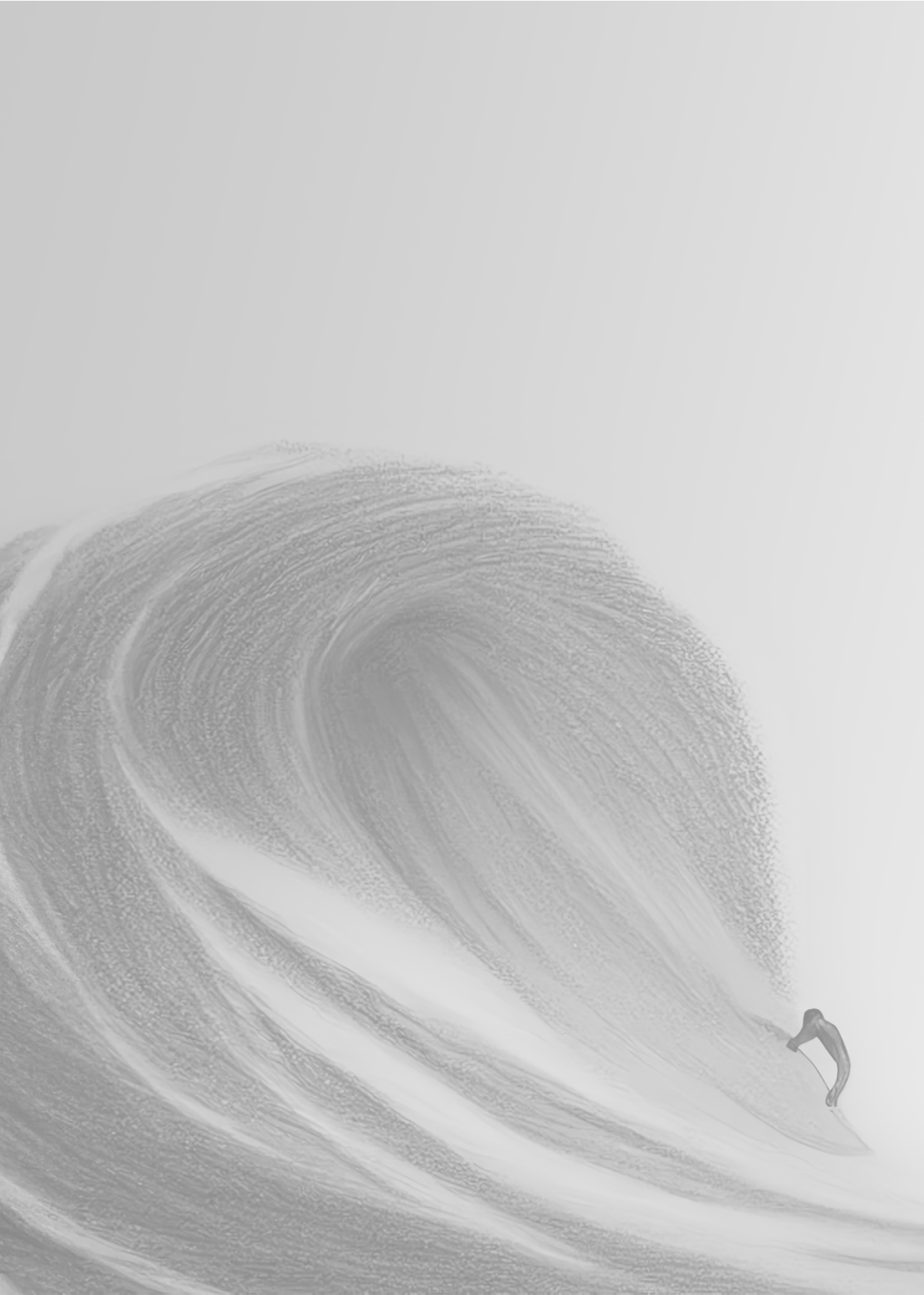
Beghi, S., Furmanik, M., Jaminon, A. M. G., Veltrop, R., **Rapp, N**., Bidar, E., Buschini, A., Schurgers, LJ. Calcium signalling in heart and vessels - Role of calmodulin and downstream dependent protein kinases. *Int. J. Mol. Sci.*, *23(24)*, 16139 (2022)

In preparation

Rapp, N.; Akbulut, C.; Jaminon, A.M.G. Furmanik, M.; Kramann, R.; Jahnen-Dechent W.; Stöhr, R; Brandenburg; V; Reutlingsperger; C., Schurgers, L.J. Combining iPSC derived human vascular cells and serum to study and predict vascular calcification – the BioHybrid 2.0 *(in preparation)*

Rapp, N.; Akbulut, C.; Jaminon, A.M.G.; Furmanik, M.; Kramann, R.; Schurgers, L.J. Utilizing iPSC derived human vascular cells to study uremia related vascular calcification *(in preparation)*

Rapp, N.; Jaminon, A.M.G.; van Gorp, R.; Halder, M.; Leenders, P.; Bauwens. M.; Mottaghy. F.; Reesink, K.; Kramann, R.; Dijkgraaf, I.; Reutlingsperger, C.; Schurgers, L.J. Development of an animal model to study effects of medial calcification as risk factor for atherosclerosis *(in preparation)*



Appendix

Acknowledgements

Many places I have been
Many sorrows I have seen
But I don't regret
Nor will I forget
All who took that road with me

Contrary to the title of the song by Billy Boyed from which I borrowed the lines above, this shall not be "The Last Goodbye"! However, I will at last turn to paths that lead to new adventures. I express my sincere gratitude towards all the amazing people I had the honour to get to know and which more than anything else shaped this experience for me.

Firstly, I want to express my heartfelt gratitude towards my promotor Prof. Dr. Schurgers. Dear **Leon,** thank you for the chance to be part of your fantastic group. The team you have built and the spirit that drives it are truly amazing. Much have I learned from you during our discussions about cutting-edge science over a coffee and life over a beer alike. You have a fire of curiosity and love for science burning in you, which I deeply admire. During our talks this fire never failed to ignite a spark within me, which propelled me a long way on this journey. You are a real mentor to me, a trusted adviser, guiding me academically and personally. I am sorrowful for our ways are parting here and it is my great hope that our paths, however long and winding, will cross again. Thank you for leading by example in many ways!

Cordial thanks also to my colleague, friend, and co-promotor (I hope you enjoyed that cookie) Dr. Jaminon! Dear **Armand**, since I started, and we both were PhD students, we have done countless projects together. Your scientific accomplishments also laid the foundation for many of my laboratory endeavours, closely tying our paths. You grew into an important pillar, not only for me, but for the entire group. You were always the first go-to person for anything lab related, providing me with many skills crucial for success and you taught me much of what I know today about the ways of science. Knowing I was able to rely on you, tap into your wisdom and your experience relieved me of a great mental load. Thank you for always having an open door, always being able and, most importantly, willing to help in every possible way.

Herewith I would like to also thank the assessment committee, namely **Prof. Dr Delhaas, Dr. Noels, Dr. Matic, Prof. Dr. Hemmelder** and **Prof. Dr. Stoll** for taking the time to read and critically assess this dissertation.

The man, the myth, the paranymph - Dr. Akbulut! Dear **Cengiz**, man of the first hour I met before my contract even started. I know you never overcame the disappointment that I have cost you your personal office and corner desk. Even more do I appreciate the friendship we have developed, and the memories collected over the years: road tripping through the lowlands, the Christmas we spent together and WU-TANG, just to name a few. You taught me much in terms of science and life alike, swing dance being one of them. I would like to take the opportunity to deny what you claimed in your booklet – swing dance will likely never be a leisure activity I would pick by free will. But you were

right about one thing - I had fun, because you made it fun. Like you made many things fun and memorable, by your magical talent to fill every void with a song – of your own making or not -, random facts, often controversial & conversation starters, or by ranting on London style. I will dearly miss my Movember mate who was always just a turn away. Thank you for not only being part of this adventure, but for shaping it and for having my back, to the very last moment!

Dear **Bibi**, should I ever be asked which 'strong women' influenced me, you are going to rank high on the list. Your passion for self-development impresses almost as much as your focus on your goals. How you pursue them with dedication and perseverance, be it in your career or a flunky ball game, in both of which you demonstrated, the fence stopping you has yet to be invented. I am sure if you keep doing science, like you hit the gym now, you will make the cover of 'Cell: Stem Cell' like you featured the 'PIÑA' poster. Everywhere you go you immediately make friends and so it was with me too. Nothing less is to be expected from an ENFJ-A - and that I know what those letters mean is entirely result of your questionable influence. Thank you for never failing to "tiramisu" in the literal and culinary sense, and for having my back to the very last moment of this adventure.

Many, many, many thanks to the much-appreciated backbone of our group. Dear **Petra**, you always have everything under control, you think at least 2 steps ahead and spot pitfalls miles in advance. Thank you for your great sense of humour and being the lab manager we may not have deserved, but we all needed. Dear **Cecile**, last minute orders of special chemicals from weird companies? After 4 years still asking how to do this, or where to find that? Never a problem for you. Thank you being a calm anchor in sometimes restless waters! Dear **Niko**, my namesake. I will miss the confusion it created at times. The dedication and commitment you show for teaching, always trying to improve the course for the students, even though the students will never know, impresses me much! Thank you for the honest conversations we often had during cell culture! Dear **Peter**, you always have everything under control with the animals, which is a huge help. Knowing these projects safe in your hands is priceless. Thank you for taking a big mental load of my mind and often lighting up my day with your shirts! Dear **Lisette**, thank you for always being willing to help wherever possible. A huge THANK YOU to **all of you**, for being the glue that holds our group together!

My gratitude also extends to our groups' more senior members: Prof. Dr. Reutlingsperger, dear **Chris** your sharp intellect and your laughter are inherent to our group. Thank you for many fruitful discussions and much appreciated advice! Dr. Furmanik, dear **Gosia**, your next hairstyle or trouser pattern were always a much-awaited surprise. The countless discussions we had about science and beyond always propelled my research and shaped my mind. Thank you for making my PhD time more colourful! Dr. Rupérez, dear **Celia**, you added yet another layer of multiculti to our group. Muchas gracias por animarme a aprender español!

A special thanks also to the more junior members of our group, who often shared my sorrows, and who always had an open ear, which helped tremendously. Dear **Rogier,** in your double role as office mate and fellow CaReSyAn ESR we shared much of our path in the past years. Thanks for all the stories you shared, they never failed to lift my spirit**.** Many thanks also to **Vanessa**, **Berta** and **Minke** for making H4.348 the – by far – best office in the history of offices, maybe ever. Of course, also **Sofia**, **Nasim**, **Femke**, **Pepijn**, **Lia**, **Baixue**, **Francesco** and many more that over the time were part of our fantastic group coined my time in Maastricht. All of you contributed significantly to making my time as part of this group a unique and fantastic experience, by always standing together, helping and supporting each other, laughing together, celebrating victories and learning from mistakes. Thank you all for all of this, and much more! I wish you most success in following your own path and all the best for the future.

Dr. Wasilewski, dear **Greg,** many alliances were formed, and battles won, many tents assembled and adventures experienced, in and outside of the Limburg forests. Your beautiful mind always was an inspiration. Thank you for teaching me, that sometimes 'it is what it is'!

Dr. Jooss, **liebe Natalie**, unsere Kaffeepausen und Gespräche über Moos und britischen Humor waren immer eine willkommene Abwechselung. Danke, für deine Empfehlungen diverser Comedians und Shows, und dafür, dass du eine Brücke in die Heimat und zu meinem Dialekt warst. Ich weiß, was du jetzt denkst, ich bin aus Baden, du aus Schwaben... aber das sind doch bürgerliche Kategorien!

Dear wonderful people of the **Biochemistry department,** I have never worked in a place with such a fantastic spirit. Many thanks for great discussions during our meetings, many joyful chats on the hallway and memorable celebrations on many occasions. You are truly Maastricht University's finest!

Dear **Trees** & **Lidewij**, you two are the heart and soul of the biochemistry department, equipped with deepest knowledge and experience about all processes in and around the university. Thank you for often checking in and being great supporters during my PhD time.

Many thanks to my fellow CaReSyAn ESRs **Giulia, Eleni, Tianlin, Jeppe, Irene, Ana, Sofia, Valeria** and **Sam**. You all were there from the beginning, and you always knew exactly what I was going through. Thank you for providing a fantastic framework of scientific and personal support, I am glad we were on this adventure together!

None of us still dwells in the city we met, but you all contributed so much to the fond memories of my time in Maastricht. Dr. Kenkhuis, dear **Marlou-Floor**, I was much impressed with the ease and relaxation you manoeuvred through your PhD, a great model to learn from. With your help I had my most Dutch experience of all time: Visiting the Efteling and buying tickets with "zegels" from AlbertHeijn. Thank you for being my bridge into the Dutch culture! Dr. Rostamitabar, dear **Matin**, - entrepreneur, scientist, leader - the dedication and energy with which you not only pursued your PhD, started a company, but also improved the life of your peers by leading the PhD Academy are

inspiring and a great example to follow. You are truly a 'Don'! Thank you for introducing me to many extraordinary musical and Persian culinary treats! (Soon to be) Dr. García Velásquez, dear **Carlos**. I will never, ever forget to listen to the avocado. Sustainability is not just a job for you, but you live and spread it, which I adore. You are practically the incarnated spirit of Christmas and in that sense, we complement each other perfectly. Thank you for showing me how to pineapple through life! Many thanks to my '**expats family**' for making the time in Maastricht unforgettable.

Größten Anteil daran, dass ich heute bin, wo ich bin und wer ich bin, hat meine Familie. Liebe **Mama & Andreas**, lieber **Papa & Daniella**, ihr habt mir immer ermöglicht meinen Weg zu gehen und mir dabei alle Freiheit gelassen. Ohne eure ständige Unterstützung wäre ich niemals so weit gekommen. Ihr seid mein Sicherheitsnetz, auf das ich mich immer verlassen kann und mein Anker in einer unruhigen Welt, der mir immer ein Gefühl der Sicherheit und Heimat gibt. Ihr habt auch meinen Horizont als Biochemiker dahingehend erweitert, dass ich gelernt habe, dass DNA nicht das ist, was eine Familie ausmacht. Danke, dass ich meinen Weg gehen durfte. Liebe **Lisa**, seit 31 Jahren kann ich mich jeden Tag auf meine große Schwester verlassen. Zahllose Geburtstage wären schon an mir vorbei gegangen, wenn du mich nicht unermüdlich erinnern und Geschenke organisieren würdest. Du hast mich sehr zum Guten geprägt und ich schätze es sehr, dass wir auch in unruhigen Zeiten immer zusammengehalten haben! **Liebe Familie**, ihr seid mein Fixpunkt, auf den ich mich immer stützen kann und eine Brücke zu meinen Wurzeln, von Herzen danke dafür!

Liebste **Sophie**, fast vom ersten Monat an hast du an meiner Seite alle Höhen und Tiefen gesehen, die ein PhD Projekt so mit sich bringt. Zu allem Übel habe nicht nur ich dir mein Leid geklagt, sondern alle aus der ‚expats family'. Und trotzdem hattest du immer ein offenes Ohr und hast mir mit deiner unermüdlich positiven und verständnisvollen Art, und gelegentlich einer Flasche Wein, geholfen die Täler zu durchschreiten und den Blick nach vorne zu richten. Mindestens genauso wichtig war es, zusammen die Erfolge zu feiern, sei es mit einem Schmankerl aus der Patisserie um die Ecke oder mit einem Strauß Karotten und -am wichtigsten- einer ‚goed gedaan' Karte. Jedes Tal haben wir zusammen durchschritten und jedes (PhD Academy) Fest zusammen gefeiert. Damit und natürlich mit dem höchst akribischen Editieren dieses Büchleins, hast du dir den „Ehren PhD" mehr als verdient! Danke, dass du an meiner Seite bist, für dieses und alle Abenteuer, die noch kommen!