CHAPTER 1

GENERAL INTRODUCTION, AIMS AND THESIS OUTLINE
**Chronic kidney disease (CKD)**

The first description of kidney disease was made by Richard Bright in the early 19th century, who linked several clinical cases with oedema and albuminuria to the renal morbid anatomy. Nowadays, chronic kidney disease (CKD) is generally defined as ‘kidney damage or glomerular filtration rate (GFR) <60 mL/min/1.73m² for three months or more, irrespective of cause’, and can be considered a general term to describe loss of renal structure and function. Worldwide more than 10% of the population suffers from CKD and this number is still rising. Studies investigating gender differences among CKD patients show generally a higher prevalence of severe CKD in woman than men. Also, the majority of CKD patients is diagnosed in the more progressive stages (CKD stages III-V), which accounts for approximately 79% of the CKD population, and the awareness of the disease remains low. CKD is classified based on the GFR (i.e., urinary or plasma clearance capacity) and the levels of albuminuria, and can be divided in five stage (I-V) (Figure 1). The first stage of CKD (stage I) is considered a normal or high kidney function. Disease progression to stage II and III is indicated as mild to moderate loss of kidney function. From stage IV onwards, renal function becomes severely impaired, and stage V is considered as complete kidney failure. In the final stages of CKD, renal replacement therapy is required (i.e., renal transplantation or dialysis), to compensate the loss of renal function.

![CKD classification diagram](image)

**Figure 1: Classification of chronic kidney disease (CKD) based on the glomerular filtration rate (GFR) and levels of albuminuria.** Stages range from stage one to five (I-V). Disease severity increases with stage. GFR is expressed in mL/min/1.73m² and can be estimated based on plasma creatinine levels. Data are based on previous reports and the figure is created with BioRender.com.

The origin of CKD is multifactorial. The most common cause contributing to CKD is diabetes mellitus, as hyperglycemia is detrimental to the nephrons in the kidney. Additionally, CKD may be caused by hypertension, glomerulonephritis, cystic kidney disease and inherited
renal malfunction, but also by environmental pollution or medicaments\textsuperscript{8,11,12}. Although symptoms are largely absent in the early stages of CKD, as the disease progresses patients start to suffer from physiological problems including uremia, hypertension (which is both a cause and consequence of CKD), and oedema. Symptoms affecting the quality of life are also included such as pain, itching, fatigue and sleeping disorders\textsuperscript{11,13}. Additionally, it has been shown that CKD patients are at increased risk for development of cardiovascular diseases (CVD)\textsuperscript{14}. Deterioration of kidney structure and function leads to the appearance of CVD-related risk factors, including volume overload, disturbed electrolyte metabolism (e.g., altered serum phosphate levels), inflammation and oxidative stress\textsuperscript{14–16}. Especially in patients with stage IV-V CKD, CVD-related mortality is demonstrated to be 40-50\textsuperscript{%}\textsuperscript{14,17}. Moreover, a large proportion (\approx 70-80\%) of the severe CKD patients develops vascular calcifications (VC), which manifests mainly in the coronary arteries, large arteries (e.g., femoral artery and abdominal aorta), and aortic valves\textsuperscript{18}. Due to the high incidence of VC in CKD, VC are considered major contributors to mortality in CKD, as the increased vascular stiffness impairs cardiac function\textsuperscript{18,19}. Moreover, VC in CKD are nowadays an emerging target for intervention.

**Calcium and phosphate homeostasis in CKD**

Calcium (Ca\textsuperscript{2+}) and phosphate (PO\textsubscript{4}\textsuperscript{3–}) levels are tightly regulated in body\textsuperscript{20}. When phosphate levels rise, multiple mechanisms are activated to reduce phosphate concentrations, and restore the phosphate homeostasis\textsuperscript{21}. At first, the bone-derived hormone FGF23 is released into the circulation, which acts on the parathyroid glands and the kidneys. In response to FGF23, the activity of the enzyme 1-\alpha-hydroxylase in the kidney is decreased, thereby reducing conversion of vitamin D into active vitamin D, and subsequently a decrease in calcium and phosphate absorption in the gastrointestinal (GI) tract\textsuperscript{22–24}. Second, FGF23 increases phosphate secretion by the kidney via inhibition of the reabsorption channel sodium-phosphate transporter (NaPi2a) and activation of the FGF23-Klotho axis\textsuperscript{23}, thereby reducing phosphate reabsorption by tubular epithelial cells. Additionally, FGF23 inhibits PTH secretion from the parathyroid glands, which is known to stimulate the vitamin D metabolism and triggers calcium and phosphate resorption from the bones, and promotes calcium retention and phosphate secretion via the kidneys\textsuperscript{22–24}. In CKD, the phosphate homeostasis becomes significantly impaired. Renal dysfunction leads to a reduced ability to excrete phosphate via the kidney, and as a result, FGF23 levels increase tremendously, leading to vitamin D deficiency and reduced serum calcium\textsuperscript{25,26}. Moreover, in CKD FGF23 resistance in the kidney, due to reduced Klotho levels, is also an important factor herein. High phosphate and low vitamin D levels trigger an elevation of PTH, thereby maintaining the positive feedback loop with FGF23, further supporting the disbalance of calcium and phosphate in
CKD\textsuperscript{25,26}. The detrimental consequences of elevated phosphate levels in CKD will be discussed in the next sections.

**Calciprotein particle formation**

An important consequence of a disturbed phosphate homeostasis in CKD is accelerated formation of calciprotein particles (CPPs)\textsuperscript{27} (Figure 2). When phosphate levels rise, phosphate precipitates with the circulating calcium and serum proteins such as albumin or Fetuin-A, leading to the formation of amorphous phosphate-calcium complexes\textsuperscript{28}. Initially, these circulating nano-aggregates are defined as calciprotein monomers (CPMs). CPMs can assemble and form amorphous calciprotein multimers, known as primary CPPs\textsuperscript{27–29}. When phosphate levels remain high, as is the case in CKD, primary CPPs can mature into secondary CPPs, thereby increasing in size and complexity. In contrast to primary CPPs, secondary CPPs consist mainly of crystalline calcium and phosphate and have a more spindle-like shape\textsuperscript{27–29} (Figure 2).

**Figure 2: Calciprotein particle (CPP) formation.** Elevated phosphate levels bind circulating calcium ions and serum proteins, leading to the formation of calciprotein monomers (CPMs). CPMs can assemble and form primary (amorphous) calciprotein particles (CPPs). Primary CPPs can mature and form crystalline secondary CPPs. Accelerated formation of secondary CPPs leads to toxicity, as secondary CPPs have been associated with development of cardiovascular disease, including vascular calcification. Transmission electron microscopy (TEM) pictures of both primary and secondary CPPs are shown in round photomicrographs. Complexity and toxicity increase during CPP maturation. Nanometer (nm), approximately (~). Figure is based on previous reports\textsuperscript{27–29} and created with BioRender.com.
Further characterization of secondary CPPs revealed presence of ions such as oxygen, sodium, and magnesium, albeit in low quantities, and presence of lipids, small DNA and RNA fragments, and even bacterial products\textsuperscript{30,31}. Interestingly, in healthy individuals CPP formation also takes place, where the particles function as reservoir for circulating calcium and phosphate ions\textsuperscript{32}. Both CPMs and primary CPPs (i.e., immature CPPs), as well as secondary CPPs, are rapidly cleared from the body\textsuperscript{33,34}. CPMs are relatively small (~9 nm) and can be filtered by the glomerulus. Primary and secondary CPPs exceed the glomerular filtration size limit and are mostly cleared via the liver and spleen\textsuperscript{33}. Recent studies showed that primary CPPs are mainly taken-up by the liver sinusoidal endothelial cells, while secondary CPPs are predominantly removed via macrophages in liver and spleen\textsuperscript{33,34}. However, once the phosphate balance is disturbed to such an extent as in CKD, and the particle clearance is decreased, CPP levels increase, which results in adverse effects\textsuperscript{33,35,36}. Accelerated secondary CPP maturation (i.e., increased calcification propensity, expressed as a decreased $T_{50}$ value) is associated with development of VC and an important contributor to cardiovascular mortality in CKD\textsuperscript{29,37–39}. Interestingly, increased secondary CPP levels appear to be a prognostic biomarker for development of VC and CVD in CKD\textsuperscript{36,40,41}.

**Vascular system: morphology and function**

The vascular system includes various types of blood vessels\textsuperscript{42}. Starting from the heart, oxygen-saturated blood travels from the left ventricle, via the elastic arteries, muscular arteries and arterioles to the capillaries, where oxygen (O\textsubscript{2}) and nutrients are exchanged for carbon dioxide (CO\textsubscript{2}) and metabolic waste products. From the capillaries, blood returns to the right atrium via respectively the postcapillary venules, medium-sized veins and large veins. Blood is re-oxygenated and CO\textsubscript{2} removed via the pulmonary circulation\textsuperscript{42–44}. Generally, blood vessels consist of three layers, namely the tunica intima, tunica media and tunica adventitia, with the exception of capillaries which only contain a tunica intima layer\textsuperscript{42,43}. The presence and composition of each layer depends largely on the function and location of the respective blood vessel in the vasculature\textsuperscript{42}. Figure 3 shows the morphology of a renal (muscular) artery, indicating the different aforementioned layers. The first layer is the tunica intima, which is exposed to, and interacts with, the circulating blood. The tunica intima comprises a single layer of endothelial cells (ECs) supported by a basement membrane, together covering the inner surface of the blood vessel\textsuperscript{45,46}. ECs form an important barrier which separates the circulating blood and its constituents from the surrounding tissue. Furthermore, ECs have an important function in the regulation of the vascular tone, coagulation, inflammation, gas and nutrient exchange and maintaining vessel integrity, though the extent to which these processes take place is dependent on the vessel type\textsuperscript{45,46}. The tunica media is located adjacent to the endothelial layer, and mainly consists of
vascular smooth muscle cells (VSMCs) embedded in extracellular matrix (ECM) of elastin and collagen (Figure 3). In response to factors derived from the ECs, VSMCs play an important role in regulating the vascular tone, by balancing vasodilation and vasoconstriction. VSMC-mediated vascular tone affects blood flow throughout the whole circulatory system. The most outer layer of vascular tissue is known as the tunica adventitia, and consists mainly of collagen and fibroblasts, but also contains tissue macrophages, mast cells, T and B cells, adipocytes, nerves, and micro vessels, known as the vasa vasorum network (Figure 3). The central functions of the adventitia layer are providing structural support and mechanical strength, preventing overexpansion of the vessel, and connecting the vessel to neighboring tissue. Herein, the majority of functions can be attributed to collagen.

![Figure 3: Morphology of medium-sized artery.](image)

An important structural component of blood vessels is the ECM, which comprises a complex architecture. The primary function of the ECM is providing a scaffold for vascular cells to bring structural support and preserve tissue organisation. Additionally, it has been shown that the interaction between ECM and vascular cells is also important for multiple other cellular processes including proliferation, migration, adhesion, and acquisition of a specific cellular phenotype. Each layer of the vascular wall (i.e., intimal, medial, or adventitial layer) accommodates a specific ECM structure, which...
varies among the different types and sizes of vessels. For example, in the tunica intima layer, support for the ECs is provided by a basement membrane made of glycoproteins, proteoglycans and different types of collagens\textsuperscript{53,54}. The tunica intima is separated by an internal elastic membrane, composed of mainly elastin and collagens\textsuperscript{55} (Figure 3). The major structural components of the tunica media are the elastic fibers, consisting of microfibrils and the protein elastin, which provides elasticity to the vessel, and the basement membrane structures connecting the lamellar layers\textsuperscript{53,54}. Next, to the VSMC layer is the external elastic lamina, which is an additional elastic matrix layer separating the tunica media and tunica adventitia of the vascular wall (Figure 3). Of note, the elastic laminas are only visible in the arteries and larger veins. Lastly, the tunica adventitia consists largely of collagens, mixed with glycoproteins and proteoglycans, providing stability and support\textsuperscript{53,54}.

**Development of vascular calcifications (VC) in CKD**

CKD patients are prone to develop two types of VC, namely intimal and medial VC, based on the location of the VC in the vascular wall\textsuperscript{56}. Intimal VC is initiated by high concentrations of low-density lipoprotein particles (LDL) entering the intima layer of the vascular wall. In response to LDL accumulation, ECs in this layer become activated, leading to recruitment and migration of immune cells, like monocytes and T lymphocytes, into the subendothelial space\textsuperscript{57–59}. Macrophages can take up oxidized-LDL from the intima layer, leading to cell death and foam cell formation. As macrophages continue to die, a necrotic core develops, which releases a variety of toxic molecules in the microenvironment. Next, VSMCs, which are normally located in the medial layer, gain a synthetic phenotype and migrate from the medial layer to the subendothelial space\textsuperscript{57–59}. Synthetic VSMCs secrete collagen, thereby causing fibrous cap generation and plaque progression. Additionally, micro calcifications are initiated at sites of dead macrophages and VSMCs. Intimal VC is further promoted by VSMC- and macrophage-dependent release of microvesicles, which contain increased levels of mineral complexes and decreased concentrations of calcification inhibiting proteins\textsuperscript{57,58}. In later stages of intima VC, VSMCs can acquire a bone-forming (osteoblast-like) phenotype, further promoting intimal VC\textsuperscript{57}.

As opposed to intimal VC, medial VC (also known as Mönckeberg’s sclerosis\textsuperscript{60}) progresses without atherosclerotic plaque development. Medial VC can develop both actively and passively\textsuperscript{61} (Figure 4). During active medial VC, VSMCs play an important role\textsuperscript{62–64}. In response to toxic stimuli, such as high phosphate or CPPs, VSMCs can undergo osteochondrogenic dedifferentiation, in which the contractile VSMC-characteristics are lost, and the VSMCs acquire an osteoblast-like phenotype\textsuperscript{62,64,65}. Additionally, VSMCs start to produce matrix metalloproteases (MMPs) which degrade the ECM, leading to new mineralization niches. Similarly to intimal VC, VSMCs can secrete microvesicles.
containing mineral complexes and calcification-promoting factors. Moreover, the secretion of calcification inhibiting factors decreases. Furthermore, apoptotic bodies and dead VSMCs act as hotspots for initiation of medial VC development\textsuperscript{62–64}. In contrast to active medial VC, it has been shown that medial VC can also develop passively (i.e., without active participation of the VSMCs)\textsuperscript{66–68} (\textbf{Figure 4}). As ECM proteins, such as collagen and elastin have a high affinity for calcium (Ca\textsuperscript{2+}), binding of these positively charged ions can attract phosphate (PO\textsubscript{4}\textsuperscript{3-}), initiating the mineralization process and promoting medial VC\textsuperscript{66–68}. Although both intimal and medial VC (in active and passive form) are present in CKD, medial VC is more abundant and therefore will be the main focus in this thesis\textsuperscript{69,70}.

\textbf{Figure 4: Vascular medial calcification.} Vascular medial calcification (medial VC) can develop via an active (left side) and/or passive process (right side) in the VSMC medial layer. During active medial VC, VSMCs gain an osteochondrogenic phenotype, increase secretion of pro-calcifying factors, decrease secretion of calcification inhibitors and produce mineral complex-containing microvesicles. Finally, apoptotic bodies and VSMC death lead to nucleation sites for medial VC. With passive medial VC, no active contribution of VSMCs is required, and calcium and phosphate, combined with CPPs, are passively precipitated on the extracellular matrix (ECM), leading to ECM mineralization and VC development. Figure is based on previous reports\textsuperscript{61,62,64,66,67} and created with BioRender.com.
Endothelial cells in health and disease
As previously mentioned, ECs form the inner lining of all blood vessels and play an important role in processes such as coagulation, barrier function, vascular tone and angiogenesis. Yet, in conditions of endothelial dysfunction, a state of reduced vasodilation, with pro-inflammatory and prothrombotic characteristics, ECs can be important contributors to the development of CVD. Regarding vascular tone, ECs are main producers of the gaseous compound nitric oxide (NO), via the enzyme endothelial nitric oxide synthase (eNOS/NOS3). NO induces relaxation of the VSMCs via the cGMP-dependent protein kinase G pathway. In ECs, eNOS deficiency reduces NO production and subsequently decreases the EC-dependent VSMC relaxation. Hence, impairment of the NO metabolism in ECs can result in reduced vascular relaxation, development of hypertension and hypertension-related CVD. Furthermore, generation of oxidative stress in ECs is also associated with CVD development. Oxidative stress in ECs can be generated via various pathways. First of all, there are several enzymes responsible for reactive oxygen species (ROS) production such as lipoxygenase, cyclooxygenase and NADH/NADPH oxidases. Additionally, the mitochondrial transport chain is an important source of ROS, as electrons which leak during mitochondrial respiration can react with oxygen (O2) and form superoxide radicals (O2-), in dysfunctional ECs. Interestingly, dysfunctional or uncoupled eNOS can also be a source of ROS. ROS are detrimental for ECs because they can react with proteins, DNA and other macromolecules, thereby altering their structure and function and further inducing cellular dysfunction. During atherosclerosis development, increased ROS levels can cause upregulation of endothelial activation markers intercellular adhesion molecule 1 (ICAM-1) and vascular cellular adhesion molecule 1 (VCAM-1) via a NF-κB-driven pathway. Upregulation of the EC activation markers allows immune cells to infiltrate the vascular wall, a process promoting atherosclerosis as described above. Likewise, in conditions of disturbed blood flow, inflammation or presence of toxic stimuli, the endothelial permeability increases (i.e., cellular junctions loosen or become disrupted, and distance increases between ECs), which favors immune cell transmigration and leakage of molecules into the subendothelial space and thereby promotes development of atherosclerosis. Given the multifaceted contribution of ECs to the (early) development of vascular diseases, ECs are considered important therapeutic targets in prevention or interference of CVD.

Aims of this thesis
In recent years a lot of research has been focusing on the effects of CPPs on VSMCs function, and how this might influence the development of CPP-induced medial calcifications in CKD. However, blood vessels are covered with a single layer of ECs which forms the physical barrier between the circulation and medial VSMCs. In vivo ECs, and not VSMCs, are the first cell type interacting with the circulating CPPs in the blood.
Nevertheless, the role of ECs in the pathology of CPP-induced medial VC is in its infancy and remains still elusive. As indicated in the previous paragraphs, EC dysfunction is considered an important contributor to the development of CVD. Therefore, in this thesis, the relation between CPPs and ECs will be investigated, proposing that the endothelium is an important yet overlooked cell type that actually drives the CPP-induced medial VC.

The overall aims of this thesis are as follows:
- To unravel whether CPPs induce EC activation and dysfunction
- To identify the effects of CPP-induced EC activation and dysfunction on the development of VC
- To translate the pre-clinical findings to a clinical setting and explore the potential contribution of ECs in CPP-induced medial VC in CKD patients

By investigating the potential role of ECs in the development of CPP-induced VC, the overall objective is to challenge the current dogma on the pathophysiology of VC, and placing ECs in a more central position of CPP-induced medial VC in CKD. This will lead to an improved understanding of the pathophysiological processes, with the ultimate goal to contribute to adequate treatment to prevent or reduce VC in CKD.

**Thesis outline**

This thesis consists of eight chapters, whereof a visual overview is provided in Figure 5. In Chapter 1 (this chapter), an introduction is given into the pathophysiology of chronic kidney disease (CKD), the calcium and phosphate homeostasis in CKD, the general morphology and function of the vascular system, the development of vascular calcifications (VC) in CKD, and the role of endothelial cells (ECs) in health and disease. Additionally, the overall thesis aims and the thesis outline are provided. Chapter 2 is a literature review on the current knowledge of calciprotein particles (CPPs). In this chapter, mechanisms of CPP formation are reviewed, as well as current knowledge on the role of CPPs in cardiovascular pathophysiology and the dynamics of CPPs in vivo. In the last part of this literature review, the clinical relevance, future perspectives and therapeutic implications of CPPs in cardiovascular disease are discussed.

Following the literature review, it is still unknown what the effect of CPPs on EC function is. Therefore, in Chapter 3, the first experimental chapter of this thesis, the interaction of CPPs and ECs will be investigated with the focus on the NO metabolism. Using an ex vivo porcine coronary artery model, the EC-dependent relaxation of VSMCs after CPP exposure will be evaluated. Possible alterations in the NO metabolism of ECs (including eNOS function and NO bioavailability), will be assessed in an in vitro setting. Findings will be related to a clinical setting via measurements of the calcification propensity and
nitrate and nitrite (NO\textsubscript{x}) in serum of CKD patients. Furthermore, as ECs form the first barrier interacting with the circulating CPPs in the blood, cross talk between ECs and VSMCs during CPP-induced VC development seems likely. In Chapter 4, paracrine signaling between ECs and VSMCs during development of VC will therefore be investigated, using a conditioned medium model. Proteomic analysis on the secretome of CPP exposed ECs will provide insight in the factor(s) possibly influencing the VSMC calcification process. To evaluate if the EC-derived secretome will altered VSMC function, a NanoString gene expression panel will be used to measure alterations in VSMC gene expression.

**Figure 5: Thesis outline.** Overview of thesis content per chapter. Abbreviations: calciprotein particles (CPPs), endothelial cells (ECs), vascular calcifications (VC), nitric oxide (NO), vascular smooth muscle cells (VSMCs) and chronic kidney disease (CKD).
As the outcomes of Chapter 4 indicate uptake of CPPs by ECs, in Chapter 5, we will further evaluate the effects of particle uptake by ECs on the endothelial calcium (Ca\(^{2+}\)) metabolism. Primarily, we will measure intracellular calcium concentrations in ECs after CPP exposure. Considering mitochondria as important organelles involved in the Ca\(^{2+}\)-regulation of ECs, cellular proteome analysis on ECs exposed to CPPs will be performed, to evaluate possible alterations in pathways involved calcium regulation and mitochondrial function of ECs. Additional analyses investigating mitochondrial function will be included as well. In the last part of this chapter, compounds will be screened as therapeutic opportunities to interfere with CPP-induced EC activation and dysfunction and subsequent cell death.

To translate our preclinical findings to a clinical setting, in Chapter 6 vascular tissue and serum will be collected from CKD patients and healthy kidney donors. Bulk RNA-sequencing, followed by gene set enrichment analysis (GSEA), will be performed on vascular biopsies of the CKD patients and healthy kidney donors, to identify alterations in gene expression patterns of ECs and VSMCs in the vascular biopsies. Also, gene expression data will be linked to circulating CPP levels and serum calcification propensity, to gain insights in the relation between CPPs and processes related to vascular pathology. Lastly, CKD patients will be subjected to computed tomography (CT) analysis, to evaluate presence of (macro) calcifications in the vasculature. In Chapter 7, the overall results presented in this thesis will be discussed. Our novel findings will be placed in the context of the current literature. Moreover, options for therapeutic intervention and future experiments will be discussed, and a general conclusion will be provided. Finally, Chapter 8 comprises a summary of the thesis.
REFERENCES


Chapter 1


Chapter 1


