Cardiovascular diseases (CVD), including vascular calcifications (VC), are major contributors to mortality and morbidity in patients with chronic kidney disease (CKD), as discussed in Chapter 1. CKD is characterized by a decline in renal function, which is associated with a rise in phosphate levels. Phosphate can bind calcium, and interact with serum proteins (e.g., Fetuin-A, albumin), initiating the formation of calciprotein monomers (CPMs, ~9 nm). CPMs can aggregate and form amorphous calcium and phosphate entities, known as primary calciprotein particles (primary CPPs, <100 nm). Over time, primary CPPs can mature into crystalline secondary calciprotein particles (secondary CPPs, >100 nm). This transition has been associated with increased risk for CVD. Accelerated secondary CPP maturation is associated with development of VC, and adverse outcome (increased mortality rates) in CKD. Nowadays, there is a growing body of literature describing the interaction of secondary CPPs and vascular smooth muscle cells (VSMCs). VSMCs are the primary cells affected by calcification in the vascular media and may actively participate in the development of VC. Unlike VSMCs, studies exploring the role of endothelial cells (ECs) during CPP-induced medial VC are largely lacking. In vivo, ECs rather than VSMCs, are the first cells interacting with circulating secondary CPPs. Moreover, EC dysfunction is considered an important hallmark of CVD and an inciting event in its pathogenesis. Therefore, in this thesis, the overall aim was to investigate the relation between secondary CPPs and ECs, thereby proposing that the endothelium is an important, but so far overlooked, cell type that actually drives CPP-induced medial VC. Throughout this summary, the term secondary CPPs will be referred to as CPP(s), unless specified differently.

Afore the contribution of ECs to CPP-induced VC was investigated, an extensive literature review was performed (Chapter 2), summarizing the current knowledge in the field on CPP formation, and the role of these particles in cardiovascular pathophysiology. In short, the body comprises a complex system of inhibitors that prevents extraosseous calcification (i.e., calcification processes other than in bones). Various proteins, including Fetuin-A, albumin, matrix γ-carboxylated glutamate protein (MGP) and γ-carboxylated glutamate-rich protein (GRP), bind free calcium (Ca\(^{2+}\)) and phosphate (PO\(_4^{3-}\)) and cluster in primary CPPs. Although under physiological conditions primary CPPs are considered harmless and function as calcium and phosphate scavengers in the body, conditions of phosphate overload cause primary CPPs to mature into harmful secondary CPPs. Various cell types have been described to interact with CPPs, among which macrophages, ECs and VSMCs. Studies investigating the effects of CPPs \textit{in vitro} are expanding, however, \textit{in vivo} studies appear far more challenging. Yet, several methods have been developed to study the \textit{in vivo} fate (i.e., kinetics of clearance and distribution) of the particles, including administration of (unlabeled or fluorescently labeled) CPPs to experimental animals. As CPPs appear to be an interesting biomarker for VC/CVD development, in the
human setting techniques to measure effects of serum components on CPP formation (crystallization time/calcification propensity/T_{50}) and actual serum CPP counts have been established but not yet implemented in routine clinical diagnostics. The final part of this review discussed various unresolved topics, among which the need for a detailed (molecular) description of CPPs *in vivo*, and the urgency to clarify the *in vitro*, but also *in vivo*, signaling pathways involved in CPP-induced VC.

Following the literature review, the next step was to explore the effects of CPPs on ECs in an experimental setting. Considering EC dysfunction as a driving process in CVD development, unravelling possible alterations in EC behavior and function in response to exposure to CPPs, was considered a first step in identifying ECs as critical players in CPP-induced VC. Accordingly, Chapter 3 describes studies on the interaction between ECs and CPPs, focusing on endothelial nitric oxide (NO) metabolism and oxidative stress. NO is a gaseous molecule produced by endothelial nitric oxide synthase (eNOS/NOS3) which promotes relaxation of the VSMCs and causes subsequent vasodilation. Using an *ex vivo* wire myography model with porcine coronary arteries, it was shown that CPPs significantly reduced EC-dependent relaxation of VSMCs. Furthermore, *in vitro* experiments performed to identify underlying pathways, demonstrated that both the expression and function of eNOS was reduced. Also, NO bioavailability was decreased, and this was accompanied by an increased superoxide radical (O$_2^-$) production, which is an indicative of eNOS uncoupling. Translation of the pre-clinical findings to a clinical setting demonstrated that patients with (early) CKD had an increased ability to form CPPs (*i.e.*, high calcification propensity/OD$_{650}$) which was significantly associated with decreased serum NO$_x$ levels as a measure for NO bioavailability. Collectively, this chapter showed that CPPs deteriorate EC function by impairing the NO metabolism and by inducing oxidative stress.

In Chapter 4, the interaction between ECs and VSMCs during CPP-induced VC was investigated. ECs and VSMCs are located in close proximity, and therefore it was hypothesized that paracrine signaling between these cell types might occur during development of CPP-induced VC. Paracrine EC-to-VSMC signaling was investigated using a newly established conditioned medium model, wherein ECs exposed to CPPs significantly enhanced VSMC calcification. To identify the factor(s) responsible for the observed increase in calcification, mass spectrometry was performed on the secretome of CPP-exposed ECs. Here, 84 differentially expressed proteins were detected between CPP-stimulated and unstimulated ECs. A literature search indicated that out of the differentially expressed proteins T-cadherin was of particular interest, as according to literature T-cadherin is secreted by stressed ECs, and overexpression of T-cadherin in VSMCs promotes transition towards a pro-calcifying phenotype. However, knockdown
of T-cadherin in ECs did not ameliorate the pro-calcifying effects of the EC-derived conditioned medium. Interestingly, the secretome of CPP-exposed ECs was further marked by proteins related to extracellular matrix remodeling and cation binding. To assess downstream effects of the conditioned medium on VSMCs, NanoString nCounter gene-expression analysis was performed showing upregulation of calcification-related transcription factors in VSMCs that were exposed to conditioned medium. Together, these experiments indicate that endothelial CPP exposure results in paracrine signaling from ECs to VSMCs and enhances calcification. Although the responsible factor(s) remain elusive, ECs are a potential target for intervention to ameliorate CPP-induced VC.

In Chapter 4, it was also shown that ECs take-up CPPs from the extracellular environment. In order to further understand the fate and functional consequences of CPPs once they are taken up, Chapter 5 focuses on the calcium metabolism of ECs in vitro. CPPs comprise mainly phosphate (PO\(_4^{3-}\)) and calcium (Ca\(^{2+}\)) ions, and therefore it was hypothesized that uptake of CPPs might impact the Ca\(^{2+}\) homeostasis, thereby causing CPP-induced EC dysfunction. Indeed, CPPs increased both the cytosolic and mitochondrial Ca\(^{2+}\) concentrations. The endoplasmic reticulum (ER) and mitochondria are the main cellular organelles responsible for the Ca\(^{2+}\) homeostasis. In conditions of cellular Ca\(^{2+}\) overload, Ca\(^{2+}\) levels in mitochondria can increase tremendously, leading to mitochondrial dysfunction. Using proteome analysis, expression of mitochondria-related proteins was determined. ECs showed decreased enrichment in proteins related to the mitochondrial oxidative phosphorylation (OXPHOS), specifically in respiratory complexes I-III. Functional respirometry measurements indicated impaired mitochondrial respiration in CPP-exposed ECs, accompanied by a reduced mitochondrial membrane potential, antioxidant capacity and decreased integrity of the mitochondrial reticulum. Finally, options to prevent the CPP-induced mitochondrial dysfunction were explored. In conditions of mitochondrial Ca\(^{2+}\)-overload, the mitochondrial permeability transition pore (mPTP) can be opened permanently, leading to a collapse of the mitochondrial membrane potential and diminished mitochondrial respiratory capacity. Collectively this will result in mitochondrial dysfunction. Exposure of ECs to CPPs in the presence of cyclosporin A (CSA), the inhibitor of mPTP opening, alleviated CPP-induced EC activation and promoted cell survival. Taken together, this chapter showed that CPPs indeed affect the Ca\(^{2+}\) homeostasis in ECs, which subsequently deteriorates mitochondrial function which was associated with increased EC activation and impaired survival. Interventions targeting mitochondrial dysfunction are promising therapeutic options for improving CPP-induced EC dysfunction.

The aim of Chapter 6 was to translate the preclinical findings to a clinical setting in CKD patients and explore the role of ECs in CPP-induced medial VC and vascular dysfunction.
Questions that were addressed included whether markers indicative of EC dysfunction and vascular remodeling are also observed in CKD vascular tissue, and how this relates to CPP counts in CKD patients? To answer these questions, vascular tissue was collected from healthy kidney donors (renal artery) and CKD patients (iliac artery) during kidney transplantation. Transcriptome analysis (using bulk RNA-sequencing) on healthy kidney donor and CKD patient vascular tissue allowed for gene set enrichment analysis (GSEA) and process identification. CKD vascular tissue was enriched for processes including endothelial activation, inflammation, extracellular matrix remodeling and ossification. Using computed tomography (CT) scans a pro-calcifying phenotype of CKD patients was confirmed as demonstrated by higher calcification scores in the abdominal arteries of CKD patients compared to the healthy kidney donors. Moreover, increased CPP counts (both primary and secondary CPPs), and elevated calcification propensity scores (lower T50, reduced crystallization time) were measured in sera of CKD patients. To link the pro-calcifying phenotype of CKD patients to the vascular tissue transcriptome profile, linear regression analysis was performed. Here, vascular remodeling markers were significantly associated with elevated primary and secondary CPP counts. Collectively, data presented in this chapter showed that CKD is characterized by systemic VC and a pro-calcifying circulatory environment with increased CPP counts. At the vascular tissue level this was associated with enrichment for processes related to vascular remodeling (including endothelial activation and inflammation), potentially priming for VC development.

In Chapter 7, the results described in this thesis are discussed in a broader context including the interrelationship between the different chapters and existing literature. Furthermore, still outstanding questions based on the experimental work are discussed. These include: Which EC-derived paracrine factor(s) is/are responsible for the enhanced VSMC calcification? If we can identify such factor(s), will this/these be target for intervention? Which treatment options for CPP-induced VC are currently known? Can the new findings described in this thesis contribute to development of future therapeutic interventions? Additionally, which experiments need to be performed to find answers to the outstanding questions and develop new innovative treatment options?

In conclusion, the work described in this thesis has undeniably shown that CPPs have an adverse effect on endothelium. The results furthermore indicate that ECs exposed to CPPs in vitro are able to enhance VSMC calcification. Based on the observations described in this thesis we conclude that the endothelium indeed is an important, but until now unjustly overlooked, cell type that is involved in CPP-induced VSMC calcification. We need to broaden our view towards development of CPP-induced VC in CKD, and in future research acknowledge the role of ECs in this pathology.