Chasing the perfect storm

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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