The calcium paradox

Societal impact

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Raising global awareness about the severity and impact of age-related diseases such as cardiovascular and bone disease has greatly increased in recent years[1]. Yet the deleterious effects on the elderly society are progressing and current therapies aiming to reduce bone loss and prevent soft tissue calcification are limited. The increasing ageing population brings with it the burden of lifestyle disease and thus huge socio-economic and public health care concerns[2]. Current therapies are costly, and their development is labour intensive and arrives with an adverse panel of side effects. For that reason, nutraceutical modulators are tested to elucidate their efficacy on disease progression. Dietary vitamin K attracts great attention as the "new kid on the block" in preventing debilitating symptoms of vascular calcification (VC) and reducing bone loss due to its multifactorial, yet specific roles. Therefore, supplemental vitamin K to modulate cardiovascular disease is a promising treatment.

Although calcium supplements are broadly used, their contribution as to whether they benefit bone health by reducing fracture risk and bone fragility is still a matter of debate[3]. Our review provides the latest take on the effects of calcium supplementation in post-menopausal women and the effect of calcium-based phosphate binders in patients suffering from chronic kidney disease (CKD). Here we discuss underlying molecular mechanisms of calcium intake in relation to ectopic calcification and bone metabolism. We imply that combining calcium supplements with vitamin K could reduce the risk of post-menopausal bone loss and simultaneously prevent VC by activating vitamin K-dependent proteins in both bone and vasculature. In the end, preventing the negatively associated vascular effects of calcium supplementation would be an easy, safe and cost-effective add-on for healthy living.

The increasing awareness of the presence of VC, and the notion that VC is an independent risk factor and predictor of CVD, has led to research models to investigate diagnosis and treatment of VC[4]. In chapter 3, we addressed the impact of VC using a basic research approach aiming to unravel molecular mechanisms in detail. Our pre-clinical approach would allow translation into clinical practice, to generate an impact on improving patient care. We developed a novel *in vivo* rodent model closely mimicking the clinical situation of CKD patients, known to be prone for VC. Our rodent model underwent ¾ nephrectomy receiving a high phosphate diet and was subjected to vitamin K deficiency by warfarin treatment. High phosphate levels and vitamin K deficiency are common risk factors in CKD patients[5,6]. Using our *in vitro* model we demonstrated that vitamin K2 (MK-7) co-supplementation with phosphate binders (PBs) could effectively reduce the magnitude of VC as compared to vitamin K2 or PB use alone. The use of calcium-based PBs was a standard treatment for many years, but because of the prejudicious effects via increasing VC are replaced by non-calcium-based PBs[7]. However, non-calcium based PBs lower phosphate levels yet do not improve cardiovascular disease in CKD patients[8]. This might be due to the ability of non-calcium-based PBs to bind, next to phosphate, also fat-soluble vitamins such as vitamin K. Although our in vivo study could not prove that PBs complex vitamin K thereby inducing vitamin K deficiency our data show that supplementation of vitamin K2 in combination with PBs results in less VC and cartilage calcification. This is supported by the fact that PBs increase dp-ucMGP levels, a vitamin K-dependent protein produced in vascular and cartilage tissue as a result of vitamin K deficiency[9]. To our knowledge, our rodent model is the first to address more precisely the clinical situation of CKD patients which could also be used by other researchers. Our data may serve as a scaffold for improving medical guidelines for the nephrology community and might aid standardisation of routine testing of vitamin K levels in the CKD population.

Rodent studies are an inevitable step in pharmaceutical, toxicological and nutritional research. With the advent of transgenesis, animal experimentation in these fields has even expanded, despite major efforts to reduce or substitute animal use. However, a patient is not simply a 75kg rodent, and translation of research from animals to humans is often not possible. Therefore, we embarked on using human induced pluripotent stem cells (iPSCs) cells to elucidate molecular mechanisms of bone formation *in vitro*. We implemented novel protocols for iPSCs differentiation towards induced mesenchymal stem cells (iMSC) to test the effects of vitamin K2 (MK-7) under osteogenic conditions.

A multitude of strategies trying to mitigate biological bone properties using primary cells exhibited unsatisfactory results mostly due to limited cell acquisition and availability. Generation and use of iPSCs prevent costly and time-consuming animal work and provide a limitless supply of patient-specific cells. It also circumvents the ethical implications associated with traditional primary stem cells surpassing the use of embryos or oocytes. To our knowledge, our model is the first to address a novel protocol of iPSCs differentiation using vitamin K2 (MK-7). Our iPSCs can be further translated into more advanced models, e.g. organoid cultures to glean and expand the database of molecular pathways underlying disease of interest. Of note, iPSC mediated bone formation is a noteworthy tool in the development of personalized medicine entangled to patients' specific needs as it is not subject to immune-reaction rejection.

In chapter 5, we used primary VSMCs and articular chondrocytes to assess *in vitro* molecular mechanisms of vascular and cartilage calcification. In the vasculature, VSMCs can under stress differentiate to osteo/chondrocyte-like VSMCs[10]. To unravel molecular pathological mechanisms leading to calcification could benefit clinical practice. Using our *in vitro* setup, we demonstrated the beneficial effects of vitamin K2 (MK-7) in VSMCs cultures, by preventing calcification. These data support the paradigm where vitamin K2 could be advocated as a dietary alternative treatment reducing or regressing cartilage degeneration and holding progression of vascular disease. Moreover, using this *in vitro* approach we could show that Amlodipine and Gabapentin, used in neuroscience research to inhibit calcium channel activity, reduce VSMC calcification thereby providing a novel therapy for VC.

In conclusion, my thesis puts forward a role for vitamin K in preventing VC and cartilage calcification in CKD. Moreover, I provide novel treatment options for reducing ectopic calcification by inhibiting calcium channels. Our data lay the foundation for further elucidating the context of bone loss and subsequent vascular disease progression.

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