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Review

Zinc Action in Vascular Calcification

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ABSTRACT: Although zinc's involvement in bone calcification is well-established, its role in vascular calcification, characterized by abnormal calcium and phosphorus deposition in soft tissues and a key aspect of various vascular diseases, including atherosclerosis, remains unclear. This review focuses on zinc's action in vascular smooth muscle cell (VSMC) calcification, including the vascular calcification mechanism. Accumulated research has indicated that zinc deficiency induces calcification in VSMCs and the aorta, primarily through apoptosis accompanied by a downregulation of smooth muscle cell markers. Moreover, zinc deficiency-induced vascular calcification operates independently of the action of alkaline phosphatase (ALP) activity, typically associated with osteogenic processes, but is partly regulated via inorganic phosphate transporter-1 (Pit-1). To date, research has shown that zinc regulates vascular calcification through a mechanism distinct from that of osteogenic calcification, providing insight into its dual effects on physiological and pathological calcification and thereby explaining the "zinc paradox," wherein zinc simultaneously increases osteoblastic calcification and decreases VSMC calcification.

Keywords: alkaline phosphatase, bone calcification, vascular calcification, vascular smooth muscle cells, zinc

INTRODUCTION

Calcification is the accumulation of calcium (Ca) and phosphorus (P) in body tissues, leading to tissue hardening. This process can occur normally in hard tissues, such as bone, or abnormally in soft tissues, such as blood vessels (Vorvick, 2022). The majority of Ca (>98%) entering the body is deposited in bones and teeth, with the remainder dissolved in the blood. When disorders disrupt the balance between Ca and other chemicals in the body, minerals can accumulate in various body parts, including the arteries, resulting in Ca deposits that affect blood vessel function. Atherosclerosis, initiated by Ca deposition in the arteries, progresses as pathogenic vascular calcification (Alexopoulos et al., 2012).

The precise mechanisms linking zinc to cardiovascular diseases remain under investigation. Apoptosis and inflammation, major consequences of zinc deficiency, are considered critical determinants of atherosclerosis and coronary heart disease, implying the occurrence of pathological calcification with disease progression (Beattie et al., 2008; Cho et al., 2008; Allen-Redpath et al., 2013; Ou et al., 2013). Conversely, zinc's stimulatory effect on osteoblasts and bone calcification has been reported. Specifically, insufficient cellular zinc levels disrupt extracellular matrix (ECM) calcification and matrix protein expression, thereby disturbing osteogenic calcification (Kwun et al., 2010; Alcantara et al., 2011; Seo et al., 2020; Lee et al., 2022).

In this review, we address the function of zinc in vascular calcification, including its mechanism. The review aims to provide insight into zinc's role in this pathological calcification process.

VASCULAR CALCIFICATION MECHANISM

Vascular calcification involves the deposition of calcium phosphate, forming hydroxyapatite in the blood vessels. Once considered a passive and degenerative process, vascular calcification is now acknowledged as a cellular regulatory process partly analogous to bone formation. It is increasingly evident that multiple factors and mutually exclusive mechanisms tightly regulate vascular calcification (Lau et al., 2010; Lee, 2011; Wu et al., 2013). To date, four mechanisms have been proposed to explain vascular calcification: apoptosis of vascular smooth muscle cells (VSMCs); loss of calcification inhibitors; circulat-

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ing nucleation complexes; and induction of osteogenesis, i.e., phenotypic changes in VSMCs transitioning them toward osteoblast-like cells (Giachelli, 2009; Leopold, 2015).

Apoptosis

Apoptosis is a programmed cell death mechanism crucial for development, homeostasis, and disease processes. Endothelial cell and VSMC apoptosis is implicated in blood vessel remodeling, injury, and various vascular pathologies, including atherosclerosis (Aravani et al., 2020).

Physiological calcification in bone and cartilage is believed to commence in matrix vesicles. These lipid bilayer membrane-bound vesicles, formed via "budding" of mineralizing cell membranes in various cells, such as osteoblasts, chondrocytes, and odontoblasts (Golub, 2009), contain essential calcification proteins, facilitating Ca and phosphate ion accumulation, leading to hydroxyapatite crystal formation. In atherosclerotic plaques, matrix vesicle-like structures are derived from VSMCs and contain the pro-apoptotic BCL2-associated X (BAX) protein, suggesting a link between these structures and apoptotic cells (Li et al., 2022). Although apoptotic bodies formed during apoptosis immediately suppress the inflammatory response, under pathological conditions, impaired apoptotic body clearance and increased matrix vesicle production are observed (Proudfoot, 2019). The resemblance between matrix vesicles and apoptotic bodies suggests a connection between apoptosis and calcification.

The hypothesis that VSMC apoptosis is involved in vascular calcification initiation is based on apoptotic body generation and Ca concentration. However, apoptosis alone is insufficient to induce calcification, indicating the presence of inhibitory pathways preventing calcification in normal VSMCs.

Loss of soft tissue calcification inhibitors

Physiological Ca and P levels are similar to the solubility constant of calcium phosphate in serum; thus, tissues are typically exposed to these concentrations. Therefore, a mechanism inhibiting soft tissue calcification is thought to exist, with inorganic pyrophosphate (PPi) known as a vascular calcification inhibitor. Notably, PPi is also present in bones but is degraded by the zinc-dependent enzyme alkaline phosphatase (ALP), allowing osteogenic calcification (Shanahan et al., 2011; Villa-Bellosta et al., 2011).

Other calcification inhibitors include matrix-Gla protein (MGP), osteopontin, and fetuin (Bjørklund et al., 2020). Active removal of calcification inhibitors is believed to suffice for the initiation of calcification in the ECM, as evidenced by calcification in MGP-null mice. Interestingly, such calcification does not affect the VSMC phenotype, indicating that VSMC transdifferentiation may not be a strict prerequisite for calcification (Rutsch et al., 2021). These observations emphasize the multifaceted nature of vascular calcification initiation mechanisms.

Circulating nucleation complexes

It has been proposed that vascular calcification is regulated by circulating nucleation complexes released from bone turnover (Leopold, 2015). These complexes, comprising calcium phosphate and proteins from bone remodeling, can induce vascular calcification. For example, osteoprotegerin (OPG), a receptor activator of nuclear factor kappa-β ligand (RANKL), has been associated with the presence and severity of arteriosclerosis and coronary artery disease. The association between bone and vascular calcification is evident in compounds resulting from bone turnover, and the underlying mechanisms of this interplay are currently under investigation. This bone-vascular axis for calcification may pose a potential risk akin to the osteoporosis-atherosclerosis axis observed in aging women (Demer and Tintut, 2010; Sage et al., 2010).

Induction of bone formation: VSMC phenotypic transition to osteoblast-like cells

VSMCs, highly specialized cells primarily responsible for blood vessel contraction and diameter regulation, exhibit remarkable plasticity and can undergo phenotypic changes in response to various phenotype-regulating stimuli (Bennett et al., 2016). Phenotypic switching of VSMCs evidently plays a major role in vascular calcification progression in atherosclerosis and other cardiovascular diseases (Durham et al., 2018). Our studies, among others, have revealed that VSMC transdifferentiation into osteoblast-like cells involves simultaneous downregulation of VSMC markers and upregulation of osteoblast marker genes (Alcantara et al., 2024). VSMC marker proteins, including smooth muscle 22 α (SM22α), SM α-actin, and calponin, are associated with the differentiated and contractile VSMC phenotype. Reduced expression of these markers, especially SM22α, may serve as a sensitive indicator of early transformation associated with the loss of VSMC properties. Conversely, increased expression of bone morphogenetic protein 2 (BMP2), a potent bone morphogen functioning in atherosclerotic plaque calcification, provides robust molecular evidence of active osteogenic processes contributing to vascular calcification beyond passive mechanisms (Durham et al., 2018).

VSMC phenotypic transition into osteoblast-like cells is exacerbated under high phosphate concentrations. As demonstrated by Villa-Bellosta (2021), elevated inorganic phosphate levels induce calcification in cultured human VSMCs, mediated by the sodium (Na)-dependent inorganic phosphate transporter-1 (Pit-1). Therefore, controlling both extracellular and intracellular phosphate levels can be crucial in vascular calcification and may help

mitigate VSMC transdifferentiation.

OSTEOGENIC CALCIFICATION MECHANISM

To understand their similarities, vascular calcification must be compared with osteogenic calcification, a physiological process mediated by bone-forming osteoblasts and bone-resorbing osteoclasts. Osteogenic calcification is a tightly regulated and sequential process. Bone is a dynamic tissue that undergoes constant remodeling to maintain its volume as well as calcium homeostasis (Murshed and McKee, 2010; Murshed, 2018). Its formation involves a complex cascade of osteoprogenitor cell recruitment and proliferation, ECM development and maturation, and finally, matrix calcification via differentiated osteoblasts.

Upon initiation of matrix synthesis, osteoblast marker genes are activated in a temporal sequence; ALP, collagen type I, and the parathyroid hormone/parathyroid hormone-related protein receptor are induced during early bone formation, whereas osteopontin and osteocalcin appear at later stages (Pujari-Palmer et al., 2016; Murshed, 2018). Subsequent to marker gene induction, mineralization of the collagenous ECM occurs, initiated by inorganic phosphate produced by ALP, leading to hydroxyapatite formation within the ECM.

The molecular mechanisms regulating osteogenic calcification are being elucidated, with accumulating evidence revealing the crucial roles of secreted noncollagenous proteins in ECM calcification and their structural contribution to ECM scaffolding properties. These mechanisms highlight the involvement of mineral-binding proteins, along with the scaffolding of collagen, ALP, and matrix vesicles, in either promoting or limiting calcification (Murshed and McKee, 2010). This model of osteogenic calcification contrasts with vascular calcification, which primarily occurs through passive mechanisms, such as apoptosis, suggesting active mechanisms are integral to bone formation.

ALP: A zinc-dependent enzyme

ALP is a ubiquitous plasma membrane-bound glycoprotein that catalyzes phosphate compound hydrolysis at basic pH values. It is expressed in various tissues as nontissue-specific ALP and in bone tissues as bone-specific ALP, wherein it produces inorganic phosphate that initiates bone calcification to form hydroxyapatite. As a main marker of active osteogenesis, missense mutations in the ALP gene, essential for bone calcification, disrupt bone mineralization and increase PPi concentrations (Brichacek and Brown, 2019; Vimalraj, 2020).

Initially, the role of ALP in calcification was attributed to its ability to generate free inorganic phosphate, thereby increasing inorganic phosphate levels necessary for calcification as hydroxyapatite in bone (Cho et al., 2007; Kim et al., 2009; Seo et al., 2010). Additionally, accumulated data also indicate that the primary action of ALP in soft tissue is the removal of PPi, a potent mineralization inhibitor present in the ECM (Villa-Bellosta et al., 2011). The role of ALP in vascular calcification is evident, given that ALP overexpression induces calcification in VSMCs and serum ALP is associated with coronary artery calcification (Lee et al., 2020; Abbasian, 2021).

In ECM calcification of osteoblasts, zinc partially contributes to regulating ALP activity. Indeed, zinc deficiency diminishes ALP activity, inhibiting ECM calcification (Kwun et al., 2010; Alcantara et al., 2011; Cho and Kwun, 2018a, 2018b). In contrast, zinc deficiency-related VSMC calcification is independent of ALP action, showing weak ALP activity and expression. In zinc-deficient VSMCs, P accumulation increases with increasing Na phosphate concentration but not with beta-glycerophosphate treatment, which requires ALP activity as a substrate for Pi generation. This suggests P accumulation in zinc-deficient VSMCs is independent of, or at least less affected by ALP, compared with osteoblasts (Beattie et al., 2012; Allen-Redpath et al., 2013; Alcantara et al., 2024).

Pit-1

Pit-1 regulates calcification by promoting intracellular phosphate uptake. Inorganic phosphate is a critical determinant of calcification, with its concentration regulated by both ALP and Pit-1. Although ALP, as a key regulator of osteogenic calcification, modulates local inorganic phosphate concentrations in the ECM by hydrolyzing pyrophosphate, Pit-1 regulates cellular inorganic phosphate balance through Na-dependent cotransporter activity (Crouthamel et al., 2013). Increased inorganic phosphate uptake by VSMCs via Pit-1 leads to cultured VSMC calcification, accompanied by phenotypic changes characterized by downregulation of smooth muscle cell marker proteins and upregulation of osteoblast markers toward an osteoblastic phenotype (Giachelli, 2009; Lau et al., 2010; Yao, 2010; Crouthamel et al., 2013)

In the bone matrix ECM microenvironment, both extracellular inorganic phosphate levels and cells' ability to actively transport phosphate are necessary for calcification. Although zinc's role as an essential component of ALP is well-established, its involvement in phosphate transport through Pit-1 remains unclear. Specifically, the role of zinc in regulating Pit-1 and ALP relative to vascular calcification is still uncertain. Zinc affects ALP activity, but ALP activity does not seem critical in VSMC calcification. Conversely, under zinc-deficient conditions, inhibition of calcification reduces ALP activity in osteoblastic cell models. However, zinc-deficient VSMCs exhibit enhanced Pit-1 expression, whereas zinc does not significantly affect its expression in osteoblastic cells

(Alcantara et al., 2024). These findings offer insights into the potential mechanisms underlying differential zinc-based regulation of osteogenic and vascular calcification via Pit-1.

ZINC AND THE CALCIFICATION PARADOX

As previously mentioned, although calcification is beneficial for hard tissue formation, including bone, it displays pathological aspects when associated with soft tissues, such as blood. Similarly, osteoporosis, characterized by low bone calcification and poor bone quality due to imbalanced bone metabolism, contrasts with vascular calcification as a pathological process, which is often accompanied by decreased bone mass and calcification. This paradoxical association, termed the "calcification paradox," lacks a fully understood mechanism (De Schutter et al., 2011; Zhang and Feng, 2017; Gu et al., 2020; Wang et al., 2022).

The calcification paradox raises questions regarding zinc's regulation of vascular calcification and whether zinc deficiency exacerbates or attenuates vascular calcification when regulatory mechanisms are similar under physiological and pathological conditions. Although zinc is a critical regulator of physiological processes, its role in the calcification paradox remains under investigation (Fig. 1).

ZINC ACTION IN VASCULAR CALCIFICATION

Vascular calcification exhibits distinct mechanisms compared with bone, providing insights into the "zinc paradox," i.e., zinc's ability to enhance osteoblast calcification while inhibiting VSMC calcification (Fig. 1). Zinc deficiency-induced VSMC calcification primarily results from VSMC apoptosis, and inhibition of apoptosis reduces calcification (Allen-Redpath et al., 2013; Shin and Kwun, 2013, 2014; Cho and Kwun, 2020). Additionally, zinc-deficient VSMCs undergo transdifferentiation into osteoblast-like cells, marked by downregulation of the smooth muscle cell marker SM22α and calponin as well as upregulation of osteoblast marker proteins, including runt-related transcription factor 2 (Runx2), osterix, and osteopontin (Beattie et al., 2008; Allen-Redpath et al., 2013). Prolonged exposure to zinc-deficient conditions exacerbates transdifferentiation and accelerates VSMC proliferation (Alcantara et al., 2013) (Fig. 2).

The notion that VSMC calcification occurs independently of (or is at least less affected by) ALP action, compared with osteoblasts, supports an apoptosis-mediated calcification mechanism under zinc deficiency (Allen-Redpath et al., 2013; Alcantara et al., 2024). Matrix vesi-

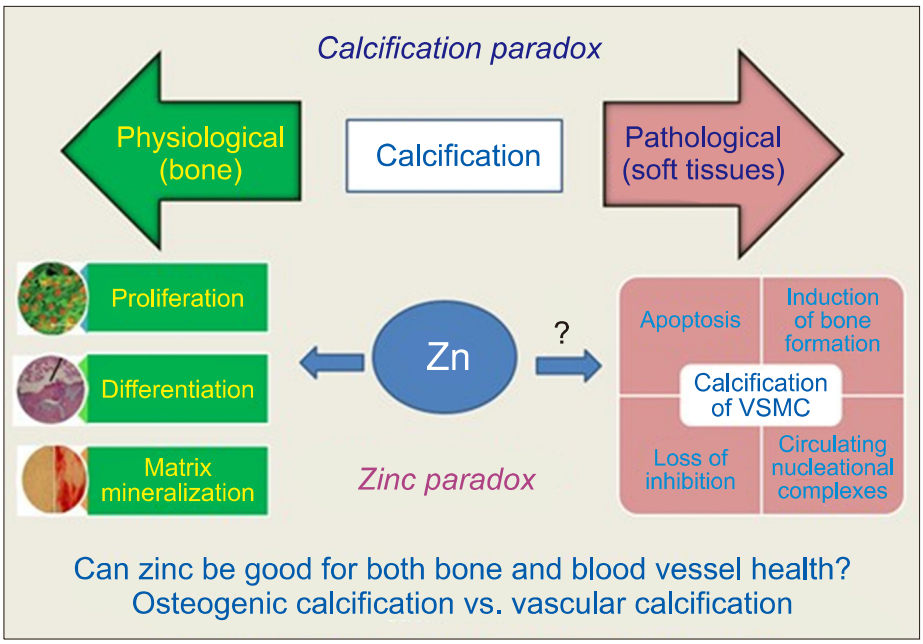

Fig. 1. Calcification paradox and vascular calcification mechanism. Zinc (Zn) is well-known for stimulating osteogenic calcification by enhancing osteoblast proliferation and differentiation while stimulating bone matrix mineralization (calcification); however, its role in vascular calcification remains unclear. Various studies have highlighted the dual action of Zn in osteogenic and vascular calcification, where Zn can promote osteogenic calcification, while decreasing vascular calcification. Proposed mechanisms for vascular calcification include the following: (1) Cell death leading to the release of apoptotic bodies by dying vascular smooth muscle cells (VSMCs), concentrating phosphate and calcium minerals and promoting vascular calcification. (2) Loss of circulating calcification inhibitor proteins normally present in soft tissues, contributing to vascular calcification. (3) Circulating nucleation complexes, primarily released during bone remodeling, potentially initiating vascular calcification. (4) Induction of bone formation characterized by phenotypic changes in VSMCs toward osteoblast-like cells, also capable of inducing vascular calcification.

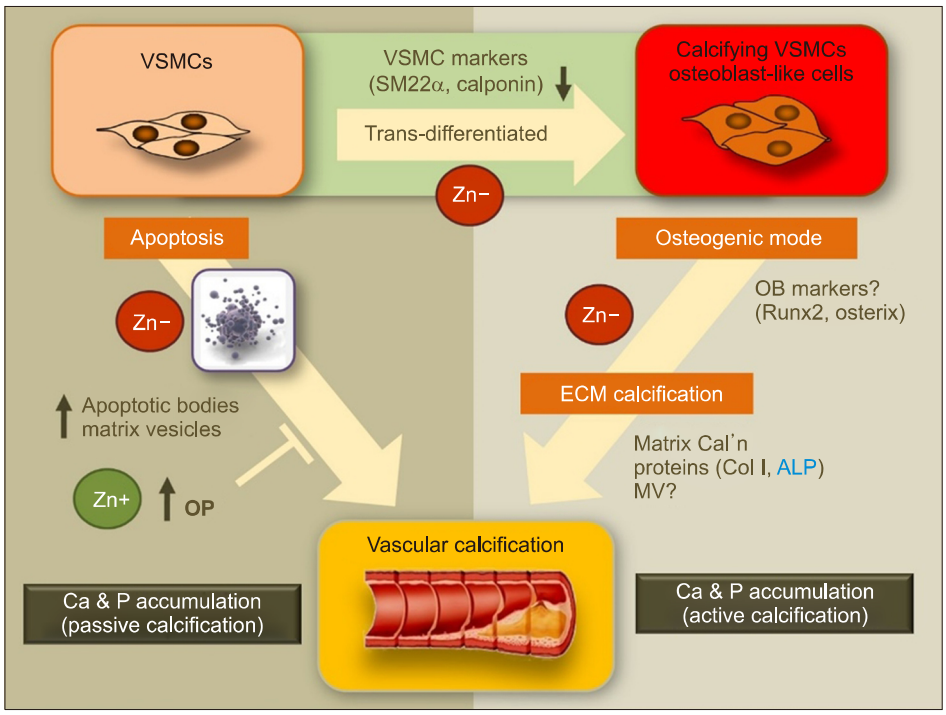

Fig. 2. Proposed model for zinc-mediated vascular calcification. (1) Vascular calcification induced by calcium (Ca) and phosphorus (P) accumulation can be triggered by vascular smooth muscle cell (VSMC) apoptosis. VSMC apoptotic bodies and matrix vesicles accumulate Ca and P, leading to VSMC calcification. Zinc (Zn) deficiency in VSMCs promotes apoptosis, thereby increasing VSMC calcification. The calcification inhibitor protein osteopotin can mitigate this calcification. (2) Zn deficiency alters VSMC characteristics toward a calcifying phenotype resembling osteoblastic cells. This alteration is marked by reduced expression of VSMC marker proteins [smooth muscle 22 α (SM22α) and calponin] and transdifferentiation of VSMCs into calcifying cells. (3) Once VSMCs trans-differentiate into calcifying cells (osteoblast-like cells), Zn deficiency further exacerbates vascular calcification, with distinct mechanisms observed for phosphate compound formation compared with osteogenic calcification. OP, osteopontin; OB, osteoblast; Runx2, runt-related transcription factor 2, a bone-specific transcription factor; ECM, extracellular matrix; Cal'n, calcification; Col 1, collagen type I; ALP, alkaline phosphatase; MV, matrix vesicle.

cle-bound ALP, a marker of active osteogenesis, is active in calcified VSMCs, indicating that ALP is not a major initiator of vascular calcification (Golub, 2009). This aligns with the notion that apoptotic bodies from dying VSMCs, structurally similar to matrix vesicles, serve as the primary initiators of vascular calcification under zinc-deficient conditions (Fig. 2).

Several epidemiological, clinical, and animal studies have associated zinc with cardiovascular disease development, with low serum zinc levels linked to coronary artery disease. Zinc is implicated in maintaining vascular endothelial cell integrity, essentially by inhibiting signal transduction pathways leading to caspase-dependent apoptosis (Seth et al., 2015), a postulated mechanism for initiating vascular calcification. Although our understanding of this mechanism is limited, accumulated data provide insights into zinc's regulation of VSMC calcification and its potential influence on vascular calcification under zinc deficiency.

Vascular calcification, a major risk factor for cardiovascular diseases, shares similarities with osteoblastic calcification, warranting attention. As zinc is crucial for osteoblastic calcification, exploring its role in vascular calcification is worthwhile. Based on the results of various studies, the potential mechanisms of zinc in vascular calcification are shown in Fig. 2.

First, *in vitro* studies show that zinc deficiency induces rat VSMC and aorta calcification via VSMC apoptosis, independent of an osteogenic mechanism. Thus, inhibition of apoptosis is associated with decreased VSMC calcification. Zinc deficiency downregulates VSMC marker proteins. *In vivo*, zinc deficiency increases aortic calcification while decreasing bone calcification, suggesting that bone mineral immobilization under zinc deficiency indirectly stimulates vascular calcification (Allen-Redpath et al., 2013; Shin and Kwun, 2013, 2014; Cho and Kwun, 2020). Second, zinc deficiency promotes rat VSMC calcification, independent of osteogenic ALP action, regulated by Pit-1. Zinc's regulation of Pit-1 may contribute to the loss of VSMC marker expression and increased calcification propensity, as blocking phosphate uptake via Pit-1 restores VSMC marker protein expression (SM22α and calponin) under zinc deficiency (Allen-Redpath et al., 2013; Alcantara et al., 2024). Third, although zinc deficiency initially reduces VSMC proliferation, it accelerates this process over the long term, suggesting zinc's involvement in mechanisms regulating VSMC proliferation and contribution to the loss of cell markers and atheroscle-

rotic calcification (Alcantara et al., 2013; Cho and Kwun, 2020).

CONCLUSION

Zinc has emerged as a critical regulator of vascular calcification. Given vascular calcification's association with several cardiovascular pathologies, zinc's protective effect against cardiovascular diseases may, in part, stem from its ability to inhibit this pathological process. Further studies are warranted to elucidate the precise mechanisms underlying this process and explore the potential of zinc in preventing both osteoporosis and atherosclerosis.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: YEC, ISK. Analysis and interpretation: JHK, YEC, ISK. Data collection: JHK, YEC, ISK, DKK. Writing the article: JHK, YEC, ISK. Critical revision of the article: DKK, YEC, ISK. Final approval of the article: all authors. Obtained funding: ISK. Overall responsibility: YEC, ISK.

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