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REVIEW

A new WNT on the bone: WNT16, cortical bone thickness, porosity and fractures

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The last decade has provided abundant data implicating the WNT pathway in bone development and in the regulation of skeletal homeostasis. Rare human mutations together with gain- and loss-of-function approaches in mice have clearly demonstrated that disrupted regulation of this pathway leads to altered bone mass. In addition to these rare human and mice mutations, large population-based genome-wide association studies (GWASs) have identified single-nucleotide polymorphisms in ~60 loci strongly associated with variations in bone mineral density (BMD) at different skeletal sites. Among the loci/genes identified by BMD GWAS, components of the WNT signaling pathway are numerous and have been shown to contribute to skeletal development and homeostasis. Within the components of WNT signaling, the gene coding for WNT16, one of the 19 WNT ligands of the human genome, has been found strongly associated with specific bone traits such as cortical bone thickness, cortical porosity and fracture risk. Recently, the first functional characterization of Wnt16 has confirmed the critical role of Wnt16 in the regulation of cortical bone mass and bone strength in mice. These reports have extended our understanding of Wnt16 function in bone homeostasis and have not only confirmed the unique association of Wnt16 with cortical bone and fracture susceptibility, as suggested by GWAS in human populations, but have also provided novel insights into the biology of this WNT ligand and the mechanism(s) by which it regulates cortical but not trabecular bone homeostasis. Most interestingly, Wnt16 appears to be a strong anti-resorptive soluble factor acting on both osteoblasts and osteoclast precursors.

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WNT Signaling and Skeletal Homeostasis

Skeletal homeostasis is maintained throughout life by the balance between bone formation by osteoblasts (which derive from mesenchymal cells) and bone resorption by osteoclasts (which have hematopoietic origin), regulated in part by the third bone cell type, the osteocyte, itself derived from osteoblasts. The adult skeleton continuously undergoes remodeling, and failure to balance these two processes can lead to skeletal diseases, such as osteoporosis, characterized by decreased bone mass, altered bone micro-structure and increased risk of fragility fractures.[1] Most studies have, however, focused on trabecular bone remodeling despite the fact that 80% of the skeleton is constituted by cortical bone.[2–4] The findings that with aging 80% of fractures are associated with cortical bone (non-vertebral fractures) indicate that cortical bone mass is a key determinant of bone strength.[2–4] Although the risk of vertebral fractures, which arise mainly at trabecular sites, is significantly decreased by the currently available anti-resorptive or anabolic treatments, the risk of non-vertebral fractures is reduced only by ~20%, confirming a dichotomy between the homeostatic regulation of the trabecular and cortical bone compartments[1,5–8]

One of the major signaling pathways involved in the regulation of bone homeostasis is the WNT signaling pathway.[9,10] Although we have learnt a lot about WNT signaling in bone in recent years, we still know little about the specificities among the various WNT ligands. In mammals, there are 19 WNT proteins that by engaging various WNT receptor complexes induce different signaling cascades to orchestrate several critical events important for the activity of mesenchymal progenitors, osteoblasts, osteocytes and osteoclasts.[11,12] WNTs are secreted cysteine-rich glycoproteins loosely classified as either 'canonical' or 'non-canonical', depending on their ability to activate β-catenin-dependent or -independent signaling events, respectively. In the canonical WNT pathway, activation of the frizzled-LRP5/6 receptor complex by WNT

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ligands leads to stabilization of cytosolic β-catenin, translocation into the nucleus and subsequent activation of canonical Wnt target genes (**Figure 1a**). Importantly, WNT ligands function with an entourage of receptors, co-receptors, agonists and antagonists that either enable or prevent Wnt signaling activation (**Figures 1a and b**).[9,11]

The role of canonical WNT signaling in skeletal homeostasis has been emphasized by the findings that in humans loss- and gain-of-function mutations in *LRP5* result in skeletal diseases characterized by low bone mass and high bone mass, respectively, and that mutations in sclerostin, an inhibitor of WNT–β-catenin signaling encoded by *SOST*, cause sclerosteosis and van Buchem disease both characterized by high bone mass.[13–16] Numerous studies in genetically modified mouse models have confirmed the phenotype seen in these human mutations and proved that while activation of the canonical WNT pathway results in higher bone mass, its inhibition is associated with decreased bone mass, although the relative impact of distinct components of this signaling machinery on bone formation and bone resorption can differ.[9]

Although the role of canonical Wnt signaling in postnatal bone homeostasis has been intensively investigated, the function of non-canonical WNT signaling, in which WNT ligands engage frizzled receptors alone or together with co-receptors such as the receptor tyrosine kinase-like orphans Ror2 or RYK, remains elusive in bone[9] (**Figure 1c**). Indeed, it has been shown that in mice Wnt7b regulates osteoblast differentiation and bone formation via the $G\alpha_{q/11}$-Pkcα non-canonical cascade[17] and that the Wnt5a-Ror2-Jnk signaling is involved in bone homeostasis.[18] Recently, a distinct β-catenin-independent cascade, the Wnt-mTORC1 (mammalian target of rapamycin complex1) signaling, has also been proposed to have a role in the regulation of bone mass.[19] Although the general thought is that β-catenin-dependent and -independent pathways activate distinct downstream signaling, it is becoming clear that: (1) several WNT ligands can signal through both pathways depending on the cellular context, the nature of receptors and co-receptors as well as the presence of specific co-activators and antagonists and (2) the interplay between different signaling cascades ultimately induces specific and distinct effects on cortical and trabecular bone mass. As the complexity of the WNT ligands and their signaling is reviewed elsewhere[9,11,12], we focus here on what is currently known about the role of WNT16 in the regulation of cortical bone homeostasis.

Genome-Wide Association Studies of Skeletal Phenotypes Reveal WNT16 as a Key Determinant of Cortical BMD and Thickness

In adults, bone mineral density (BMD) is measured at sites where osteoporotic fractures occur more often, such as lumbar spine, hip and forearm. BMD is used for the diagnosis of osteoporosis and is the best predictor of fracture risk.[20,21] Osteoporosis is a complex disease resulting from both environmental and genetic factors. More than 40 distinct GWAS and meta-analyses of GWAS for osteoporosis and related traits have been published in the last 7 years, mainly focused on variations in BMD of spine and hip. Across all these studies, ~60 loci involved in key pathways that contribute to the pathophysiology of osteoporosis, including bone mass and fracture susceptibility, have been identified and have confirmed the strong regulation exerted by the RANKL and WNT signaling pathways (reviewed in references 22–24).[25–27]

Although the role of some of the loci/genes identified by BMD GWAS lack experimental confirmation of their involvement in skeletal homeostasis, several have been shown to contribute to skeletal phenotypes and to be involved in signaling pathways regulating bone mass. Thus, known key master genes of skeletal development (such as *SOX9* and *RUNX2*), mesenchymal cell differentiation (including *RUNX2* and *SP7*) and regulation of osteoclast differentiation (*RANK-RANKL-OPG* (osteoprotegerin)) have been associated with BMD in humans.[22,23] Importantly, some of these genes/loci, including *RANK, RANKL and OPG,* have been replicated across studies[22] and their function characterized through cellular and molecular functional studies, as well as by generating knockout, knockin and conditional knockout mouse models. Among the 60 loci shown to be strongly associated with BMD variations, several loci cluster within the Wnt signaling pathway including *AXIN1,*[22] *CTNNB1,*[25,28,29] *DKK1,*[25] *LRP4,*[25,28,30] *LRP5,*[22,26,28] *MEF2C,*[25,28] *RSPO3,*[22,29] *SFRP4,*[25,29] *SOST,*[22,25,29,31] *WLS,*[22,28,32] *WNT3,*[25] *WNT4*,[25] *WNT5B*[25] and *WNT16*.[22,25,33–38]

Recently, two distinct GWAS meta-analyses for cortical bone thickness (three cohorts with 5878 European subjects) and for forearm BMD (five cohorts with 5672 samples) have shown that missense polymorphisms of the *WNT16* locus in adults are strongly associated with cortical bone thickness, forearm BMD and with osteoporotic fractures.[38] In addition, a genome-wide association scan for total-body BMD variation in 2660 children of different ethnicities and GWAS focused to identify potential genetic loci influencing peak bone mass in premenopausal women have also identified missense single-nucleotide polymorphisms located on the *WNT16* locus.[37,36] Furthermore, recent studies have further demonstrated that several novel genetic variations in and around the *WNT16* locus are strongly associated with BMD at different skeletal sites and have concluded that *WNT16* positively affect BMD and bone strength, particularly at cortical sites.[33,34,35]

Therefore, all these studies indicated that WNT16 is a key determinant of cortical bone mass and is associated with risk of fracture in humans. In addition, WNT16 might have a role in determining bone accrual and peak bone mass, known to be important determinants for the risk of osteoporosis in adulthood. Importantly, as discussed below, the functional relevance of this member of the WNT family in the regulation of bone and mineral homeostasis has now been confirmed in both *in vitro* and *in vivo* animal studies.

WNT16 and the Regulation of Bone Homeostasis

During mouse skeletal development, *Wnt16* is selectively expressed in the perichondrium and periosteum[39] and, consistent with its role in cortical homeostasis, *Wnt16* is highly expressed in cortical bone in postnatal bones.[40]

Recently, our studies have shed light on the role of Wnt16 in postnatal bone homeostasis in mice and in the mechanism(s) by which this WNT ligand affects bone biology.[40,41]

The first study demonstrating that Wnt16 contributes to cortical bone mass in mice was part of the Lexicon's Genome5000 program focused to characterize mouse knockout phenotypes of genes with pharmaceutical potential. These initial findings were included in two BMD GWAS, in which

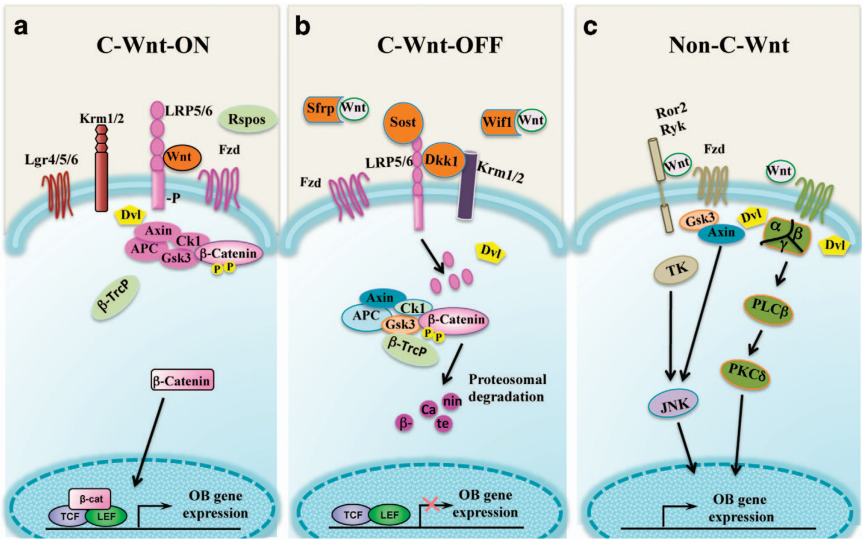

Figure 1 *WNT* signaling. (**a**) Canonical WNT signal on. Binding of Wnt ligands to the frizzled (Fzd) family of receptors activates the cytoplasmic signaling protein Dishevelled (Dvl), which in turn recruits the axin-glycogen synthase kinase 3 (GSK3) complex, leading to LRP5/6 phosphorylation. LRP5/6 phosphorylation prevents phosphorylation of β-catenin and thereby its degradation. R-spondin (Rspo) proteins are secreted agonists that enhance activation of canonical WNT signaling. Subsequently, β-catenin accumulates in the cytoplasm and enters the nucleus to initiate gene transcription. (**b**) Canonical WNT signal off. In the absence of WNTs, or when secreted WNT inhibitors such as Dickkopf1 (Dkk1), sclerostin (Sost) and secreted frizzled-related proteins (Sfrps) antagonize WNT signaling by either binding directly to the receptors or by functioning as decoy receptors for WNT proteins, the key protein β-catenin is phosphorylated by the destruction complex and degraded by ubiquitin-mediated proteolysis in the cytosol. Tcf/Lef assembles a transcriptional repressor complex to silence WNT target genes. (**c**) Non-canonical WNT signaling triggers its effects through alternative pathways including WNT/Rho-Rac and WNT/G-protein coupled receptors. In these pathways, WNT ligands signal through the Fzd receptors, or directly through membrane receptors such as Ror2 and Ryk, and dependently or independently of Dvl lead to the activation of multiple distinct downstream effectors, which eventually affect expression of genes involved in osteoblast differentiation.

the *WNT16* gene was found to be strongly associated with cortical bone thickness and fracture susceptibility in humans.[38,37] Indeed, micro CT analyses of the femoral diaphysis of 24-week-old mice demonstrated that Wnt16 deletion leads to a significant reduction in cortical thickness, cortical cross-sectional area and bone strength with a more severe phenotype seen in female than in male mice.[38]

Our recent investigations have demonstrated that global deletion of Wnt16 in mice is associated with a thinner cortical bone, consistent with the GWAS observations in humans, and suggesting that the single-nucleotide polymorphisms observed in these studies likely induce some loss of function (**Figure 2**).[19,40] In mice, we observed that this cortical thinning is mainly due to an increase in the number of osteoclasts on the endocortical surface, linked to an increased cortical *Rankl*/*Opg* ratio.[40] In addition, a lower, but non-significant, periosteal mineral apposition rate was seen during bone development that may also contribute to the thinning of the cortical bone. Importantly, the lack of Wnt16 leads to higher cortical bone porosity and lower bone strength that, together with a lower cortical thickness, results into spontaneous diaphysal fractures in these mice.[40]

Supporting the biological relevance of Wnt16 in cortical bone during skeletal growth and maintenance are the findings that the effect of Wnt16 deletion impacts cortical bone at 5 weeks of age, a time of skeletal growth in mice, and persists at 16 and 24 weeks of age, a time of skeletal remodeling.[40]

Despite its effect on cortical bone, Wnt16 deletion does not affect trabecular bone mass, suggesting that Wnt16 is a strong determinant of cortical bone mass, but it is not required for trabecular homeostasis, most likely due to the fact that other WNTs can compensate for the lack of Wnt16 in trabecular but not in cortical bone. Importantly, this differential effect of Wnt16 on cortical and trabecular bone confirms the emergent hypothesis of differential homeostatic regulation between the cortical and the trabecular bone compartments.

Wnt16 is predominantly expressed in osteoblasts and, consistent with a positive role of Wnt16 on bone homeostasis, removal of Wnt16 from the early osteoblast stage onwards (Runx2-creWnt6$^{fl/fl}$) leads to a phenotype similar to that seen with global deletion, suggesting that Wnt16 expressed by early osteoblasts during development and skeletal growth is required for proper cortical bone homeostasis but not for trabecular bone.[40] The findings that mice lacking Wnt16 in both mature osteoblasts and osteocytes (Dmp1-creWnt16$^{fl/fl}$) display a modest but significant decrease in cortical bone thickness only with aging indicate that the contribution of the osteocytes to Wnt16 production in long bones is relatively small and that Wnt16 expressed by osteocytes contributes only modestly to cortical bone homeostasis.

WNT signaling affects the activity and function of the entire osteoblastic lineage, including mesenchymal stem cell, osteoblasts and osteocytes. However, WNT ligands also directly affect osteoclasts and their precursors.[9] Importantly, the lack of Wnt16 does not significantly affect osteoblast proliferation and differentiation but decreases OPG production by these cells.[40] Conversely, treatment of osteoblasts with Wnt16 leads to increased *Opg* expression.[40] Consequently, mice lacking Wnt16 displayed normal osteoblast function but higher osteoclast number in the endosteal surface of cortical bone, a surface where Wnt16 is highly expressed. Lack of Wnt16 results in a local decrease in *Opg* within cortical bone that in turn leads to increased *Rankl*/*Opg* ratio, explaining the increase in endocortical osteoclast numbers and the decrease

in cortical bone thickness. Importantly, and in addition to the osteoblast-mediated change in the *Rankl*/*Opg* ratio, Wnt16 significantly inhibits Rankl-induced osteoclastogenesis of mouse bone marrow macrophages and human $CD14^+$ peripheral blood monocytes, indicating a direct action of Wnt16 on osteoclast precursors. Osteoclast precursors and mature osteoclasts do not, however, express Wnt16, the main local source of Wnt16 being the osteoblast. All together these results identify osteoblast Wnt16 as a key local regulator of osteoblast-to-osteoclast communication and cortical bone mass (**Figure 3**).

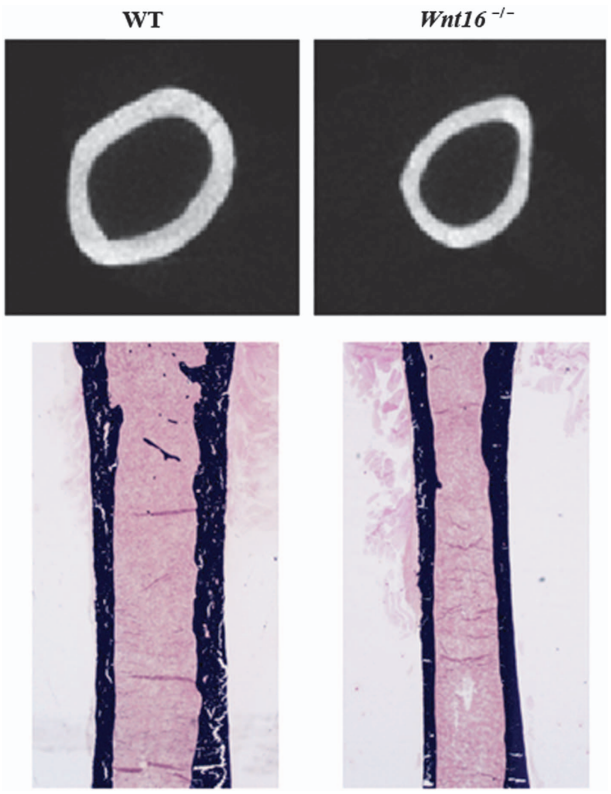

Figure 2 Deletion of WNT16 in mice decreases cortical bone thickness, representative μCT and histological images of 16-week-old WT and *Wnt16*$^{-/-}$ female mice. WT, wild type.

From a mechanistic point of view, Wnt16 can signal via both canonical and non-canonical WNT cascades. Hence, although in zebrafish, in human keratinocytes or in human hematopoietic stem cells WNT16 has been proposed to signal via the JNK non-canonical cascade, studies in synovial joints, in rat chondrocyte cell line RCS, in leukemia, in human articular cartilage and in prostate tumors have shown that Wnt16 functions in a β-catenin-dependent manner.[42,43–49] These findings imply that the signaling cascade and downstream effectors triggered by Wnt16 might depend on the cell type, as well as on the presence of specific receptors, co-receptors, antagonists and agonists. With regard to bone, we and others have recently shown that in osteoblasts Wnt16 signals via the canonical WNT cascade.[40,41] However, in contrast with the emergent hypothesis that Wnt16 has a positive effect on bone (similar to activation of canonical WNT signaling) and with the findings that Wnt16 deletion leads to decreased WNT signaling in cortical bone, Hendrickx *et al.*[34] have reported that one of the WNT16 single-nucleotide polymorphisms, which falls in the Kozak sequence of WNT16 and is associated with increased WNT16 translation and increased BMD, does not activate β-catenin-dependent signaling. This suggests that either Wnt16 is a weak canonical WNT ligand in osteoblasts and/or that its ability to activate the canonical cascade may be dependent on multiple stochastic changes in the levels of biological factors in the micro-environment and on the presence and/or variations in the levels of specific receptors, antagonists and agonists. In this context,

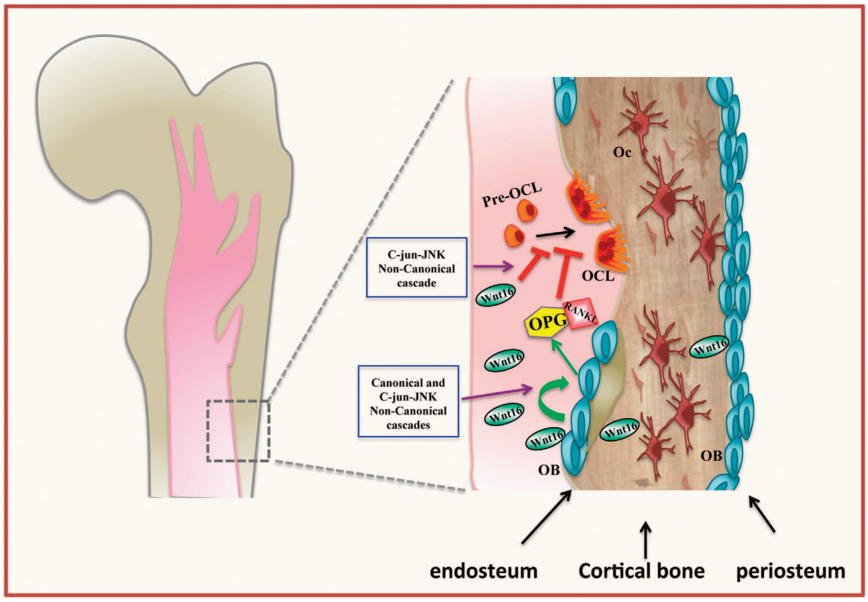

Figure 3 Model for WNT16 function in regulation of cortical bone. Wnt16 is expressed in osteoblasts lining the cortical bone and maintains cortical bone homeostasis by regulating endocortical resorption. Osteoblast-expressed Wnt16 signals via the canonical WNT pathway to regulate *opg* expression, which in turn functions as a decoy receptor for *rankl* expressed by osteoblasts and osteoclasts and regulates osteoclastogenesis. Osteoblast-expressed Wnt16 also functions directly on osteoclast precursors to regulate osteoclastogenesis via a non-canonical JNK cascade. OB, osteoblasts; Oc, osteocytes; OCL, osteoclasts.

the findings that Wnt16 can also activate the non-canonical JNK cascade in osteoblasts[40] and that it signals only via a non-canonical cascade in osteoclasts[40] indicate that Wnt16 activates both β-catenin-dependent and -independent signaling in the skeleton and that the mechanisms coordinating these two cascades have ultimately broad implications for the regulation of skeletal homeostasis by Wnt16 (**Figure 3**).

As within the last decade both human and mouse genetic data point to the positive modulation of skeletal homeostasis by the WNT pathway, both academia and industry have extensively invested in exploring several therapeutic approaches to target this pathway to increase bone mass. Thus, blocking the function of WNT antagonists, such as sclerostin and Dkk1, has emerged as a promising approach to activate canonical WNT signaling and thereby increase bone formation.[1,50] In this context, the findings that when locally delivered Wnt16 prevented inflammation-induced bone loss in mouse calvarial bone and increased BMD in rat tibiae[40] strongly suggest that Wnt16 might have a beneficial effect on cortical bone mass and on the risk of fracture, acting both as a strong anti-resorptive and an anabolic ligand.

Conclusion

These studies suggest that WNT16, a gene shown to be strongly associated with cortical bone thickness and fracture susceptibility in humans, regulates cortical bone thickness, and this primarily through inhibition of endocortical bone resorption. Our observation also supports the emerging hypothesis that the homeostasis of the trabecular and cortical bone compartments is, at least in part, differentially regulated.

The findings that in mice deletion of Wnt16 affects only cortical bone might be explained by different levels of expression of Wnt16 in the cortical and trabecular compartments. In addition, the presence of receptors and co-receptors as well as of the entourage of agonists and antagonists that keep a balanced WNT signaling might contribute to this compartment-specific response. Alternatively, given the known interplay between canonical and non-canonical WNT signaling with other signaling pathways, it is possible that in the absence of Wnt16 alternate pathways contribute to the maintenance of trabecular homeostasis but are absent from cortical bone. These different responses may also be due to the fact that the trabecular and the cortical bone contain different populations of cells generating a different cellular microenvironment, due to the presence of abundant hematopoietic and vascular cells in the trabecular compartment. Finally, it is possible that the different biomechanical loading conditions in trabecular and cortical bone are responsible for the different responses of these two compartments we observed in the absence of Wnt16.

Studies on the mechanisms by which Wnt16 affects bone homeostasis may help shed light of the molecular basis for the distinct biology of these two bone compartments. Importantly, further investigations on this key determinant of cortical bone thickness may open novel avenues for the development of therapeutic strategy for the prevention and treatment of osteoporotic-induced non-vertebral fractures, an unmet medical need.

Conflict of Interest

The authors declare no conflict of interest.

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