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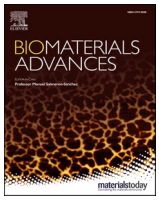
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Review

Mechanistic insights into the spontaneous induction of bone formation



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ABSTRACT

The grand discovery of morphogens, or "*form-generating substances*", revealed that tissue morphogenesis is initiated by soluble molecular signals or morphogens primarily belonging to the transforming growth factor-β (TGF-β) supergene family. The regenerative potential of bone rests on its extracellular matrix, which is the repository of several morphogens that tightly control cellular differentiating pathways, cellular matrix deposition and remodeling. Alluringly, the matrix also contains specific factors transferred from the heterotopic implanted bone matrices initiating "*Tissue Induction*", as provocatively described in Nature in 1945. Later, it was found that selected genes and gene products of the TGF-β supergene family singly, synchronously, and synergistically mastermind the induction of bone formation. This review describes the phenomenon of the spontaneous and/or intrinsic osteoinductivity of calcium phosphate-based biomaterials and titanium' constructs without the applications of soluble osteogenetic molecular signals. The review shows the spontaneous induction of bone formation initiated by Ca++ activating stem cell differentiation and up-regulation of *bone morphogenetic proteins* genes. Expressed gene products are embedded into the concavities of the calcium phosphate-based substrata, initiating bone formation as a secondary response. Pure titanium's substrata do not initiate the spontaneous induction of bone formation. The induction of bone is solely dependent on acid, alkali and heat treatments to form apatite layers on the treated titanium surfaces. The induction of bone formation is achieved exclusively by apatite-based biomaterial surfaces. The hydroxyapatite, in its various forms and geometric configurations, finely tunes the induction of bone formation in heterotopic sites. Cellular differentiation by fine-tuning of the cellular molecular machinery is initiated by specific geometric modularity of the hydroxyapatite substrata that push cellular buttons that start the ripple-like cascade of "*Tissue Induction*", generating newly formed ossicles with bone marrow in heterotopic extraskeletal sites. The highlighted mechanistic insights into the spontaneous induction of bone formation are a research platform invocating selected molecular elements to construct the induction of bone formation.

1. Introduction

As we composed this review on how to engineer self-inductive osteogenic matrices, the journal Science published a special review issue on the "*Powerful and momentous potential of cell engineering*" [1]. We thus decided to use Science's cover story to start this review by quoting the platform ahead for the new era of biological engineering, which uses the modularity of cells as building blocks to translate cellular engineering [2]. The review reports one of the basic principles of cell engineering, i.e. how the programming of biological functions is to controllably "push a cell's button" [2]. In context, the geometric modularity of the substratum is an example that triggers regulatory gene expression changes to drive the induction of bone formation.

The induction of bone formation is at the crux of the basic cell engineering paradigm, whereby soluble osteogenic signals pre-combined with insoluble substrata and/or signals [3] initiate the programming to push cell's buttons [2] to activate the cascade of "Tissue Induction" [4] (Tables 1, 2).

In his Nature paper on "Tissue Induction" in 1945, Levander [4] "made the intellectual connection between the induction of bone in mesenchyme and the studies on embryonic development by Hans Spemann of Freiburg University, who had recently received the 1935 Nobel Prize for Medicine. Spemann and his student Hilde Mangold had shown that a region of the gastrula embryo, called the organizer, could

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Table 1
Abbreviations.

μCT	Micro-computed tomography
ALP	Alkaline phosphatase
BMSCs	Bone marrow mesenchymal stem cells
BMP	Bone Morphogenic Protein
Ca	Calcium
DBM	Demineralised bone matrix
hSC	Human stem cells
MSC	Mesenchymal stem cell
qRT-PCR	Quantitative Reverse Transcription PCR
OP	Osteogenic Protein
PDGFR	Platelet derived growth factor receptor
RUNX2	Runt-related transcription factor 2
SSPC	Skeletal stem progenitor cells
TGF	Transforming Growth Factor
VEGFA	Vascular endothelial growth factor A

Table 2
The transforming growth factor-β (TGF-β) supergene family of proteins:

Specificity and Redundancy	
BMP-1	neg. [133]
BMP-2	pos. ++ [134]
BMP-3	neg. [135]
BMP-4; BMP-5	pos. + [136]
BMP-6	pos. ++ [137]
BMP-7 (OP-1)	pos. ++ [15]
OP-2	pos. +++ [138]
TGF-β1, TGF-β2	pos. +++++ [17]†
TGF-β3	pos. ++++++ [53,77]†
Activin, Inhibin	??
DPP	++ [79]
60 A	++ [79]
Vg-1	?
GDF-1, −2, −3, −9	?
BMP-9	+ [139]*
BMP-12; BMP-13	neg. inhibition [140]
Naturally derived highly purified osteogenic fractions	pos. ++ [35–37]

† Only in primates' species.
* In vitro osteogenic differentiation.

differentiate neighboring cells into different tissue types. He called this process embryonic induction" [5].

Systematic experimentation on the *de novo* bone formation by self-inducing biomimetic matrices has mainly focused on the insoluble signal, which initiates the spontaneous and/or intrinsic induction of bone formation [6–10]. Fundamental research for the biological understating of the "Bone induction principle" [11] stressed that mechanical reconstitution of osteogenic soluble molecular signals with different insoluble signals and/or substrata initiate the induction of bone formation. [3,12–18].

Incisive contributions by Reddis' experimentation reported the crucial role of responding cells and stem cells [3], translated in "*Tissue transformation into bone* in vivo" [19]. The reported tissue transformation highlighted how soluble signals, mechanically reconstituted with insoluble substrata or signals, could be manipulated to generate heterotopic ossicles, including newly formed mandibles and femoral heads. These ossicles were formed by injecting highly purified naturally derived osteogenic proteins within rodents' *rectus abdominis* muscle flaps inserted into tissue moulds replicating long bones and mandibles [19].

A classic article that should be quoted when reviewing the hydroxyapatite-induced osteogenesis model or the spontaneous and/or intrinsic induction of bone formation, defined in three papers from our research laboratories [9,20,21], is the paper of Hulbert and co-workers [22] that reported on the capability of ceramic materials to act as permanent implantable skeletal prostheses [22]. The manuscript first established the biological concept of the "*physiological compatibility with ingrowth of natural bone*" into ceramic constructs in canines' femoral defects. Such ceramics have interconnected porous networks to facilitate bone growth and/or invasion within the porous spaces [22].

The paper reports on a crucial study highlighting the growth of bone in ceramic pellets following implantation in dog femurs [22]. The study reported that the ideal bone ingrowth into the bioceramics occurred in specimens with pore size over 100 μm (i.e., 100–150 and 150–200 μm). The study showed that the ceramic material was highly compatible with the recipient bone [22]. A concluding statement opened the biological horizon to the future hydroxyapatite plasma-sprayed technologies. This insight provided biological coatings for implantable constructs in animal models with translational capacity in human patients [22–24].

At the same time, however, using the word "*inert porous ceramic materials*" failed to convey the critical role of bioceramics' bioactivity [22]. The concept of bioceramics' bioactivity indeed revolutionized tissue engineering strategies, setting into motion the identification of newly designed macroporous bioreactors instilled with the remarkable capability to initiate heterotopic induction of bone formation [25–34].

Of great interest, Hulbert et al. [22] reported that the larger pores sizes, i.e. 300 μm, allowed for a greater number of blood vessels and vascular invasion (Fig. 1A). Macroporous spaces were highly vascular

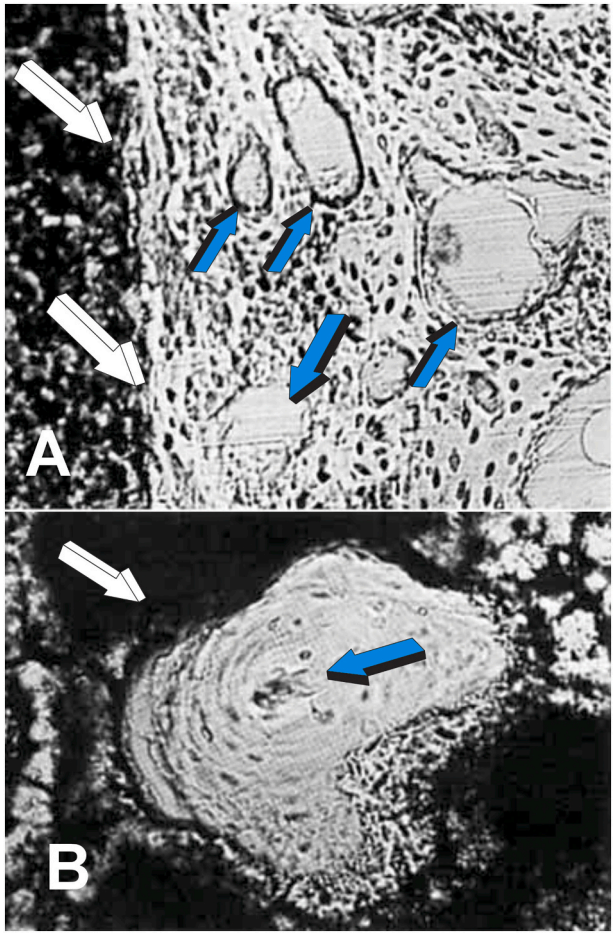


Fig. 1. Fibrovascular invasion, bone ingrowth and remodeling into macroporous spaces of calcium aluminate ceramics implanted in canine femoral defects [22]. (A) Prominent vascular invasion and capillary sprouting (*blue* arrows) within the macroporous spaces of the ceramics (white arrows), 4 weeks after intrafemoral implantation [22]. (B) Bone invasion by conduction within a macroporous space 11 weeks after implantation [22]. Invaded bone in direct contact with the ceramic substratum has remodeled into lamellar osteonic bone surrounding the central blood vessel (*blue* arrow). Images A and B reproduced from Ref. [22] (Figs. 2A and 7A with permission, the J Biomed Mat Res 1970). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and yet not mineralized bone or osteoid occupied about 50 % of the inner pore volume (Fig. 1B) [22]. Significantly, Hulbert [22] noticed the formation of lamellar osteonic bone in macroporous spaces of 300 μm or greater (Fig. 1B), understanding that the basic cellular unit of the osteonic bone, i.e., the concentric lamellar osteonic bone forming around the central blood vessel (Fig. 1B blue arrow) could also initiate within macroporous spaces of orthotopically implanted macroporous hydroxyapatites [22].

This review aims to present mechanistic and morphological data supporting the spontaneous induction of bone formation in animal models, and to critically review the potential of non-calcium phosphate-based biomaterials i.e., titanium constructs and implants to induce bone spontaneously. The review uniquely provides elemental insights that define the spontaneous and/or intrinsic induction of bone by certain biomaterials matrices presenting novel molecular and cellular biological paradigms that mechanistically resolve the spontaneous induction of bone formation.

2. Osteoinductive calcium phosphate-based macroporous bioreactors

The most significant contributions to the spontaneous and/or intrinsic induction of bone formation, or the hydroxyapatite-induced osteogenesis model [9,20,21], have been reported by heterotopically implanting a range of calcium phosphate-based bioreactors in animal models, i.e. canines, rodents, lagomorphs, ovine and non-human primates' species [26 for review]. These experiments often resulted in different and debatable amounts of bone, ranging from none to florid induction, within or surrounding the intramuscularly implanted calcium phosphate-based constructs [9,26,31].

Following the first morphological observations of extensive bone induction in long-term studies of coral-derived calcium phosphate-based and sintered crystalline bioreactors implanted in *Papio ursinus* rectus abdominis muscle. (Fig. 2) [6–10,17,26] and the reported heterotopic bone formation by granular porous hydroxyapatites after intramuscular implantation in canines (Fig. 3) [32,33], research into calcium phosphate-based induction of bone formation developed globally.

The extended research provided communications describing the induction of bone by calcium phosphate-based bioreactors when implanted in heterotopic sites of animal models, primarily intramuscularly [6–10,25–34]. The available morphological studies hypothesized a variety of parameters that may or may not differentiate osteogenic cells initiating the deposition of bone against the calcium phosphate-based biomaterial constructs.

Despite the explanations that wished to resolve the calcium phosphate-based induction of bone formation (for reviews [26,31]), the global research effort resulted in the publication of several papers proposing a variety of hypotheses. However, these contributions lacked vital mechanistic data (for reviews [26,31]).

Biomimetic matrices of calcium phosphate-based constructs soon became biomaterials of choice for replacing bone mass by filling bone defects in patients acutely in need of bone formation for craniofacial and orthopedic applications. [23,24].

Fundamental papers that highlighted the critical role of hydroxyapatites with different shapes, crystallinity, macro- and micro-topography followed the critical papers of Hulbert [22] and Urist and co-workers [35]. The latter provided evidence for the binding and thus of partial purification of bovine bone morphogenetic proteins by hydroxyapatite adsorption chromatography [35].

The purification of osteogenic proteins or "osteogenins" from demineralized and chaotropically extracted bovine and baboon bone matrices [36,37] revealed why calcium phosphate-based bioreactors, derived from corals, or sintered crystalline hydroxyapatites initiated spontaneous and/or intrinsic bone formation [6–8,38]. The loading and eluting of osteogenic protein fractions by hydroxyapatite Ultrogel adsorption chromatography [35–37] indirectly explained the

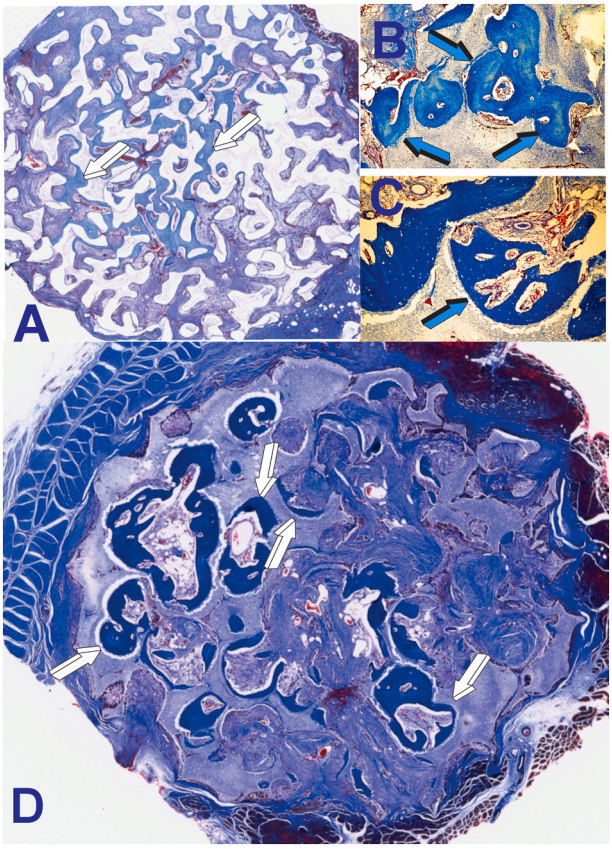


Fig. 2. Long-term induction of bone formation by geometric cues of coral-derived and sintered crystalline macroporous hydroxyapatites. (A) Substantial induction of bone morphogenesis (white arrows) on day 90 after *rectus abdominis* implantation of a coral-derived macroporous bioreactor in *Papio ursinus*. (B,C) Intrinsic induction of bone formation by repetitive concavities (*blue* arrows) of the macroporous spaces of coral-derived bioreactors 90 days after intramuscular *rectus abdominis* implantation [56]. (D) Self-inducing sintered crystalline macroporous hydroxyapatite 90 days after heterotopic implantation. Bone initiates against concavities of the substratum (white arrows) [38]. Decalcified sections cut at 4 to 6 μm stained with Goldner's trichrome. Images B and C reproduced from Ref. [56] (Fig. 3C and D with permission, Biomaterials 2012). Image D reproduced from Ref. [38] (Fig. 7 with permission, South African J of Science 1999). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

spontaneous induction of bone formation by coral-derived macroporous bioreactors following heterotopic implantation in the Chacma baboon [6–8]. The adsorption of osteogenic fractions onto hydroxyapatite Ultrogel [39] correlated with bone induction [6–8], leading to the hypothesis that implanted coral-derived bioreactors would literally adsorb BMPs present within the extracellular matrix of the surrounding and enveloping rectus abdominis muscle [6–8].

The adsorption strategy of osteogenic proteins from the extracellular matrix onto calcium phosphate-based bioreactors was not strictly correct, and several years of experimentation were needed to mechanistically resolve the intrinsic and/or spontaneous induction of bone using calcium phosphate-based bioreactors [25,26,31,38,40,41].

It transpired that Ca^{++} was at the crux of the bone induction by the intrinsic osteoinductive bioreactors [25,26,41]. Osteoclastic activity occurring on the surface of the heterotopically implanted calcium phosphate-based substrata would release Ca++. Released Ca++sustained angiogenesis, together with the differentiation of mesenchymal cell populations into osteoblasts, with these cells attaching to the hydroxyapatite substratum [25,41]. Differentiating osteoblasts would express and secrete osteogenic TGF-β supergene family

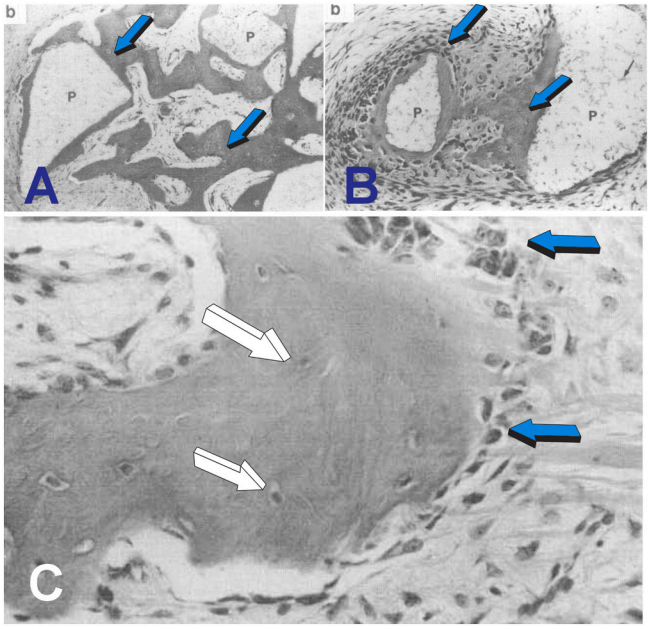


Fig. 3. Induction of bone formation after heterotopic implantation of porous hydroxyapatite ceramic granules in canine subcutaneous sites. (A) Ceramic granules implanted in the sub-cutis of canines harvested on day 90 [32]. Newly formed trabeculae of bone (*blue* arrows) between hydroxyapatite granules or particles (P) also surrounding the granular hydroxyapatites. (B) Differentiating osteoblast-like cells lining newly formed bone attached to particulate (P) ceramic granules connected by newly formed bone (*blue* arrows). (C) Detail of the induction of bone formation by ceramic granules harvested on day 90 showing newly formed bone surfaced by osteoblasts (*blue* arrows). Newly formed bone shows intra lacunar osteocytes (white arrows). Images reproduced from Ref. [32]. Images reproduced from Ref. [32] (Figs. 3B, D and 4 with permission, the Jap J Oral Biol, 1990). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proteins later embedded onto the substratum, thereby initiating the induction of bone formation as a secondary response [25,26,41,42].

The embedding of osteogenic proteins onto the hydroxyapatite matrices is a clear example of cell engineering, whereby a variety of factors, viz. bioreactor crystallinity and macro- and micro-topography; Ca++ release, angiogenesis, and expressed and secreted BMPs, push molecular buttons [2] to drive the bone induction cascade.

3. The importance of geometry

The critical role of geometry in bone formation is a fascinating biological concept of tissue engineering and regenerative medicine that has yet to receive much attention despite early incisive contributions defining the vital role of inductive substratum geometry in regulating bone induction and tissue morphogenesis [43–45].

The studies of Nelson et al. [46] reported that the geometry of the tissue defines mammary branching morphogenesis and that geometric cues direct the differentiation of mesenchymal stem cells [47]. Comprehensive reviews on the geometric induction of bone [26,48] proposed that surface geometry and topography controlling gene expression and cellular differentiation present a unified theme of geometry research [25,26,48].

Geometry has been defined as a theme that unifies a series of cues to functionalize matrices [48,49], which is critical for controlling tissue induction and morphogenesis. Bioreactor geometry that has been described as pits, nanotopographic surface alterations, concavities or micro-concavities, or sintered surface microstructures are all cues that define configurations capable of inducing cellular differentiation in tissue induction [26,48].

Mechanistically reviewing the induction of bone formation by naturally derived and/or recombinant human BMPs pre-loaded onto calcium phosphate-based biomaterials revealed the critical role of geometry regulating the induction of bone formation. Osteogenic fractions purified from bovine bone matrices were loaded onto coral-derived porous hydroxyapatites in particulate granular (400–620 μm in diameter) and disc design of 7 mm diameter and 3 mm [50]. The osteogenic phenotype was confined to macroporous hydroxyapatites in disc configuration (Fig. 4). Highly purified osteogenic proteins, in

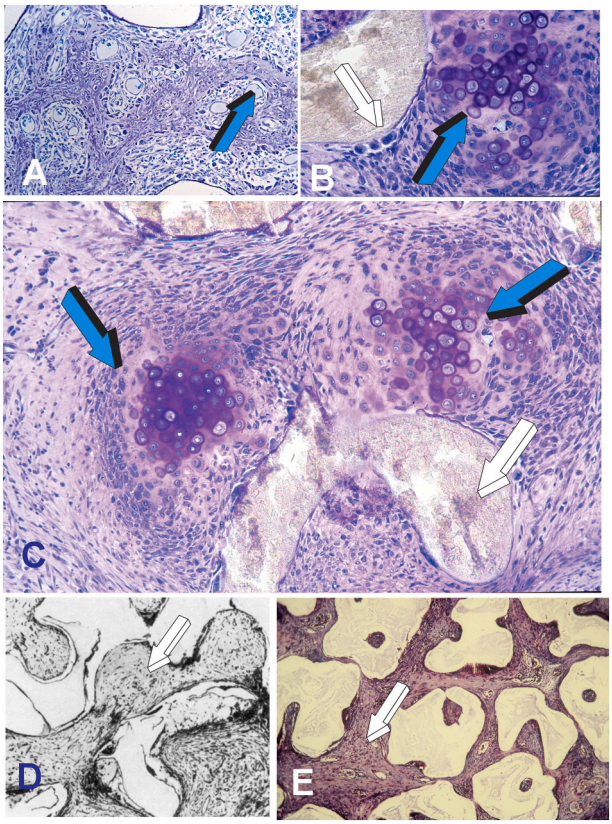


Fig. 4. The critical role of the geometry of the hydroxyapatite substratum on the induction of bone by naturally derived highly purified osteogenic fractions. (A) Highly purified bovine osteogenic fractions (50 μg in 50 μl of 5 mM HCL acid), purified greater than 50,000-fold [36], were combined with coral-derived constructs in disc and granular configurations [50].
(A). Morphogenesis of delicate trabecular-like bone formation by induction (*blue* arrow) within the macroporous space of the coral-derived construct on day 7 after heterotopic implantation in the rodent subcutaneous bioassay. (B) Chondrogenesis (*blue* arrow) on day 11 with the induction of chondroblastic cells attached the coralline substratum (white arrow). (C) Islands of chondrogenesis (*blue* arrows) on day 11 again with chondroblasts differentiating when attached to the substratum (white arrow). (D) Particulate granular coral-derived constructs reconstituted with 50 μg osteogenic fractions and harvested on day 21 [50]. Lack of bone formation within the macroporous spaces (white arrow). (E) Identical particulate granular and cylinder geometrical configurations but without osteogenic fractions were also implanted in the *rectus abdominis* muscle of non-human primates' *Papio ursinus* [20]. Harvested specimens on day 60 and 90 showed the induction of bone formation in coral-derived constructs in cylinders' configuration at both periods [30]. Granular particulate hydroxyapatites showed fibro vascular tissue ingrowth (white arrow) without the spontaneous induction of bone formation. (A-C) Undecalcified Historesin embedded sections cut at 3 μm. (D,E) Decalcified sections cut at 4 to 6 μm. Images A and D reproduced from Ref. [50] (Figs. 1B and 4C with permission, Matrix Biology 1992). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

combination with particulate granular porous hydroxyapatite, with identical pore dimension and surface binding characteristics to bioreactors in this configuration, failed to induce the induction of bone formation (Fig. 4D) [50]. The study showed that the geometry of the substratum overruled the molecular signals of the osteogenic proteins of the TGF-β supergene family (Fig. 4D) [50].

Further confirmation of the fundamental role of substrata geometry, the hydroxyapatite-induced osteogenesis model in the Chacma baboon *Papio ursinus* [7,8] was utilized to explore the influence of geometry and pore size. Different geometric configurations of the substrata were implanted in into surgically prepared heterotopic pouches in rectus abdominis muscle of the Chacma baboon [20]. Substrata were cylinders of macroporous coral-derived hydroxyapatite with a pore size of 200 and 500 μm and hydroxyapatite in granular particulate form of 400 to 620 μm diameter and pore size of 200 and 500 μm [20].

Except for a minute amount of bone within a concavity of two particulate granular specimens, the induction of bone formation occurred solely in substrata of cylinders' configuration of either pore size at 60 and 90 days after intramuscular implantation (Fig. 4E) [20].

Wang et al. [51] showed that the architectural porosity of the hydroxyapatite scaffold contributed not only to the extent of bone induction after heterotopic implantation in canines but particularly to the extent of vascularization and angiogenesis within the spaces of the macroporous scaffold [51].

Further comprehensive research analysed serial cellular events initiated during bone formation in calcium phosphate ceramics [52]. Histological analyses showed bone induction by standard osteoinductive tricalcium phosphate ceramics with submicron surface topographies (TCPs). Of interest, Guo et al. [52] depleted macrophages' invasion of TCP macroporous spaces with peri-implant injections of clodronate liposomes (LipClod). Explants at eight weeks after implantation with LipClod injections showed a lack of bone formation. The research study proposed a cellular cascade of events initiating with the activation of macrophages concomitant with osteoclastogenesis, targeting cell differentiation into osteogenic-like cells [52].

Mechanistic insights underlying the induction process were provided by Klar et al. [41]. The experiment described osteoclastogenesis in coral-derived bioreactors as critical for driving spontaneous bone induction. Morphologically coral-derived bioreactors loaded with 0.24 mg bisphosphonate zoledronate (Zometa®) showed significantly inhibited bone formation (Fig. 5).

Coral-derived bioreactors harvested at 15, 30, 60 and 90 days after heterotopic implantation in *Papio ursinus*, showed the critical role of the induction of mesenchymal collagenous condensations against the macroporous surfaces [6–8]. The induction of collagenous condensations is preceded by osteoclastic activity, acting to prime macroporous surfaces for the induction of bone formation [31,41]. Osteoclastic activity along the macroporous spaces leads to nanotopographical geometric configurations, resulting in the generation of self-inducing nanopatterned surface modifications within the confined spaces of the concavities of the substratum [25,26,40–42].

The critical role of osteoclastogenesis nanotopographically priming the self-inductive geometric landscape of the coral-derived surfaces has been indirectly shown by heterotopically implanting coral-derived bioreactors preloaded with 0.24 mg of the osteoclast inhibitor zoledronate [25,26,40,41]. Morphological analyses showed a lack of bone differentiation (Fig. 5). The morphological landscape showed the altered pattern of mesenchymal collagenous condensations, with a lack of cellular invasion, capillary sprouting and bone differentiation (Fig. 5) on day 90 (D,E), 60 (G) and 15 (F). Molecularly, qRT-PCR showed *BMP-2* down-regulation with concomitant up-regulation of the BMPs' inhibitor *Noggin* gene [25,26,40,41].

Molecular gene expression analyses showed that the induction of bone formation by macroporous bioreactors solo is related to high osteogenic protein-1 (OP-1) expression [40]. The intrinsic induction of bone formation was further investigated by applying recombinant

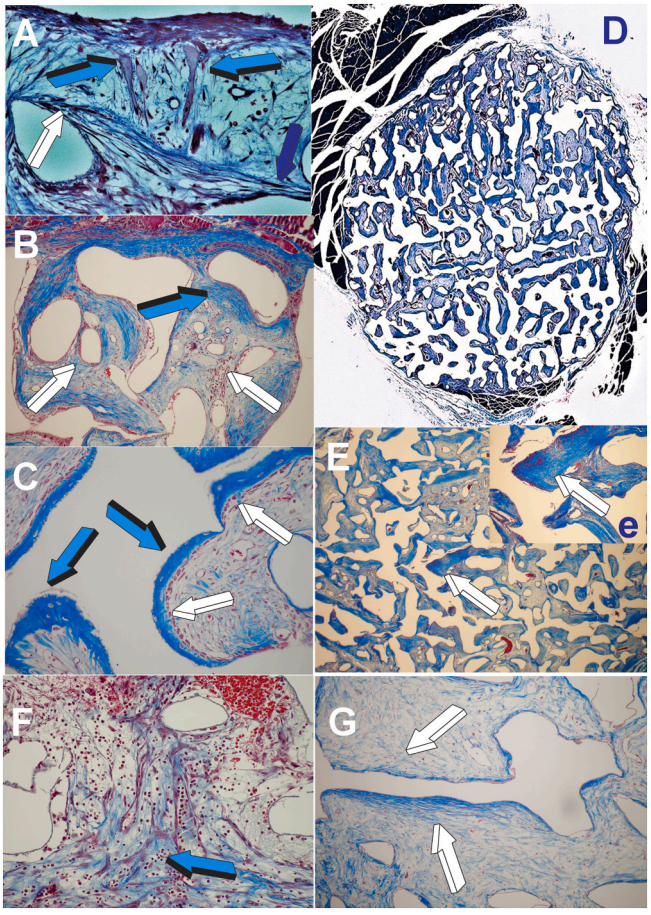


Fig. 5. Spontaneous and/or intrinsic induction of bone formation and its inhibitory modulation by the osteoclastic inhibitor bisphosphonate zoledronate: comparative morphological analyses. (A) High power view of a coral-derived macroporous space with capillaries (*blue* arrows) cut fortuitously longitudinally by the microtome in tight relationship with the differentiating mesenchymal condensation on day 15. White arrow points to collagenic fibers originating from a capillary perivascular space attaching to the coral-derived substratum (*dark blue* arrow). Note how the fibers provide collagenic material for cell riding across the intercapillary space [40]. (B) Peripheral area of a coral-derived bioreactor on day 15 without the addition of zoledronate showing differentiating mesenchymal condensations (*blue* arrow) and a prominent capillary invasion within the macroporous spaces as early as day 15 (white arrows). (C) Induction of bone formation within concavities (*blue* arrows) of the coral-derived substratum on day 60 after heterotopic implantation in *Papio ursinus*. Note the elegant induction of bone formation within the concavities of the substratum (*blue* arrows). Newly formed bone is covered by contiguous osteoblasts (white arrows). (D) Tissue induction and tissue patterning by coral-derived bioreactors pre-loaded with 0.24 mg zoledronate 90 days after heterotopic implantation in the *rectus abdominis* muscle of *Papio ursinus*. Formation of non-inductive collagenous condensations with improper alignment with lack of bone differentiation. (E) Non-inductive tissue invasion and mesenchymal condensations in coral-derived bioreactors pre-loaded with 0.24 mg zoledronate and harvested on day 90. Lack of bone differentiation with inactive collagenous condensations (inset e). (F) Tissue invasion in coral-derived bioreactor pre-loaded with 0.24 mg zoledronate and harvested on day 15 showing limited fibrovascular invasion as compare to untreated controls (A and B). (G) 0.24 mg zoledronate treated specimen harvested on day 60 after *rectus abdominis* implantation. Induction of non- osteoinductive condensation (white arrows) with limited vascular invasion. Decalcified sections cut at 4 to 6 μm. Decalcified sections cut at 4 to 6 μm. Images of Fig. 5 reproduced from Ref. [26, 41]. Image 5 A (Fig. 4.4C with permission, CRC Press. Images B, C and D reproduced from Ref. [41] (Figs. 2B, L and 6G with permission, J Cell Mol Med 2013). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

human Noggin onto the macroporous hydroxyapatite-based bioreactors before heterotopic implantation in *Papio ursinus* [42]. Macroporous bioreactors loaded with 125 or 150 μg hNoggin completely blocked bone formation (Fig. 6) [25,42,53]. This observation provided evidence that intrinsic bone induction is initiated via the BMP gene expression pathway [41,42,53].

Blocking tissue patterning of collagenous condensations at the hydroxyapatite border leads to inhibition of bone induction. Adding hNoggin inhibits the sequential morphological cascade of the induction of collagenous condensation (Fig. 6), thereby blocking the induction of bone formation [25,26,42,53]. Of interest there is the induction of haphazardly patterned non-productive collagenous condensations, indicating that BMP expression and secreted proteins are essential for the construction of the osteoinductive collagenous condensations (Fig. 6). Elegantly, doses of hNoggin preloaded together with doses of recombinant hOP-1/BMP-7 blocked the bone induction cascade as initiated by hOP-1 (Fig. 6E,F).

Because of the critical role of osteoclastogenesis in the induction of bone formation, released Ca^{++} ions are available within the macroporous spaces and particularly within the concavities of the substrata, supporting angiogenesis and cellular differentiation [25,26,41]. To indirectly study the role of Ca++ ions, 500 μg verapamil hydrochloride in a liquid vehicle was preloaded onto coral-derived constructs. Bone induction was substantially reduced in verapamil hydrochloride-treated specimens (Fig. 7) [41,53].

Lack of bone differentiation correlated with *BMP-2* downregulation and *Noggin* overexpression [41,42]. Doses of verapamil hydrochloride inhibited bone formation by blocking the patterning of mesenchymal collagenous condensations (Fig. 7C,D) [41]. There was a direct

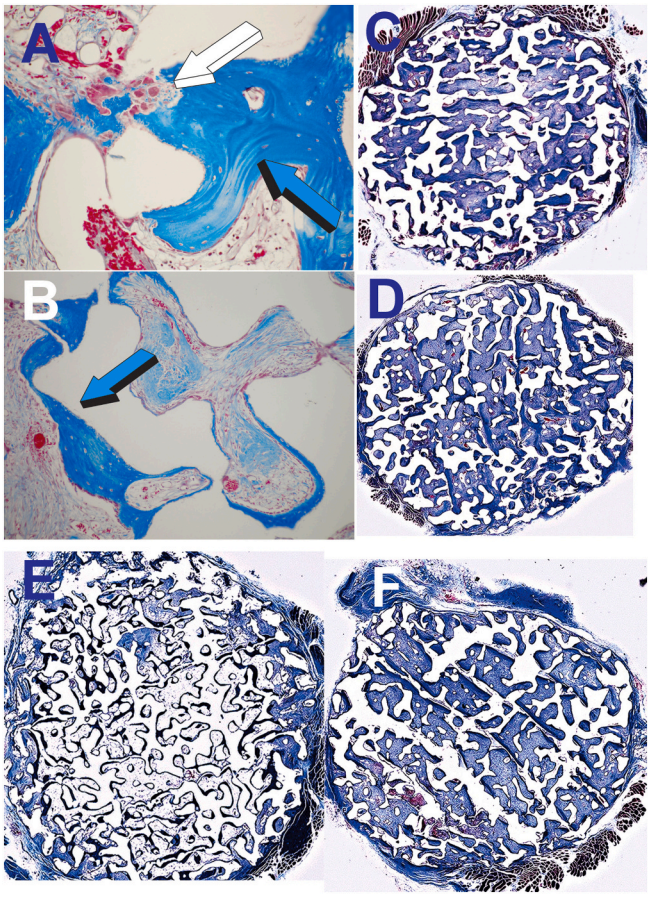


Fig. 6. Modulation and inhibition of the spontaneous and/or intrinsic induction of bone formation by doses of human recombinant Noggin (hNoggin). hNoggin at doses of 125 or 150 μg were pre-loaded onto coral-derived macroporous bioreactors implanted in heterotopic intramuscular *rectus abdominis* sites of adult *Papio ursinus* [25,26,53]. (A,B) Induction of bone formation on days 90 (A) and 60 (B) (*blue* arrows) after heterotopic implantation with remodeling of the newly formed bone on day 90 (*blue* arrow in A) with prominent osteoclastic activity of the newly formed bone (white arrow in A). (C,D) Low power views of coral-derived bioreactors pre-loaded with 125 (C) and 150 μg hNoggin (D). The addition of doses of hNoggin blocks the induction of bone formation via the induction of poorly patterned collagenous condensations with limited vascular invasion. (E) Induction of bone formation across the macroporous spaces of the coral-derived bioreactor pre-loaded with 125 μg recombinant human osteogenic protein-1 (hOP-1 - hBMP-7). (F) Pre-loading coral-derived bioreactors with 125-μg hOP-1 together with 125-μg hNoggin blocks the induction of bone formation [26,40,42]. Undecalcified sections cut at 4 to 6 μm stained with Goldner's trichrome. Images 6 A and 6B reproduced from ref. [41]. (Fig. 5H and 2H with permission from Ref [41], J Cell Mol Med 2013). Image 6F reproduced from ref. [26]. (Fig. 4.9d with permission CRC Press Vol. 2021 ref. [26]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

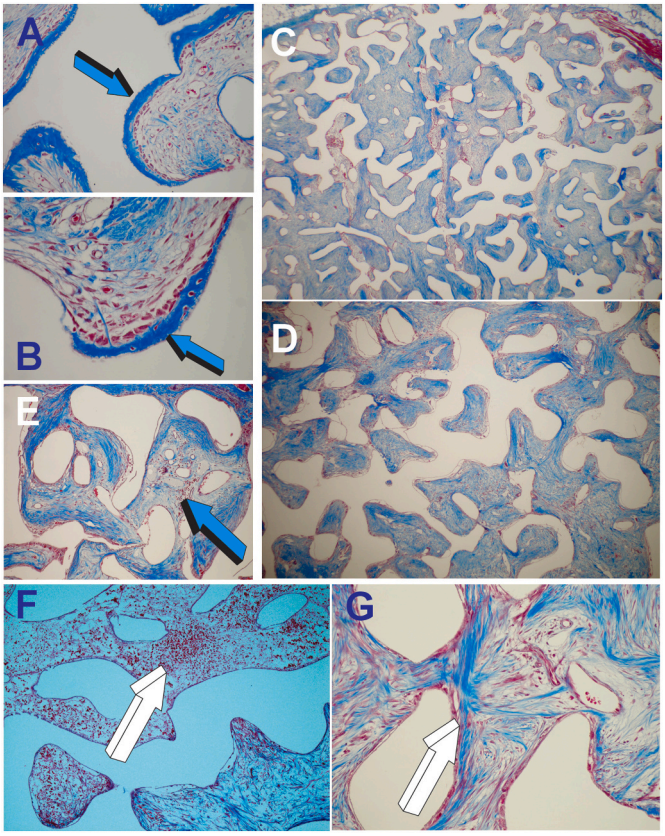


Fig. 7. Inhibition of the induction of mesenchymal condensations and inductive patterning by Ca^{++} channel blocker verapamil hydrochloride. (A,B) Bone formation (*blue* arrows) within concavities of the substratum by day 60 after heterotopic implantation. (C,D) Pre-loading coral-derived bioreactors with verapamil hydrochloride blocks the spontaneous and/or intrinsic induction of bone formation. There is the induction of poorly organized patterned collagenous condensations on days 90 (C) and 60 (D) after intramuscular *rectus abdominis* implantation. (E) Properly induced collagenous condensations with prominent vascular invasion and angiogenesis within the macroporous spaces (*blue* arrow) of a coral-derived macroporous bioreactor on day 15 after heterotopic implantation. (F,G) Disorganized and poorly patterned collagenous condensations in coral-derived bioreactors harvested on day 15 pre-treated with verapamil Ca^{++} channel blocker. Disorganized condensations leading to lack of bone differentiation on days 60 (D) and 90 (C). Undecalcified sections cut at 4 to 6 μm stained with Goldner's trichrome. All Images apart from 7F reproduced with permission from ref. [41], J Cell Mol Med 2013. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

correlation between the lack of bone differentiation with BMP-2 downregulation and upregulation of the BMPs' inhibitor *Noggin* gene [26,41].

Fig. 8 schematically represents the known molecular and cellular circuits and mechanistic insights of the spontaneous bone induction by calcium phosphate-based devices. The differentiation of osteoclastic cells and Ca^{++} release initiates vascular invasion and differentiation of stem cells along the osteoblastic cell lineage [41,42]. Cellular differentiation of osteoblast cells occurs in the concavities of the substratum, leading to the expression of BMP genes and secretion of proteins. The proteins embed in the substratum, and this leads to the induction of bone formation (Fig. 8).

4. Does titanium metal initiate bone indution?

The importance of the geometric configuration is not limited to macroporous calcium phosphate-based bioreactors but also encompasses the design of solid titanium prostheses for orthopedic and dental applications [26,54–59].

Sintered crystalline hydroxyapatite coating of concavities assembled on titanium implants inserted in heterotopic sites of the Chacma baboon *Papio ursinus* resulted in the induction of bone formation within the concavities of the geometrically designed substratum (Fig. 9) [26,54–59].

Currently, most titanium implants inserted in clinical settings are titanium constructs lacking sprayed hydroxyapatite coatings, which often results in implant failure [60,61]. The preparation of titania surface geometries with topographical modifications capable of initiating bone formation is at the forefront of topographic surface science to prepare highly osteophilic, if not osteoinductive titania constructs for clinical applications [55,56,62]. A critical question in biomaterial surface science is: Can bone spontaneously form by titania substrata without plasma-sprayed hydroxyapatite coatings? To answer these questions, it is paramount to review several concepts and terminologies that define osteoinduction, osteoconduction, osteointegration, and biomimetic macro- and micro-topographies.

5. Surface geometries and osteoinduction

The available up-to-date literature on the spontaneous and/or intrinsic induction of bone formation by calcium phosphate-based biomimetic matrices predominantly describes the morphological formation of bone on histological sections most often prepared from decalcified tissue blocks of harvested heterotopic specimens from different animal models [9,25–34,63,64]. Cellular and molecular studies to mechanistically resolve the spontaneous osteoinductivity of calcium phosphate-based biomaterials were not only delayed, but the published contributions ascribed the induction of bone to secondary factors, i.e. sintering time and temperatures, macro- and micro-porosity, crystallinity and nanotopographies or additional compounds of the macroporous

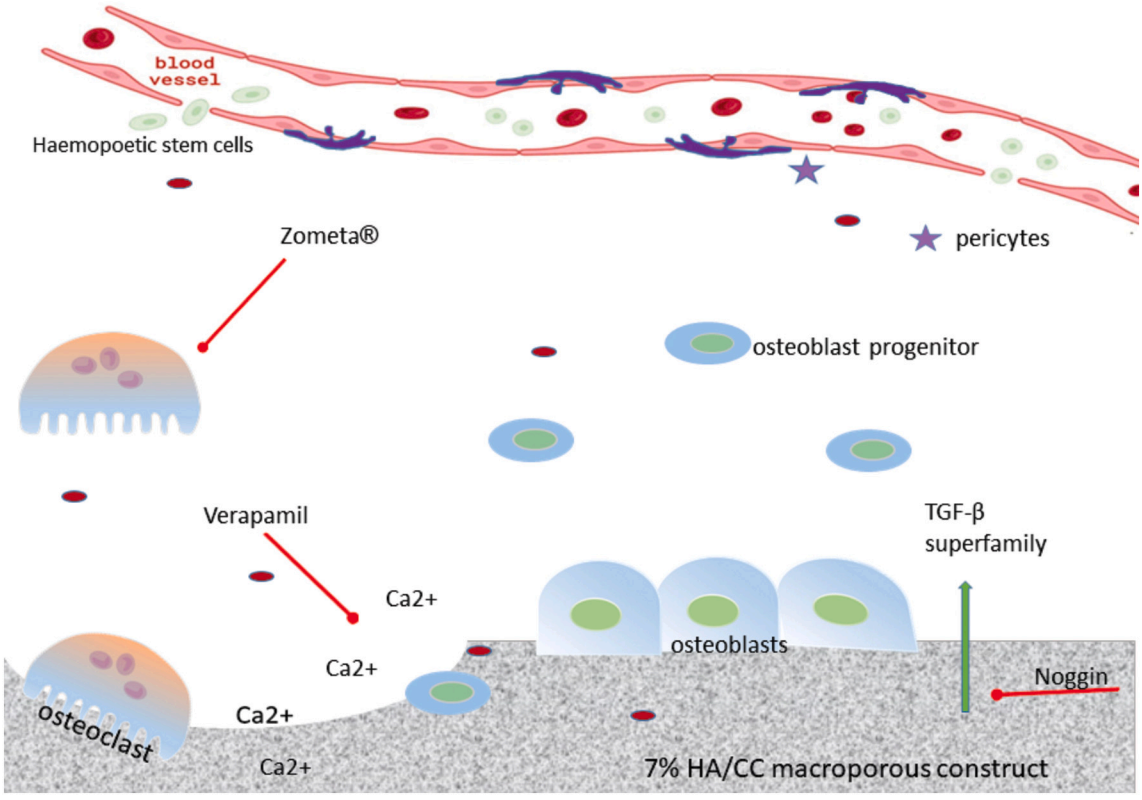


Fig. 8. Mechanisms initiating the intrinsic induction of bone formation by coral-derived macroporous bioreactors. Recruitment of monocytes forms a progenitor pool for osteoclasts and osteoblasts' differentiation. Osteoclastic activity on the calcium-phosphate surface leads to release of Ca^{++} ions and activation of signaling pathways differentiating mesenchymal stem cells resting within the cellular and extracellular matrix niche of the concavity. There is prominent angiogenesis with capillary sprouting with upregulated *collagen IV* expression. Gene profiling shows that the intrinsic osteoinductivity of the macroporous bioreactors is via the *bone morphogenetic proteins* (*BMPs*) pathway. Pre-treatment of the bioreactors blocks the induction cascade [21,26] The importance of early priming events was demonstrated by the use of the Zoledronate and Verapamil-HCL. Pre-treated bioreactors with the bisphosphonate zoledronate Zometa, an inhibitor of osteoclastic activity, blocks the induction of bone formation by inducing poorly patterned and inactive collagenous condensations. Molecularly, zoledronate Zometa-treated samples show upregulated *Noggin* and lack of *BMP*-2 expression [41,42,53]. Verapamil Ca^{++} channel blocker treated bioreactors show disorganized inactive collagenous condensations. Gene expression analyses show pronounced *BMP-2* downregulation and concomitant *Noggin* upregulation resulting in lack of bone differentiation [52]. Key events underlying the intrinsic induction of bone formation are detailed in several papers referenced in the text [8,25,26,31,38,40–42,53,62].

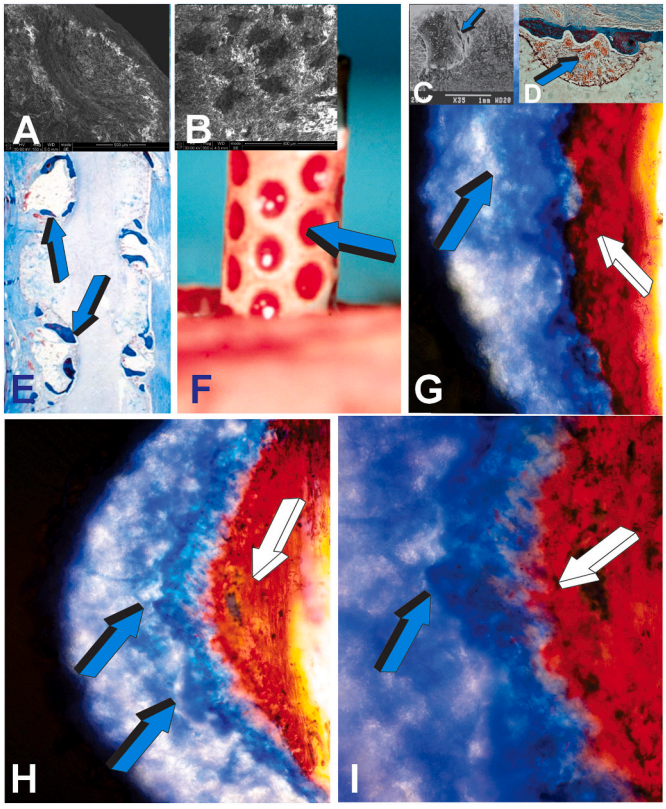


Fig. 9. Morphological and geometric landscape of the spontaneous and/or intrinsic induction of bone formation by self-inducing morphogenetic concavities. Concavities were prepared in titanium bioreactors later coated by crystalline plasma sprayed hydroxyapatites. (A) Scanning electron microscopy (SEM) analyses image of a concavity prepared in the linear titanium surface later coated by crystalline plasma spayed hydroxyapatite. (B) High power view illustrating the micro-concavities of the plasma sprayed construct and the overall geometric configuration of the coated-titanium bioreactor. (C) Five days after *rectus abdominis* implantation, coated titanium bioreactors were harvested, fixed and coated for SEM analyses. SEM evaluation showed patterning organization and tractional forces aligning patterned collagenic fibers across the edges of the concavity (*blue* arrow). Patterning of collagenic fibers across the concavity predates the transformation of collagenic fibers (C) into the induction of bone formation across the edges of the concavity with substantial angiogenesis as shown in (D). (E). Sintered solid discs of crystalline hydroxyapatite were implanted in the *rectus abdominis* muscle of *Papio ursinus* and harvested on day 90 [48]. Histological analyses on transversal sections showed the induction of bone formation solely within the concavities of the substratum (*blue* arrows). (F) Translational pre-clinical bioassay of the concavity into solid titanium geometric bioreactors with concavities coated with crystalline hydroxyapatite (*blue* arrow). Geometric implants were implanted in edentulous mandibular ridges and in the *rectus abdominis* muscle of *Papio ursinus* [56]. Bioreactors harvested 31 months after heterotopic implantation showed the induction of bone formation within the concavities of the substratum (G,H,I). Mineralized bone formed attached to the sintered hydroxyapatite coating (*blue* arrows in G, H,I) with the induction of osteoid tissue (white arrows). Images of Fig. 9 reproduced with permission from Biomaterials ref. [56]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

constructs [27–30,65–70]. These contributions seldom reported the critical role of endogenously expressed BMPs that need to be expressed and secreted to initiate bone formation as a secondary response [25,26,31].

A recent paper describing the macropore regulation of osteoinduction by porous hydroxyapatite scaffolds via a microfluidic pathway is worth mentioning [71]. The study reports that the size and shape of the hydroxyapatite macro porosity affect the microfluidic pathway within the scaffold, resulting in altogether different inductions of bone formation and bone pattern distributions [71]. The communication does not allude to the fundamental role of BMPs in initiating bone formation.

Immuno-localization analyses of harvested heterotopic bioreactors implanted in heterotopic sites suggested that "the hydroxyapatite substratum may act as a solid-state matrix for adsorption, storage and controlled release of bone morphogenetic proteins which locally initiate the induction of bone formation" [8,38].

Lander, in his Cell paper states, "Whether morphogens gradients cross threshold values at which genes are turned on or off, or influence each other triggering the spontaneous emergence of stable, long-range patterns of morphogen activity, only Morpheus unbound can trigger the cascade of pattern formation resulting in tissue induction and morphogenesis" [72]. "Morpheus unbound" thus needs to bind to extracellular matrices or other substrata to induce bone formation. Heterotopic implantation of calcium phosphate-based biomimetic matrices sets into motion the synthesis and expression of BMPs' genes. Secreted proteins are embedded into the macroporous spaces of the concavities initiating the induction of bone formation as a secondary response [25,26,38,40,41].

The mechanical reconstitution of a soluble signal with an insoluble signal is the fundamental tenet of regenerative medicine and tissue engineering [3,12,13,15]. This reconstitution protocol is also the tenet of cell engineering, whereby both soluble and insoluble signals push cellular' buttons [2] to initiate tissue morphogenesis.

Bone formation occurs without osteogenic proteins of the transforming growth factor-β (TGF-β) supergene family [17,74–78]. In primates, the mammalian TGF-β isoforms induce the substantial induction of bone [75–81]. Notably, the capacity and/or prerogative to initiate de novo bone formation extends to additional family members, the decapentaplegic (DPP) and 60 A proteins in *Drosophila melanogaster* [79].

RUNX-2 is up-regulated by TGF-β signaling together with Osteocalcin up-regulation and expression on day 15 after rectus abdominis intramuscular implantation of relatively high doses of hTGF-β3 in the non-human primate *Papio ursinus* [53,78,80]. The initiation of bone formation by hTGF-β3 in *Papio ursinus* is via the BMPs pathway; the recombinant morphogen controls the induction of bone formation by regulating the expression of *BMPs* genes and gene products via *Noggin* expression, eliciting bone induction by up-regulating endogenous BMPs [42,78,80].

The discovery of the osteogenic proteins of the TGF-β supergene family stemmed from the molecular dissection of the extracellular matrix of bone [3,10,81]. Incisive research from the laboratories of AH Reddi reported that the osteoinductive morphogens are in the solubilized component of the chaotropically extracted demineralized bone matrices.

Importantly, the morphogens need to be reconstituted with a carrier matrix to restore the osteoinductive activity, which is lost after dissociation of the matrix [3,10,12,13,16,81].

The operational reconstitution of naturally derived or recombinantly produced molecular signals of the TGF-β superfamily with an insoluble signal or substratum was the fundamental step to identify, purify and isolate the solubilized bone morphogenetic morphogens or proteins present within the extracellular matrix of bone [12,13,36,37]. The operational reconstitution additionally defined the heterotopic bioassay to test a variety of proteins and newly designed matrices that may or may not initiate the induction of bone formation [3,12,13,17,81].

The acid test defining osteoinduction is to induce de novo bone formation after heterotopic implantation of newly developed proteins and/or biomimetic devices. Osteoinduction is confined to proteins and/or devices that de novo initiate the induction of bone formation where there is no bone, i.e. in heterotopic sites. An osteoinductive matrix is a device bearing osteogenic activity per se; its discriminatory action is osteogenesis [10]. The above biologically denies bone formation by proteins and/or devices implanted in orthotopic sites [3,77,81], where

bone can form from viable bone interfaces and grow by osteoconduction into the macroporous spaces [17,22].

As previously described [10], an "*osteoconductive biomaterial is not inherently osteoinductive*" and only directs the growth of bone onto its porous spaces, as described by Hulbert et al. [22]. Osteoconduction was a fundamental biological step towards incorporating macroporous prosthesis and titanium devices when implanted into viable bone.

Fundamental studies by Albrektsson et al. [82] resulted in experimental research studies in animal models that provided evidence for the definition of a new term, i.e. osteointegration. Osteointegration is the "direct structural and functional connection between living bone and the surface of a load-bearing artificial implant" [82], primarily a titanium substratum [82]. Such a functional connection is essential for the stability of the implanted construct and, thus, for the loading and long-term success of osteointegrated titanium implants.

Topographical surface modifications of biomaterials' surfaces, particularly of titanium constructs, stemmed from the biological need to fabricate osteoinductive titanium implants that per se would set into motion the induction of bone formation [54–59]. Besides the inherent biological value of such experiments, osteoinductive biomaterials and particularly osteoinductive titanium substrata would dramatically enhance its use in clinical context by providing therapists and patients with titanium implants per se endowed with the prerogative of initiating new bone. New bone would form without the exogenous application of the recombinant osteogenic proteins of the TGF-β supergene family [54–59]. This would improve osteointegration, thereby shortening loading masticatory times in clinical contexts.

A plethora of research experiments were then published to try unravel titanium' surfaces with "*Biomimetic microtopography to enhance osteogenesis* in vitro" [83]. Experiments were designed to study both in vitro and in vivo topographic surface characteristics that would either induce, stimulate and/or enhance molecular and cellular pathways of bone induction and morphogenesis [83–96].

The communications showed that alterations of micro topographical geometric configurations of titanium surfaces or sintered crystalline hydroxyapatites resulted in the spatial control of cellular differentiation with up-regulation of TGF-β/BMP signaling [67,69,83,91–94]. McNamara et al. proposed different cellular responses when cultured on functionally different titania nanotopographies [90].

In vivo studies summarizing the extensive surface geometric topography regulating the expression of osteogenetic genes showed that "*nanopatterned titanium implants*" and "*bioactive three-dimensional graphene oxide foam/polydimethylsiloxane/zinc silicate scaffolds*" accelerate [95] and enhance [96] bone formation *in vivo* and osteoinductivity for bone regeneration [96].

The contribution of Greer et al. [95] on nanopatterned titanium implants accelerating bone formation *in vivo* may indeed offer a "*promising route for translation to future clinical orthopaedic implants*". Engineered implants with disordered nanotopographies presented as pillars with 15–25 nm height and 100 nm diameter induced an osteogenic gene expression pathway upon seeding the geometric surfaces with STRO-1-enriched human skeletal stem cells [95].

Similarly, scaffolds of bioactive graphene oxide m/polydimethylsiloxane/zinc silicate, when seeded with bone marrow mesenchymal stem cells (mBMSCs), micro-patterned surface geometric configurations showed that the cells/graphene oxide composite induced mBMSCs proliferation and osteogenic differentiation, with expression of selected genes including ALP, RUNX2, VEGFA and Osteopontin [96]. This data suggests that the cells/graphene oxide composite "is a promising alternative for application in bone regeneration" [96].

To summarize, all these in-depth molecular and cellular experiments with the expression of osteogenic genes upon seeding with stem cells and/or osteogenic cell precursors showed that the geometry of the substratum is critical to induce a gene expression pathway equal to the osteogenic cascade as seen in vivo by osteoinductive biomaterials as defined above.

Osteoprogenitor cells from marrow biopsies or other mesenchymal stem cells (MSCs), now redefined as pericytes [97], are needed to initiate the induction of bone. The extensive data available, however, does not yet report that titanium' surface geometric nanotopographies initiate the induction of bone, where there is no bone, and in absence of soluble osteogenic molecular signals and/or precursor osteogenic cells.

A vital contribution showed the bioactivity of titanium bioreactors prepared by alkali and heat treatments inserted into rabbit tibial bones [98]. Histological and mechanical analyses showed that pre-treated titania' surfaces revealed breakage of the treated layer in the sodium-free alkali- and heat-treated titanium group, indicating superior osteointegration and bone bonding capacity. It was concluded that sodium removal accelerated the in vivo bioactivity of pre-treated implants and achieved faster bone bonding capacity [109]. These data followed the experimentation of Kobuko et al. [99] which showed the formation of bone-like apatite on the surface of titanium in simulated body fluids after a combination of alkali and heat treatment [99].

The experiment reported that apatite formation on the titanium surface is critical for bioactivity, i.e., "*direct bone bonding*" to the titanium constructs. Further experiments showed that alkali- and heath-treated titanium and titanium alloys could directly bond to bone [100,101].

Later, Fujibayashi and his team reported the induction of bone by a porous bioactive titanium metal after implantation in the dorsal musculature of adult beagle dogs [102]. Of note, the authors stated "*that even a non-soluble metal that contains no calcium or phosphorus can be an osteoinductive material when treated to form an appropriate macrostructure and microstructure*". Of interest, however, the paper reported that untreated pure titanium did not yield bone formation by induction [103].

The manuscript further states that osteoinduction by porous bioactive titanium metal "*may elucidate the nature of osteoinduction, and lead to the advent of epochal osteoinductive biomaterials for tissue regeneration*" [102]. The statement may be part of the hype of the tissue engineering dream, but the paper also reports "*that the surface forming after chemical and thermal treatments plays an important role in the osteogenesis*" [102]. This is due to the in vitro apatite formation of chemically and thermally treated titanium' surfaces [102]. "*An* in vitro *apatite-forming ability may be a prerequisite for biomaterials to be osteoinductive materials* in vivo". The authors further state that apatite was deposited along the surface of the bioactive titanium after chemical and thermal treatment. It is worth stating again that untreated pure titanium did not yield the induction of bone when implanted in heterotopic sites in the dorsal musculature of canines [102].

Additional studies by Kyoto University showed the effect of sodium removal by dilute HCl treatment [103]. The HCl, together with alkali and heat treatment showed superior osteoinductive activity, with bone formation within three months, compared to 12 months by chemically and thermally treated porous titanium blocks [102,103]. The authors concluded dilute HCl treatment "*is a promising candidate for advance surface treatment of porous titanium implants*" [103]. The previous work of Fujibayashi et al. showed that bone initiated only in the chemically and thermally treated porous blocks at 12 months [102]. Macroporous blocks of pure titanium do not form bone by induction within the macroporous spaces [102,103].

It is noteworthy to analyse the above statements further. Non-treated titanium macroporous blocks with equal macroporous interconnected structures do not initiate bone formation [102]. It is worth reporting that "*An* in vitro *apatite-forming ability may be a prerequisite for biomaterials to be osteoinductive materials* in vivo" [102]. Bioactive titanium was engineered after specific chemical and thermal treatments with the formation of hydroxyapatite crystals, ultimately responsible for the induction of bone.

An important question is: "*Can uncoated yet geometrically designed titanium substrata form bone with concavities along its linear surfaces?*" [57]. A review of the studies of Fujibayashi's group shows that bone formation by titanium substrata is dependent on acid, alkali and heat-

treatments to form apatite layers on the treated titanium surfaces and that pure untreated titanium does not initiate the spontaneous and/or intrinsic induction of bone formation [98–103].

To further study osteointegration and osteoinduction by geometric titanium bioreactors with concavities across the planar surfaces without the coating of crystalline hydroxyapatite, geometric titanium' bioreactors were implanted in large mandibular defects regenerated after implantation of 250 μg hTGF-β3 delivered by human demineralized bone matrix in adult *Papio ursinus* (one g human DBM per 250 μg hTGF-β_3) [104]. Mandibular tissue blocks with regenerated mandibular defects were harvested at 9 and 12 months after mandibular regeneration and 15 and 30 days after titanium constructs' implantation [104].

Generated μCT scans of newly engineered mandibular bone by the hTGF-β_3 showed limited osteointegration of the inserted uncoated titanium implants with minimal, if any invasion of new bone in the concavities of the implanted substrata at both 15 and 30 days after implantation (Fig. 10) [104]. Morphological analyses of undecalcified sections showed limited formation of bone within the concavities even on day 30 after implantation (Fig. 10D). μCT scans confirmed the limited bone formation within the concavities of the substrata at 15 (Fig. 10A,B) and 30 days (Fig. 10C).

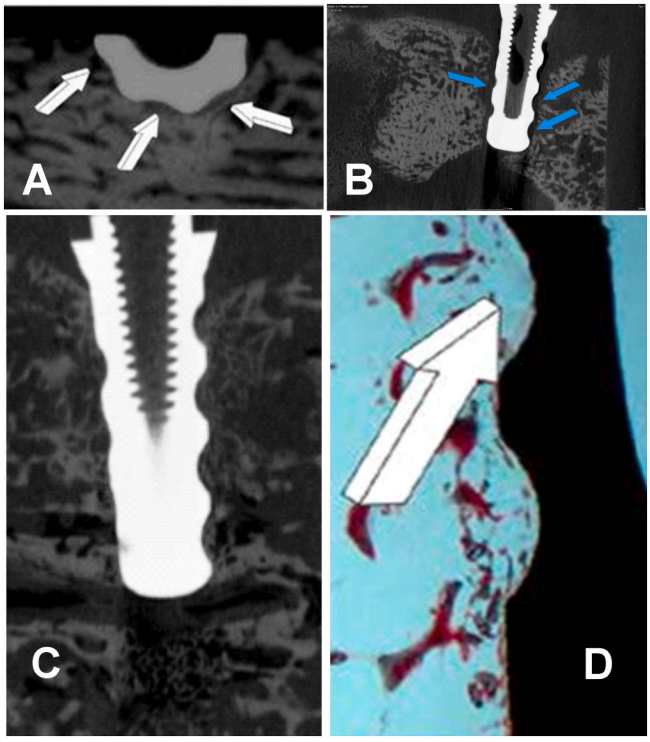


Fig. 10. Is pure titanium metal spontaneously osteoinductive? Titanium implants with a series of repetitive concavities along the planar surfaces but without sintered crystalline hydroxyapatite coating were inserted into mandibular regenerates after implantation of transforming growth factor-β_3 (hTGF-β_3) 8 and 9 months after mandibular regeneration [104]. (A) Transversal μCT scan of a mandibular regenerate 8 months after implantation of 250 μg hTGF-β_3 [104]. White arrows show limited direct osteointegration of the concavities of the titanium construct harvested 15 days after implantation into the mandibular regenerate. (B) Longitudinal μCT scan with a geometric titanium implant inserted 15 days before tissue harvest and histological processing showing limited osteointegration and direct contact of bone across the concavities of the substratum (*blue* arrows). (C) μCT scan of a geometric implant inserted into a mandibular regenerate 9 months after mandibular implantation of hTGF-β_3 inserted into the mandibular regenerate 30 days before tissue harvest and histological processing [104]. Limited if any osteointegration also confirmed by undecalcified sectioning on the Exakt diamond saw grinding and polishing equipment. (D) Limited bone formation and osteointegration within the concavities of the titanium substratum (white arrow) cut and stained on the Exakt diamond grinding and polishing equipment [104]. Images of Fig. 9 reproduced with permission from ref. [104] J of the Dent Association of South Africa). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The present review shows that a variety of naturally derived or sintered macroporous constructs of hydroxyapatite and/or calcium phosphate-based biomaterials have the unique capacity to de novo initiate the induction of bone formation in heterotopic extraskeletal sites of a variety of animal models. The available data show that the spontaneous and/or intrinsic induction of bone formation is solely achieved by hydroxyapatite calcium phosphate-based biomaterials, and that hydroxyapatite in its various forms and geometric configurations finely tune the induction of bone formation in heterotopic sites.

Whilst this review primarily focuses on engineering pro-osteogenic matrices for the induction of bone formation, the following subheading highlights some of the hype in regenerative medicine and tissue engineering that also affects newly designed pro-osteogenic matrices initiating bone formation.

6. Stem cells and regenerative therapies without the osteogenic proteins of the TGF-β supergene family

Recently, several investigative reviews have highlighted the potential role of different cellular phenotypes and induced pluripotent stem cells in tissue engineering and regenerative medicine. We express a word of caution when reading published papers stating that the promise of tissue regeneration and tissue engineering is close [14,105,106]. We suggest that the "*hype in regenerative medicine is a series of statements based on a promise of organ regeneration, which are simply based on hypotheticals, not proven, but only biologically and molecularly generated by several results from the bench top with minimal if any experimentation in preclinical contexts*" [14,106].

The extraordinary hype in tissue engineering and regenerative medicine has been driven by the discovery of the induction of pluripotent stem cells from differentiated somatic cells [107–109], instrumental in the 2012 Nobel Prize winning award.

The promise of regenerative medicine initiated by the extraordinary crescendo of discoveries resolved several pending issues in tissue biology, and led to strides in understanding tissue induction, morphogenesis and pattern formation [72,73,107,110,111,112]. The combined research efforts discovered signaling molecules, or "morphogens", first defined by Turing as "*forms generating substances*" [113]. As previously stated, [114] "obedient to the classic recapitulation evo-devo' rule, developmental events that initiate in embryogenesis can be redeployed and recapitulated postnatally in tissue induction and thus regeneration" [4,110,114,115].

The promise of regeneration and *restitutio ad integrum* of tissues and organs has been, however, yet to be delivered [116]. Similarly, the promise of tissue restoration of cranio mandibulo facial defects has yet to be fulfilled, including mandibular regeneration. Despite several advances in understanding the basic molecular and cellular biology mechanisms of cellular and extracellular matrix cross talk and interactions, complex disfiguring cranio mandibulo facial defects and congenital anomalies remain a grand unsolved challenge [117].

The use of induced pluripotent stem cells is also still a promise for *de novo* and *ex-novo* engineering and assembling tissues and organs, though research experiments are still under evaluation. An important discovery has been the published work of Crisan et al. which showed that the mesenchymal stem cells (MSCs) of developed organs are of perivascular origin [97].

After extensive analyses, the communication concludes that blood vessel walls harbour a reserve of progenitor's cells, and that the "*archetypal multipotent progenitor's cells*", the MSCs, are in fact, pericytes and that isolated pericytes retain chondrogenic and osteogenic potential [97].

Further studies showed that activation of skeletal stem progenitor

cells (SSPCs) responsible for bone regeneration is masterminded by the tyrosine kinase cell-surface receptor for the platelet-derived growth factors (PDGFRβ) [118]. Together with hSSCs, the sub-heading will also discuss the angiogenic critical role of several peri-vascular cells. The "archetypal multipotent" mesenchymal stem cells (MSCs) are indeed of perivascular origin and are now defined as pericytes [97,119]. Obviously, the angiogenic wall of invading capillaries harbours several progenitor cells in para-vascular location including pericytes and myogenic endothelial cells [120], with pleiotropic differentiation capacities during the induction of bone formation.

The fundamental work of Discher's laboratory has revealed how cells feel and respond to the stiffness of their substrate [121] and how mechanistically, "Stem cells feel the difference" [122]. The work presented by Discher's laboratory is a step ahead on how cells and stem cells respond to the stiffness of the substratum [121–124]. We believe that the published work is a critical contribution to cellular differentiation by various substrata, showing how mesenchymal stem cells commit to cellular differentiation with high sensitivity to nuances of substrata' elasticity [123]. The following statements by Engler et al. [123] are perhaps the most advanced results on cellular differentiation that show the critical role of self-organizing substrata programming biological functions, i.e. "soft matrices that mimic brain are neurogenic, stiffer matrices that mimic muscle are myogenic, and comparatively rigid matrices that mimic collagenous bone prove osteogenic" [123]. The mechanical regulation of cell functions was also evaluated in depth by Fu et al. [125]. The data showed how elastomeric substrata geometrically modulate the function of cells. The data indicate the possible use of novel coating methods to engineer diverse bioreactors promoting different cellular responses to biomaterials' surface microstructures [125].

The discovery that the vessel wall retains several progenitor cells is not new. Still, the presented work showed as the capillaries crosstalk and interact with several stem cells, including endothelial cells, myoendothelial cells and pericytes, the latter enveloping the vessels and the invading capillaries [97,126–128]. This has indicated that invading sprouting capillaries provide a continuum of viable stem cells migrating along tissues' invading capillaries where regenerative phenomena or remodeling is underway.

The identification of human stem cells (hSSCs) has revealed some of the mechanisms that regulate the interaction of hSSCs with the extracellular matrix to resolve the regenerative potential of hSSCs possibly, and to induce novel therapeutic strategies in clinical contexts [126].

Stem cell technologies have been highlighted by a series of papers that propose the promise of multiple regenerative scenarios for treating several human disorders, including oligodendrocyte and astrocyte disorders [127]. Again, the isolation and cloning of selected stem cells and their localization "continue to provide pivotal groundwork for future clinical applications of stem cell technology" [127]. The promise of regenerative tissue engineering with stem cells "is advancing incredibly rapidly, and with the powerful potential for this technology in the treatment and cure of various hitherto life-threatening disorders, the feasibility will soon be at our doorstep" [127].

Studies on stem cells and their niches also resulted in a special issue of Development: Stem cells and regeneration, where Development published several leading manuscripts also celebrating the work of Sir John Gurdon and Shinya Yamanaka, the recipient and co- recipient of the 2012 Nobel Prize in Medicine and Physiology [128].

The interest in stem cell technology and more recently the in-depth studies on stem cells' niches and their microenvironments have once again pushed stem cell biology towards stem cell-based therapies for regenerative medicine. Novel therapies following the injections of stem cells to treat complex human disorders, including cancers, Parkinson's disorders, and other neuropathologies, have been suggested. [127–130].

The promise of tissue regeneration has been responsible for the rapid expansion of regenerative medicine. Such biological expansion has resulted in many extraordinary discoveries in the molecular and cellular biology of tissue regeneration. Published research has shown extraordinary results in animal models, including non-human primate studies, where single proteins combined with different matrices as carriers resulted in the induction of previously unknown tissue regenerative phenomena, including bone formation [53]. However, translation in clinical contexts has been difficult, and the promise of regenerative medicine and tissue engineering remains a promise [131,132].

7. Conclusion

Translational research in clinical contexts is the ultimate challenge of the induction of bone formation and the development of pro-osteogenic matrices spontaneously initiating the induction of bone formation. Rigorous research studies should be implemented to analyse the binding and release kinetics of the osteogenic proteins of the TGF-β supergene family for the predictable induction of bone formation by newly designed self-inductive pro- osteogenic matrices. Further studies should focus on novel micro-topographic surfaces to differentiate invading mesenchymal stem cells into bone cells, initiating the induction of bone formation within the macroporous spaces.

Identifying and developing self-inducing pro-osteogenic matrices initiating the induction of bone formation has been a step ahead in tissue engineering. Focused research still needs to be implemented to resolve the induction of bone formation in human patients mechanistically, and to prepare pro-osteogenic bioreactors self-inducing bone formation in clinical contexts.

CRediT authorship contribution statement

Ugo Ripamonti: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Validation, Visualization. **Raquel Duarte:** Formal analysis, Investigation, Validation.

Declaration of competing interest

Authors have no conflicts of interest, nor employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding from any source whatever with the exclusion of the University of the Witwatersrand, Johannesburg.

Data availability

Data will be made available on request.

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