Acta Biomaterialia 145 (2022) 1–24

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actbio

Review article

# Sustained local ionic homeostatic imbalance caused by calcification modulates inflammation to trigger heterotopic ossification

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ARTICLE INFO

*Article history:*
Received 13 December 2021
Revised 30 March 2022
Accepted 31 March 2022
Available online 6 April 2022

ABSTRACT

Heterotopic ossification (HO) is a condition triggered by an injury leading to the formation of mature lamellar bone in extraskeletal soft tissues. Despite being a frequent complication of orthopedic and trauma surgery, brain and spinal injury, the etiology of HO is poorly understood. The aim of this study is to evaluate the hypothesis that a sustained local ionic homeostatic imbalance (SLIHI) created by mineral formation during tissue calcification modulates inflammation to trigger HO. This evaluation also considers the role SLIHI could play for the design of cell-free, drug-free osteoinductive bone graft substitutes. The evaluation contains five main sections. The first section defines relevant concepts in the context of HO and provides a summary of proposed causes of HO. The second section starts with a detailed analysis of the occurrence and involvement of calcification in HO. It is followed by an explanation of the causes of calcification and its consequences. This allows to speculate on the potential chemical modulators of

*Abbreviations:* 2D, Two-Dimensional; 3D, Three-Dimensional; ABG, Autologous Bone Graft; ABCC6, ATP-Binding Cassette sub-family C member 6 protein; ALP, Alkaline Phosphatase; ASTM, American Society for Testing and Materials; BCP, Biphasic Calcium Phosphate (mixture of $\beta$-TCP and HA); BGS, Bone Graft Substitute; BMP, Bone Morphogenetic Protein; BMSC, Bone Marrow-Derived Stem Cell; BPOP, Bizarre Parosteal Osteochondromatous Proliferation; CaSR, Calcium-Sensing Receptor; CD, Clusters of Differentiation; CDHA, Calcium-Deficient Hydroxyapatite; DB, Demineralized Bone; DC, Dystrophic calcification; DISH, Diffuse Idiopathic Skeletal Hyperostosis; eNTPD1, EctoNucleoside TriPhosphate Diphosphohydrolase-1; FGF, Fibroblast Growth Factor; FOP, Fibrodysplasia ossificans progressive; G-CSF, Granulocyte Stimulating Factor; GLAST-CreER, Bacterial Artificial Chromosome (BAC) transgenic line that expresses inducible CreER (Cre recombinase (Cre) fused to a mutant estrogen ligand-binding domain (ER)); GPRC6A, G-protein-coupled receptor 6A; HA, Hydroxyapatite; HO, Heterotopic Ossification; IFN-$\gamma$, Interferon gamma; IGF, Insulin-like Growth Factor; IL, Interleukin; ISO, International Organization for Standardization; MNGC, MultiNucleated Giant Cell; MSC, Mesenchymal Stem Cell; NCX, Na-Ca exchange ion channel; Nf-K$\beta$, Nuclear factor Kappa-light-chain-enhancer of activated B cells; NKA, Na +, K + -ATPase ion pump; NSAID, Non-Steroidal Anti-Inflammatory Drug; OCP, OctaCalcium Phosphate; PDGFR, Platelet-Derived Growth Factor Receptor; PIEZO1, Mechanosensitive ion channel protein encoded by the gene PIEZO1; PMCA, Plasma-membrane $Ca^{2+}$ ATPase transport protein; POH, Progressive Osseous Hyperplasia; PTH, Parathyroid Hormone; RANKL, Receptor Activator of Nuclear factor Kappa-B Ligand; Runx2, Runt-related transcription factor 2; SBF, Simulated Body Fluid; SCI, Spinal Cord Injury; SLIHI, Sustained Local Ionic Homeostatic Imbalance; SSA, Specific Surface Area; TBI, Traumatic Brain Injury; Tie2, Angiopoietin-1 receptor; $\alpha$-TCP, $\alpha$-Tricalcium Phosphate; $\beta$-TCP, $\beta$-Tricalcium Phosphate; TGF, Transforming Growth Factor; TNAP, Tissue-Non-specific Alkaline Phosphatase; TRPV, Transient receptor potential vanilloid; VEGF, Vascular Endothelial Growth Factor; VGCC, Voltage-gated calcium channels; $\Delta$(CaP), Consumption of calcium and phosphate ions; $C_f$, Chemical factors responsible for mineralization; $S_m$, Surface of the implant on which mineralization can occur = surface of implant material.; m, Implant mass; $SSA_m$, Specific surface area of the material; $p_i$, Implant porosity; $\rho_{th}$, Theoretical density of the implant material; $V_m$, Volume of the implant material; $V_i$, Implant (apparent) volume; $S_E$, External surface of the implant ("envelope" of the implant); $S_{Eo}$, Open external surface ("pore surface").

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[1] Dr. Marc Bohner was an editor of the journal during the review period of the article. To avoid a conflict of interest, Prof. William R. Wagner acted as editor for this manuscript.

https://doi.org/10.1016/j.actbio.2022.03.057

*Keywords:*
Apatite
Bone
Bone Morphogenetic Protein
Bone Graft
Heterotopic ossification
Inflammation
Osteoinduction

inflammation and triggers of HO. The end of this second section is devoted to *in vitro* mineralization tests used to predict the ectopic potential of materials. The third section reviews the biological cascade of events occurring during pathological and material-induced HO, and attempts to propose a quantitative timeline of HO formation. The fourth section looks at potential ways to control HO formation, either acting on SLIHI or on inflammation. Chemical, physical, and drug-based approaches are considered. Finally, the evaluation finishes with a critical assessment of the definition of osteoinduction.

**Statement of significance**

The ability to regenerate bone in a spatially controlled and reproducible manner is an essential prerequisite for the treatment of large bone defects. As such, understanding the mechanism leading to heterotopic ossification (HO), a condition triggered by an injury leading to the formation of mature lamellar bone in extraskeletal soft tissues, would be very useful. Unfortunately, the mechanism(s) behind HO is(are) poorly understood. The present study reviews the literature on HO and based on it, proposes that HO can be caused by a combination of inflammation and calcification. This mechanism helps to better understand current strategies to prevent and treat HO. It also shows new opportunities to improve the treatment of bone defects in orthopedic and dental procedures.



## 1. Introduction

Heterotopic ossification (HO), defined as the formation of mature lamellar bone in extraskeletal soft tissues [1–3], is considered an adverse event (Fig. 1). Although HO has been associated with genetic [4], traumatic [5–8], and neurogenic [9–11] predispositions, soft tissue injury appears to be a unifying feature for all types of HO [12]. HO can be triggered locally at the location of muscle injury [13–15], close to bone injury [5–8], or in the mesentery or scar tissue of trauma patients or in patients whom have had abdominal surgery [16–22]. HO can also form at a distance from the primary injury, such as in the legs of patients with spinal cord injury (SCI) or traumatic brain injury (TBI) [23,24]. Increased joint stiffness, limited range of motion, warmth, swelling, localized pain, tenderness, vascular stasis, edema, and erythema are the principal clinical signs of HO [2,3,25]. The prevalence of HO has been reported to be as high as 65% following hip replacement [26], 65% following wartime high-energy extremity injuries [8], 98% following cervical disc arthroplasty [27], 37% following ulna and humerus fracture [7], 53% following spinal cord injury (SCI) [23], and 23% following traumatic brain injury (TBI) [24]. Since TBIs and cervical disc arthroplasties account for approximately 1 million cases each year in the US alone [24,28,29], the incidence of HO likely reaches several million cases yearly worldwide. Clinically significant HO is typically observed only in single to low double digit percentages of HO patients [27,30]. However, complications in the clinically affected patients can be excessive, including severe restrictions in joint motion or ankylosis [30]. Complications can be even more dramatic in HO associated with genetic disorders such as Fibrodysplasia ossificans progressiva (FOP), a rare genetic condition causing congenital malformations and progressive HO induced by soft tissue injury leading to life threatening immobility [31]. Medical treatments for HO include the use of anti-inflammatory drugs, irradiation, and bisphosphonates. However, the efficacy of these treatments is limited and surgical intervention to explant ectopic bone is often necessary with the exception of FOP [2,30,32–37]. As a result, there remains a substantial unmet medical need for better HO prophylaxis and medical therapies.

Efforts to develop new therapies to prevent and treat HO have been hampered by a lack of understanding of the etiology of HO. However, the data suggests that the pathophysiology of HO requires the presence of osteoprogenitor cells, soluble factors, and a permissive environment [38]. The soluble factors are generally assumed to be bone morphogenetic proteins [39], or in the case of FOP, activins. The concept of a permissive environment was first proposed by Chalmers who had observed that demineralized bone induced bone formation in muscle and fascia, but not in the liver, spleen, and kidney [38]. Based on these observations, the permissive environment was proposed to be an inflamed subcutis or muscle [40,41]. Adams associated the permissive environment with hypoxia, inflammation, growth factors, and calcification [34].

Spatially controlled induction of bone formation is required in many orthopedic and dental applications. These include the treatment of segmental diaphyseal bone defects [42], metaphyseal bone defects [43], vertebral compression fractures [44,45]), osteotomies [46,47], tooth extraction sockets to allow placement of dental implants [48,49], spinal arthrodesis [50–52]), or to treat non-unions [53,54]. Bone regeneration is achieved with the combination of appropriate surgical technique and autologous, allogenic, or xenogenic bone grafts, plant-derived bone graft materials, or synthetic bone graft substitutes [42,55,56]. The current standard to stimulate bone formation is autologous bone graft (ABG), which combines cells, an osteoconductive matrix, and osteoinductive and osteogenic factors. However, the volume of available ABGs can be limited and there is the potential for donor site morbidity [57–59]. Bone morphogenetic protein (BMP)/carrier combinations have been used successfully to induce spine fusion [60,61]. BMP/carrier combinations are also approved for use in trauma and oral maxillofacial indications. However, the results are not as consistent as in spine fusion [62–64]. BMPs can also be combined with bone graft substitutes (BGS). Limitations associated with the use of BMPs include relatively high cost and local adverse events associated with the use of excessive BMP concentrations in off-label indications [65]. BGS are generally considered inferior to ABG and BMPs for the repair of large bone defects because they lack osteoinductive capacity. However, multiple reports have shown that few cell-free, drug-free polymers, ceramics, and metals effectively trigger the formation of bone ectopically in animal models [66–72]. The ability to induce HO may be considered proof of their osteoinductive potential. Various mechanisms have been proposed to explain the osteoinductive effects of these different materials. These include the adsorption of growth factors on the implant surface and subsequent release during resorption [68,73], the release of calcium and phosphate ions during resorption [74], and surface topography [75–77] (Fig. 2). However, the fundamental mechanism responsible for the ability of certain materials to trigger bone formation ectopically has remained elusive [70,72,78], which strongly hinders evolvement of knowledge and its use to improve bone regenerative therapies.

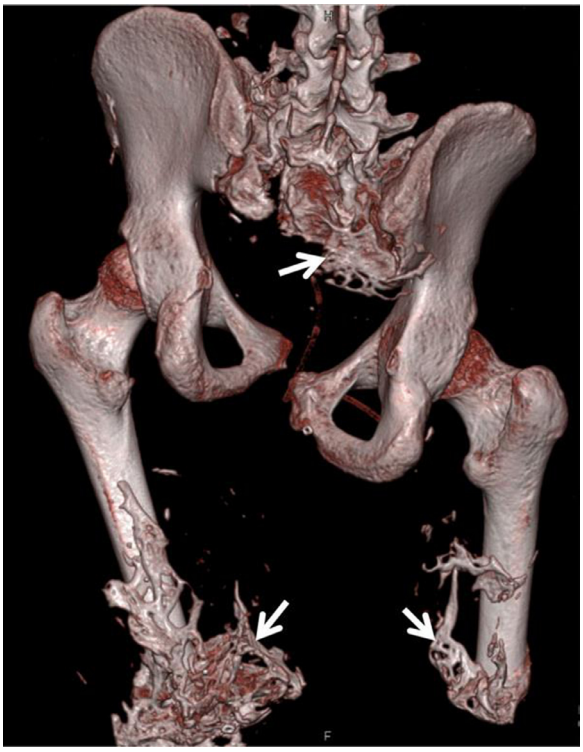

**Fig. 1.** 3D computed tomographic reconstruction of the pelvis and residual femora of a combat-injured patient. White arrows, heterotopic ossification. Copyright: [450].

A new mechanism was recently proposed to explain material-induced HO [79]. Local consumption of calcium and phosphate ions to grow crystals during calcification was hypothesized to be large enough in some instances to maintain a sustained local depletion of calcium and phosphate levels within the mineralizing area. This sustained local ionic homeostatic imbalance (SLIHI) was proposed to be the trigger for a cascade of biological reactions leading to HO formation. This mechanism could explain (i) why not only calcium phosphates but also polymers, metals, and non calcium phosphate ceramics can trigger an osteoinductive response, (ii) why ectopic bone often forms within the core of the implanted porous BGS rather than at the periphery [74,80] (exceptions: [81,82]), (iii) why bone induction is positively influenced by a decrease in macropore size [80] or an increase of the implant volume [83], or (iv) why it may take months for bone to form ectopically in the absence of administered osteogenic factors. Even though the authors describing this new mechanism did not focus on HO, a number of analogies between material-induced HO and injury-induced HO were noticed. These include the importance of mineralization, inflammation, and implant/injury volume [79] (Table 1).

The aim of the present work was to assess the hypothesis that the mechanism of SLIHI described earlier for material-induced HO [79] is also an essential component in the pathogenesis of pathology-induced HO. The evaluation is based on a literature study of HO including the pioneering work of Urist on demineralized bone (DB) and BMPs [39,84]. Based on this analysis, it is suggested that HO can be considered as a cascade of events in which the combination of inflammation and SLIHI plays a central role. In the absence of any of these two components, no ectopic bone forms. Traumatic brain injury or tissue damage and associated necrosis can be considered to be upstream in the cascade. Osteoclastogenesis and BMP release can be considered to be downstream. This approach raises a number of questions including the correct definition of osteoinduction induced by materials and allows for a better understanding of current strategies to prevent HO, to treat existing HO, and to improve the treatment of bone defects in orthopedic and dental procedures.

The evaluation contains five main discussions. Relevant concepts in the context of HO and a summary of proposed causes of HO are discussed in Section 2. Calcification is discussed in Section 3 starting with a detailed analysis of the occurrence and involvement of calcification in HO. This is followed by an explanation of the causes of calcification, the consequences of calcification and speculation about the potential chemical triggers for HO. The end of this section is devoted to *in vitro* mineralization tests used to predict the osteoinductive potential of bone graft substitute biomaterials. Section 4 reviews the biological cascade of events occurring during pathological and material-induced HO, and proposes a

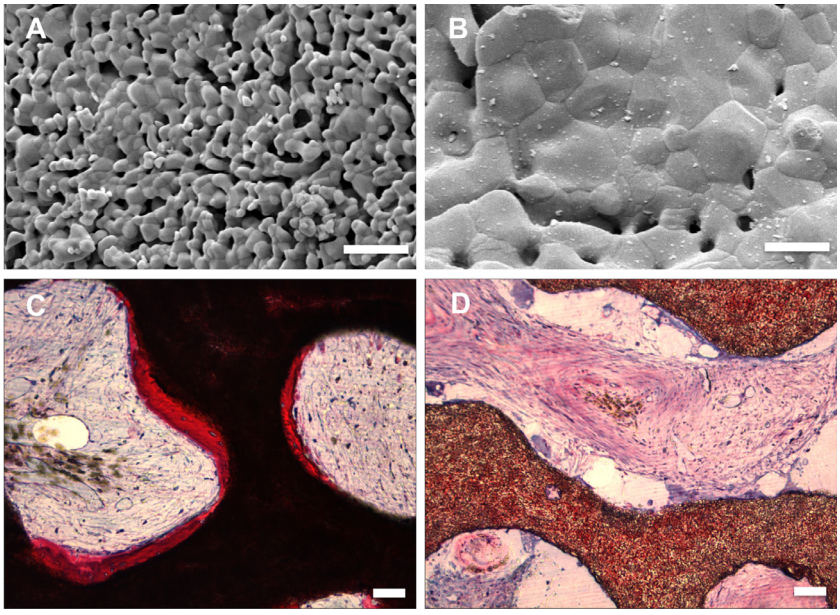


**Fig. 2.** Physicochemical properties of calcium phosphate ceramics influence their osteoinductivity. Osteoinductive biphasic calcium phosphate ceramic with small grains and micropores (A) induces bone formation (in red in the figure) upon 12-week intramuscular implantation in a goat (C), in contrast to its non-osteoinductive counterpart with larger grains and fewer micropores (B) that is only infiltrated by fibrous tissue (D). Scale bar: in (A) and (B) 5 µm, in (C) and (D): 100 µm. Figure taken with permission from [451].

**Table 1**
Similarities and differences between material-induced and pathological heterotopic ossification (HO). NSAID = non-steroidal anti-inflammatory drugs.

| Parameter | Material-induced HO | Pathological HO |
|---|---|---|
| Mechanism | Unclear | Unclear |
| Time to bone formation | Weeks to months | Weeks to months |
| Calcification | Calcification before ossification | Calcification before ossification |
| Volume effect | More bone with more implanted material | More bone with increased trauma area |
| Inflammation | Swelling, edema prior to bone formation | Swelling, edema prior to bone formation |
| Treatment | NSAID drugs reduce/prevent bone formation | NSAID drugs reduce/prevent bone formation |
| Mechanism of bone formation | Mostly intramembraneous | Mostly endochondral |
| Bone location | Mostly inside the implanted material (e.g. inside pores, between granules) | Formation of a bone shell, but unclear location compared to the edge of the injured tissues |

quantitative timeline of HO formation. Potential methods to control HO formation, either acting on SLIHI or on inflammation are discussed in Section 5. Chemical, physical, and drug-based approaches are considered. A critical assessment of the definition of osteoinduction is presented in Section 6.

## 2. Definitions and HO causes

### 2.1. Definitions

It appears relevant and important to review the definition of important terminology used in the context of HO and bone substitution, namely bone, BMP, calcification, HO, osteoinduction, osteoconduction, and osteopromotion.

Bone: Bone is a complex organ composed primarily of collagen and hydroxyapatite as structural elements. Cellular elements include mesenchymal stem cells, osteoblasts, osteocytes, osteoclasts, bone lining cells, hematopoietic stem cells, hematopoietic bone marrow lineage cells, adipose cells supported by vascular and neural networks. Bone can be described in different ways, for example according to its function (locomotion, organ protection, mineral exchange, hematopoiesis and metabolic regulation), its structure (cancellous, cortical, composite), or its composition (collagen, apatite, and cellular components). In the context of HO, ossicles of bone, formed in extraskeletal locations, contain all the structural and cellular components of normal bone [85].

Bone morphogenetic protein (BMP): First described by Urist and Strates in 1971 [39], "*Bone morphogenetic proteins*" are "*the osteogenetic chemical components of the matrix of bone, dentin, and other hard tissues that are deinsulated by demineralization and associated intimately with collagen fibrils*". They added that "*BMP guides modulation and differentiation of mesenchymal cells of muscle into bone and bone marrow cells*". In other words, BMPs are factors capable of initiating the entire process of bone formation in skeletal and extraskeletal locations. BMPs, secreted by multiple cell types including osteoblasts and immune lineage cells are sequestered in the matrix of bone, dentin, and other hard tissues. BMP signaling is required for bone repair and regulation of bone homeostasis by Wnt (Wingless/Integrated) and parathyroid hormone. BMPs also participate in organ patterning in embryogenesis and subsequent organ differentiation and in hematopoiesis. BMPs guide the modulation and differentiation of mesenchymal cells contained in the bone marrow, periosteum, muscle, and vascular elements into bone.

Calcification: (Dystrophic) calcification (DC) refers to the physico-chemical process of calcium phosphate salt deposition in damaged, inflamed or necrotic soft tissues. It should be distinguished from "*ossification*" which is a much more complex process (see below). Calcification remains in essence a physico-chemical process, while ossification involves a biological process to generate bone tissue. Ossification is technically the formation of mineralized bone which by definition is a combination of cellular and physico-chemical processes. Calcification is the physico-chemical formation of an acellular mineral deposit within or on existing structures.

Heterotopic ossification: HO has been defined as "*the formation of mature lamellar bone in extraskeletal soft tissues*" [1], "*the formation of the bone outside the skeletal system*" [3], "*the formation of histologically regular bone in an abnormal extraskeletal location*" [9], "*the ossification of extraskeletal tissues into true bone*" [86], or "*the presence of lamellar bone at locations where bone normally does not exist*" [2]. Various alternative terms have been used interchangeably to describe HO including "*ectopic bone formation*", "*ectopic ossification*", "*myositis ossificans*", "*neurogenic ossifying fibromyopathy*", "*paraosteoarthropathy*", "*periarticular ossification*", and "*heterotopic ossification*" [1,2]. HO has been further characterized by some investigators into subgroups based on location including "*myositis ossificans*" and "*fasciitis ossificans*" in muscles and in or along fascia, respectively [25]. Other investigators have characterized HO based on causalities including trauma ("*post-traumatic HO*", "*traumatic myositis ossificans*", "*myositis ossificans circumspecta*" [1]) or genetic ("*fibrodysplasia ossificans progressive*" (FOP) and "*progressive osseous hyperplasia*" [3]). "*Myositis ossificans traumatica*" has been further divided into periosteal, intermediate and intramuscular depending on the location of the newly formed bone. In the first two types, bone-forming cells from damaged periosteum have been proposed as the source of HO. However, the intramuscular subgroup is seen within the muscle, apparently separated from bone and bone-derived cells [14,87]. Other investigators disagree with this subgrouping contending that HO leads to bone formation independent of bone-derived cells, but can subsequently fuse to the periosteum [25]. Despite the various attempts to characterize HO, some investigators consider that there is no consensus on the definition and classification of HO [2].

Osteoinduction: The definition of "*osteoinduction*" has evolved over the years. The term "*induction*" was initially used to refer to the ability of a transplanted tissue to induce the formation of new tissue [88–90]. Grobstein [88] stated "*inductive tissue interaction takes place whenever in development two or more tissues of different history and properties become intimately associated and alteration of the developmental course of the interactants results*". Huggins [91] reported the ability of the epithelium of the urinary tract to induce bone formation when transplanted under the skin of the groin of a dog in 1931. In 1967, Urist [85] stated that "*when cellular differentiation is attributed to the physicochemical effect of one tissue upon and in contact with another, the mechanism is known as induction*". In 1971, Urist [39] proposed a slightly different definition with "*the process of tissue differentiation initiated by close contact and mutual interaction of cell populations of diverse origins*". Even though the meaning and use of "*osteoinduction*" is quite obvious in the context of a bone induction, for example in the report of Friedenstein in 1968 [92], the term "*osteoinduction*" was used for the first time in the scientific literature in 1972 [93,94]. With the discovery of the importance of soluble differentiation factors such as BMPs for osteogenesis, the definition of "*osteoinduction*" evolved. In 1982, Urist [95] proposed that "*the recruitment of perivascular connective tissue cells (pericytes) from a fibrogenetic to an osteogenetic pathway of development is known as osteoinduction*".

In 1985, Glowacki and Mulliken described "*osteoinduction*" as "*the phenotypic conversion of connective tissue into bone by an appropriate stimulus*". The 1987 consensus conference proposed a broad definition: "*the process by which osteogenesis is induced*" where "*osteogenesis*" is the "*whole process of development and formation of bone*" [96]. Albrektsson and Johansson [97] proposed that the definition of osteoinduction "*means that primitive, undifferentiated and pluripotent cells are somehow stimulated to develop into the bone-forming cell lineage*". The American Society for Testing and Materials (ASTM) developed a much narrower definition of osteoinduction in 2015 in the ASTM 2529 standard based on the cause ("*a substance*") and the location ("*an implant site*") [98]. They defined osteoinduction as "*the ability of a substance to stimulate cells to differentiate along some osteoprogenitor pathway resulting in cells capable of synthesizing and secreting components essential to the formation of bone at an implant site*". It is currently accepted by most investigators that the ability to induce bone at an ectopic site independently of any pre-existing bone is direct proof of an osteoinductive process. In this document, an osteoinductive material refers to a cell-free, drug-free material that induce ectopic bone formation.

Osteoconduction: Osteoconduction was defined by Urist in 1982 as "*the process of extension of bone repair growth from previously differentiated bone cells of the host bone*" [95]. This term generally applies to bone conducted from an existing bone site into an implanted matrix. The most common example is bone formation into matrices implanted within metaphyseal bone defects or adjacent to the proximal or distal bone ends in diaphyseal segmental defects. Osteoconductive matrices fail to induce bone in an ectopic site and fail to conduct bone formation over large distances, which explains why osteoinductive BGS may be benefitial for treating large bone defects.

Osteopromotive: The term "*osteopromotive*" was first used in 1991 in the context of "*guided bone regeneration*" to describe the positive action of membranes on bone formation [99]. According to this definition, an "*osteopromotive*" substance enhances bone formation in an orthotopic bone site, but does not induce bone formation in an ectopic site independent of bone [100]. Osteopromotion has more recently been used to describe the ability of "smart" matrices to enhance bone formation at either a bone or ectopic site. The ability of an osteopromotive matrix to induce bone formation has been attributed to the sequestration of circulating BMPs, BMPs produced by adsorbed cells, or by the surface architecture of the matrix.

### 2.2. HO causes

HO is generally associated with an injury, but an injury does not always lead to HO. HO can be induced by a wide range of conditions, but is most often seen following musculoskeletal trauma, arthroplasty (also a musculoskeletal trauma in itself), or injury of the central nervous system (Fig. 3). McCarthy and Sundaram [3] classified the causes of HO as genetic, post-traumatic, neurogenic, post-surgical, and as distinctive reactive lesions most often seen in the hands and feet.

Genetic causes of HO include Fibrodysplasia Ossificans Progressiva (FOP) and Progressive Osseous Hyperplasia (POH) [1,4,101–104]. These are both rare genetic conditions resulting in extensive bone formation in all soft tissues [1]. HO has also been reported in a patient suffering from Albright's hereditary osteodystrophy, a pseudohypoparathyroidism [3,105]. It has been speculated that the HLA $\beta$7 gene associated with spondyloarthritis favors HO [10,106,107]. Patients with spondyloarthritis are often given non-steroidal anti-inflammatory drugs (NSAIDs) prior to arthroplasty surgeries. Additionally, HO has been related to a number of other

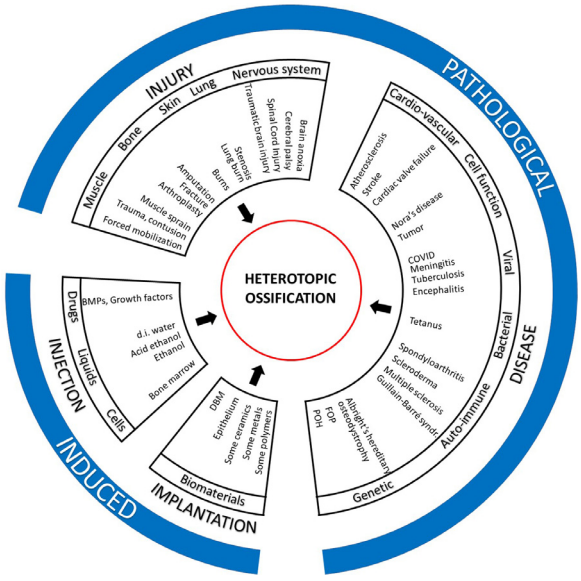


**Fig. 3.** some of the causes of heterotopic ossification / ectopic bone formation. Pathological HO should be prevented, but being able to induce HO in a controlled manner is a highly wanted property for bone repair purposes. A difference is made between injury-related and disease-related pathologies leading to HO.

auto-immune diseases including scleroderma [108], multiple sclerosis [109], or the Guillain-Barré syndrome [109].

Traumatic injuries that may lead to HO include burn injuries [5,110–113], blast injuries [1,6], lung injuries [114,115], bone injuries [7,11,27,116], abdominal injuries [16,17,19,117] and amputations [1,8,118,119]. Generally, the risk of HO reflects the severity of the injury, such as the size and the occurrence of multiple injuries [5,8,110,112,113,116,120]. However, smaller injuries, like muscle sprains and contusion, may also provoke HO [1,3,126,127,13–15,121–125], particularly if the injuries are repetitive [86]. Michelsson et al. [125,128–132] demonstrated in rabbit studies that the mobilization of a previously immobilized knee joint may also cause HO in the neighboring vastus intermedius muscle. Importantly, traumatic HO is generally seen at the location of the injury, contrary to neurogenic HO.

Neurogenic conditions preceding HO include traumatic brain injury (TBI) [1,9,11,34,109] and spinal cord injury (SCI) [9–11,32,34,109,133,134]. Non-traumatic neurological disorders such as brain stroke [109,135], cerebral anoxia [109], meningitis [136], meningoencephalitis [9], encephalitis [137], cerebral palsy [109] can also lead to HO [9]. Even though some investigators like Salisbury et al. [138,139] speculate that nerve-derived signals/neuroinflammatory processes are involved, most investigators consider that other factors are more important such as vascular stasis, edema and prolonged swelling [3], extensive passive exercises leading to injury to ligamentous attachments [140], and respiratory alkalosis favoring the deposition of salts of calcium and phosphate leading to HO [141]. Shahidi et al. [142] studied the alterations of muscle tissue after TBI in a mouse model and concluded that TBI causes soft tissue damage in the form of muscle fiber degeneration. In fact, Li et al. [12] consider that muscle injury is the unifying feature for all types of HO.

Arthroplasty surgeries represent a main cause of post-surgical HO [3]. The risk is typically low and complications are rare. High-risk patients (e.g. those with spondyloarthritis and diffuse idiopathic skeletal hyperostosis [143], among others) are treated prophylactically with NSAID drugs. The most common reactive lesions in the hands and feet is bizarre parosteal osteochondromatous proliferation (BPOP), also known as "*Nora's lesion*". The lobular prolif-

eration of reactive bone and cartilage in BPOP forms an exophytic mass adjacent to the bone (benign tumors).

A number of pathologies not mentioned herein are associated with the cardio-vascular system, including mechanical failure of cardiac valves [144], and atherosclerosis [145,146]. As in other pathologies, calcification is seen prior to ossification, and not all calcified occurrences lead to ossification [144,146]. In addition to the pathological causes listed above, HO can be induced by the injection of liquids [147–149], the implantation of cells, tissues, drugs, demineralized bone, and materials including polymers [66], metals [67,150,151], and ceramics [68,69]. The term "osteoinduction" is generally used in this context. Importantly, HO risk is higher for men [25,152–154] and in younger adults [154,155].

## 3. Calcification

This section is devoted to calcification. A detailed analysis of the occurrence and involvement of calcification in HO is presented. The causes of calcification and its consequences are then discussed. Potential chemical triggers for HO are proposed. The end of this section addresses *in vitro* mineralization tests used to predict the ectopic potential of materials.

### 3.1. Occurrence of calcification and its involvement in HO

The role of calcification in the process of ossification has been the subject of considerable debate. For example, prior to his discovery of BMPs, Urist investigated tissue calcification and ossification following liquid injection such as ethanol, acid ethanol mixtures, calcium chloride, and phosphate solutions [149,156]. Like others, Urist observed that tissue calcification could sometimes lead to HO [147–149]. In 1965, Urist [84] demonstrated that demineralized bone (DB) was much more potent than unprocessed bone at inducing HO. Since DB was found to remineralize (= calcify) upon implantation, Urist evaluated the potential effect of calcification on osteoinduction. Although recalcification of DB was observed in several instances, Urist concluded that recalcification of DB rarely coincided with areas of osteogenesis [84]. The lack of correlation with recalcification of DB with osteogenesis was supported by the addition of toluidine blue (blocks carboxylic acid groups and binding of calcium ions) which prevented recalcification of matrix but did not inhibit osteogenic induction [84]. With the discovery of BMPs, Urist attributed the osteogenic effect of DB to be the sole result of BMPs [39]. Several investigators argued that calcification might be another trigger of DB-related HO [157]. Nimni [158] observed that the ability of DB to induce the formation of new bone, and its capacity to calcify after its bone-inductive ability is destroyed by glutaraldehyde fixation. In other words, preventing calcification prevented osteoinduction. Yamashita and Takagi [159] showed that DB calcification always preceded DB ossification in the formulation they tested, in agreement with various other authors [160–164]. An assessment of the experimental data leads to the conclusion that it is much easier to explain the osteoinductive properties of DB by considering two mechanisms of osteoinduction, one based on BMPs and one based on calcification (supplementary files).

More recently, the team of Schoenecker [165,166] tried to demonstrate that the persistent presence of minerals in muscles leads to HO. Specifically, Mignemi et al. [165] noted that an injury of the muscles of mice suffering from congenital plasminogen deficiency provoked muscle calcification and eventually HO. Injecting bisphosphonate weekly, starting two weeks pre-op, strongly reduced calcification and no HO was observed. Mignemi et al. [165] concluded that persistent dystrophic calcifications represent a potential osteoinductive mechanism for HO. Meyers et al. [25] came later to the same conclusion. In a different study, Moore-Lotridge et al. [166] induced locally the precipitation of hydroxyapatite crystals by injecting a cardiotoxin in the muscles of ABCC6-deficient mice (this mouse strain features low serum pyrophosphate levels, a calcification inhibitor). They observed removal of the minerals within 4 weeks and no HO. However, when liposome laden bisphosphonate (clodronate) were injected weekly from the time of injury until sacrifice to attenuate macrophage phagocytic activity, the mineral remained and HO occurred. Fournier et al. [167] observed both HO and dystrophic calcification (DC) of spinal tissues in diffuse idiopathic skeletal hyperostosis (DISH) patients and postulated that the two forms of ectopic mineralization may reflect different disease processes or perhaps distinct stages in the pathogenesis of DISH. In material-induced HO, surface calcification is often seen prior to bone formation [66,67,72,74,168–170] and the associated ionic imbalance is considered to be a pre-requisite for HO [150,151,169–176].

One of the main arguments against a calcification-triggered mechanism of HO is related to the observation that persistent calcification does not necessarily lead to ossification, as shown for example by Moore-Lotridge et al. [166]. An explanation for this observation was recently proposed by Bohner and Miron [79] who stated that ossification is triggered by calcification only when calcification overwhelms the homeostatic potential to maintain calcium and phosphate concentrations locally, leading to sub-physiological calcium and phosphate concentrations (sustained local ionic homeostatic imbalance; SLIHI). This hypothesis can also explain why ossification is more likely to occur in e.g. larger implants, larger animals, or bone graft substitutes with smaller pores [70,80,83]. Having established that calcification is a trigger for (at least some) HO, it is important to explain how calcification can occur *in vivo*.

### 3.2. Causes of calcification

Thermodynamic data reveal that soft tissue calcification could spontaneously occur. Indeed, the human body is supersaturated towards hydroxyapatite formation [79,177–180] with physiological concentrations of $Ca^{2+}$ and $PO_4^{3-}$ favoring precipitation of calcium phosphate crystals [181]. In general, calcification does not occur spontaneously because the human body contains many calcification inhibitors such as proteins and ions. These include for example magnesium [182,183], pyrophosphate [184–187], citrate [188–191], and phosphocitrate ions [190,192,193], as well as fetuin [105,146,194], and Matrix gla protein [146] (Fig. 4). Posner [195] proposed three mechanisms that can lead to calcification. The first mechanism is a local increase of the supersaturation towards biological apatite to levels that would cause spontaneous biological apatite precipitation. To this first mechanistic category belong the injection of a calcium chloride solution [149,156], the implantation of a soluble calcium and/or phosphate salt [196], systemic metabolic changes (e.g. hypercalcemia) occurring for example after traumatic brain injury, spinal cord injury, burn, and immobilization, and an increase of pH [179,197–203]. Providing substances that create nucleating sites or remove barriers to these sites constituted the second mechanism for calcification. Examples of this second mechanism include cell apoptosis, pyroptosis, necroptosis or necrosis, and the associated release of cell debris, including matrix vesicles, tissue necrosis, as well as the implantation of a material [79,204,205]. Removing or neutralizing bone mineral inhibitors was the third mechanism proposed by Posner for inducing calcification. An example for this third mechanism is the local reduction of pyrophosphate levels during bone apposition by osteoblasts [185] or the work with ABCC6 mice mentioned earlier [166]. In this context, one may point out that Porter et al. [206] reported that burn and possibly spinal cord patients, two populations prone to HO, suffer from hypophosphatemia and hypomagnesemia (magnesium ions are mineralization inhibitors).

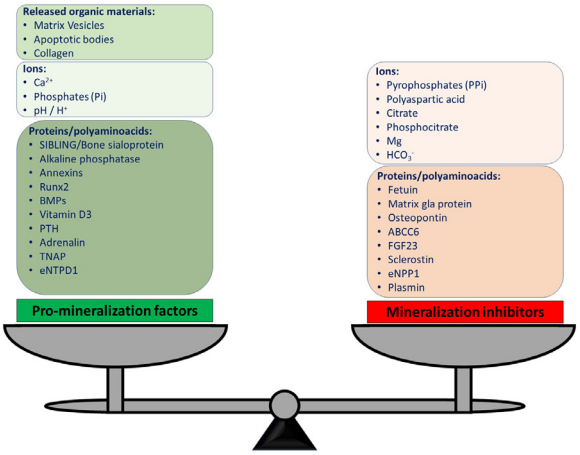


**Fig. 4.** List of some pro-mineralization and anti-mineralization factors present *in vivo*. Some have mostly a physical effect by promoting heterogeneous nucleation, e.g. collagen matrix [207,208], matrix vesicles [452–454], and apoptotic bodies [453,455]. Some have mostly a chemical effect, such bone sialoprotein [456–458], annexins [454], magnesium [182,183], pyrophosphate [184–187], citrate [188–191], and phosphocitrate ions [190,192,193], as well as fetuin [105,146,194], and Matrix gla protein [146]. Some compounds have mostly a biological effect. Among pro-mineralization factors, these include alkaline phosphatase [185,276,278], runx2 gene proteins [459], BMPs [460–462], Vitamin D3 [463], PTH [276,278], adrenalin [184], tissue-nonspecific alkaline phosphatase (TNAP) [185,454], and Ectonucleoside triphosphate diphosphohydrolase-1 (eNTPD1) [186]. Mineralization inhibitors include osteopontin [464], ATP-binding cassette sub-family C member 6 protein (ABCC6) [465,466], Fibroblast growth factor-23 (FGF23) [463], sclerostin [467–469], ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) [186,293,465], and plasmin [165]. The true effect of some factors involved in biomineralization are still under debate, for example osteocalcin [469,470], whereas other may have a dual effect, such as citrate [188] and polyaspartate ions [471]. An in-depth discussion of *in vivo* calcification, and in particular calcification promotors and inhibitors, can be found here [209].

Interestingly, osteoblasts might use all three calcification mechanisms to mineralize collagen. These include increasing the local phosphate concentration [185] and the pH, releasing matrix vesicles and producing the collagen network [207,208], decreasing the pyrophosphate ion concentration [185] and preventing by size exclusion the penetration of fetuin, a 48 kDa inhibitor of apatite growth, into the collagen network [194]. An in-depth discussion of *in vivo* calcification, and in particular calcification promotors and inhibitors, can be found here [209]. The consequences of calcification are discussed in the next section.

### 3.3. The consequences of calcification on SLIHI and its time-scale

Hydroxyapatite generally mineralizes in the form of nanometric crystals with a very large specific surface area. Bone apatite crystals have dimensions of ~1–5 nm in thickness, ~10–40 nm in width, and ~20–100 nm in length [210–213] with a specific surface area (SSA) close to 80 $m^2/g$. This means that the growth of a small quantity of hydroxyapatite crystals can consume very large amounts of proteins and ions. For example, the growth of a 1-nm thick apatite layer on 1g of bone-like hydroxyapatite crystals (≈ 2 $cm^3$ for a typical BGS) would completely deplete 25 mL of serum of its calcium and phosphate ions. Since depletion never reaches 100% due to kinetics and solubility considerations (e.g. -30% drop of calcium levels in [214]), the volume of serum that could be potentially affected by calcifications is very large. The possibility to disrupt local ionic homeostasis into a SLIHI is then related to a "*supply and demand*" problem. Demand is controlled by the consumption of ions based on crystallization kinetics including crystal nucleation and growth rate. Supply of ions is controlled by ion concentrations in the systemic circulation and the circulation rate. Once calcium and phosphorus homeostasis are disrupted, chemical gradients are formed. As a corollary, not detecting mineralization does not necessarily mean that there is no mineralization, but can simply mean that the characterization techniques are not powerful enough for its detection.

In bone formation, the duration of calcification is controlled by geometric confinement [215]. Once the interfibrillar space between collagen fibers is filled, calcification stops [160]. In tissue calcification, there is no confinement, so calcification can theoretically continue for a long time provided the calcification trigger is not removed by phagocytosis [166] or is fully coated by crystal growth inhibitors for example. In fact, sintered non-resorbable hydroxyapatite (HA; $Ca_5(PO_4)_3OH$) microporous pellets implanted in non-osseous sites of hamsters was reported to gain more than 50% weight over a year period [216], without bone formation. A small weight gain was reported for $\beta$-tricalcium phosphate ($\beta$-TCP; $Ca_3(PO_4)_2$) samples implanted for 4 and 12 weeks in the muscles of rabbits, followed by a small loss at 24 weeks [217].

Even though calcification may proceed during years [216], the appearance of bone within weeks suggest that the combination of inflammation and SLIHI can trigger HO on a much shorter time scale. Guo et al. [218] compared the biological reactions of non-osteoinductive and osteoinductive $\beta$-TCP granules. Bone was only observed associated with the osteoinductive $\beta$-TCP granules within the pores and between granules after 3 weeks. If a material can only elicit a SLIHI within its core, it might take months to observe HO since vascularization occur at a rate well below 1 mm/day [219,220]. Various investigators have reported an enhanced resorptive activity after HO [221,222]. This is likely due to bone remodeling activities following primary bone formation. Therefore, it appears likely that the SLIHI is reduced or disappears once ectopic bone is formed.

### 3.4. A potential chemical trigger for HO

Calcification can provoke a sustained local reduction of ions and proteins. It also leads to the creation of physical surfaces that cells can sense and respond to. Accordingly, local concentration changes, protein adsorption, and surface-cell interactions have been extensively discussed as potential cause of material-induced HO. Unfortunately, tracking local chemical changes in solution at the microscale is technically very demanding [223,224] and to the best of our knowledge has only been performed very recently in bone marrow [225]. Also, the distinction between a chemical and a physical cause for a given reaction is very difficult. The next paragraph addresses various potential triggers for HO, including calcium ions, phosphate ions, proteins, and topography.

Calcium ions have a generalized effect since they can modulate the function of the parathyroid gland, thyroid gland, kidney, and other organs and cells via the calcium-sensing receptor (CaSR) [181,226,227]. The CaSR is considered to regulate bone remodeling [228–230], including both osteoclastogenesis and osteoblastogenesis [229,231–233]. Extracellular $Ca^{2+}$ gradients represent potent chemical signals for cell migration and differentiation [234,235] of macrophages [236,237], osteoblasts [237,238], peripheral blood monocytes [239,240], hematopoietic stem cells [241], and bone marrow progenitor cells [242]. A number of investigators postulated that calcium levels modulate inflammation [236,243–245].

*In vitro* and *in vivo* studies devoted to sub-physiological extracellular calcium levels (i.e. less than 1.0-1.3 mM for ionized calcium [79]) are very limited because most studies address an increase rather than a decrease of Ca and phosphate [174,232,246]. However, sub-physiological Ca levels were found to strongly increase osteoblastic differentiation [247], decrease osteoblast proliferation and mineralized nodule formation [248,249], and lead to apoptosis [230,249]. Sadowska et al. [250–252] observed in

various co-cultures that osteogenesis was favored in the presence of a Ca-depriving solid like calcium-deficient hydroxyapatite (CDHA; $Ca_9(HPO_4)(PO_4)_5OH$) compared to a more inert solid like $\beta$-tricalcium phosphate. In co-cultures of osteoblast-like cells and bone marrow, a sub-physiological Ca level led to an increase in the number of osteoclasts [253,254]. Chen et al. [255] analyzed the effect of $\beta$-TCP extracts on macrophages, and subsequently the effect of macrophages pre-conditioned with $\beta$-TCP extracts on bone marrow-derived stem cells (BMSCs). These authors observed that the inclusion of $\beta$-TCP in the cell culture medium lowered the calcium and phosphate concentration, favored the M2 macrophage phenotype, and upregulated BMP2 expression. Macrophages preconditioned with $\beta$-TCP extracts triggered BMSCs osteogenic differentiation. Li et al. [256] compared the effect of two $\beta$-TCP samples varying in osteoinductive potential on macrophage polarization. The $\beta$-TCP sample with the highest mineralizing ability *in vitro* [256] and *in vivo* [170] was the most potent at polarizing M0 macrophages towards the M2 phenotype and at inducing HO [256]. Interestingly, cellular differences evoked by these two $\beta$-TCP samples were already noted after 1 day of implantation, which was confirmed in a more recent study [218]. In *in vivo* studies, bone loss occurred when the extracellular Ca level was reduced by giving a low-Ca diet [257–260]. However, these changes were accompanied by an increase in alkaline phosphatase (ALP) [257–259], 1,25(OH)2D3 [257], parathyroid hormone (PTH) [259], and osteoblast numbers [257,258].

Osteoclasts are sensitive to extracellular calcium concentration ($[Ca^{2+}]_e$) levels [261–263]. Sensing is made mostly via CaSR [264–268], but other receptors have been identified, such as the notch receptor and cadherins [269,270]. A change of $[Ca^{2+}]_e$ affects osteoclasts intracellular calcium levels [266,271,272], attachment [266,272,273], resorptive activity [265–268], apoptosis [233,274], differentiation [233], and genesis [265]. Accordingly, Hwang and Putney [275] suggested that $[Ca^{2+}]_e$ regulates the resorptive activities of osteoclasts. Most studies investigated the effect of an increase of $[Ca^{2+}]_e$ and concluded that an increase leads to a reduction of osteoclastic resorptive activity [265–268], perhaps due a reduction of attachment [266,272,273]. One may thus expect that the opposite occurs with a reduction of $[Ca^{2+}]_e$. In fact, Xiang et al [273] suggested that osteoclasts attach to bone surfaces where $[Ca^{2+}]_e$ is low. Also, a sub-physiological Ca level triggered an increase in the number of osteoclasts in co-cultures of osteoblast-like cells and bone marrow [253,254].

Another potential mode of action of SLIHI is via phosphate signaling. Any imbalance in phosphorus homeostasis may impact bone health in general, and bone cells in particular [276,277]. For example, phosphate levels affect and are regulated by ALP and PTH levels, which both play a key role in bone metabolism [276,278]. Beck [279] demonstrated that inorganic phosphate signaling facilitates the temporal coordination of expression and regulation of multiple factors necessary for mineralization. The same author suggested that inorganic phosphate levels induce osteopontin gene expression [280]. Unfortunately, only few studies are devoted to effects of low levels of inorganic phosphates on cells compared to low calcium levels. Farley et al. [247] noticed a decrease of ALP activity by osteoblastic cells with a decrease of phosphate concentration.

In addition to the involvement of calcium and/or phosphate ions, the mode of action of SLIHI could also include the adsorption of other ions or proteins on apatite. Indeed, apatite can readily interact with ions and proteins [281–286]. Hydroxyapatite crystals can adsorb ions at their surface (e.g. Mg) or even incorporate ions (e.g. Sr) in their lattice structure. Some of these ions, such as Sr, are biologically relevant and are used therapeutically, in particular in the context of (osteoporotic) bone [287–289]. Ripamonti [73] proposed that the osteoinductive action of coral-derived hydroxyapatite is related to the ability to adsorb and subsequently release growth factors.

During calcification, new surfaces are created indicating the mode of action of SLIHI could be of a physical nature. Numerous studies have demonstrated that the topography and stiffness of an implanted material can control the fate of cells on its surface, including differentiation into an osteogenic lineage [290–292]. Surface topography is considered to be critical in determining the material osteoinductive properties of biomaterials [70,71]. Nevertheless, it is not easy to distinguish between the chemical and topographical effect of a surface, since a topographical change has an effect on the chemical interaction between the material and its surroundings. An attempt to decouple the effect of topography and chemistry has not provided conclusive results in favor of topography [251,293]. HO is most often seen in the core of (porous) implanted materials even though the material topography is the same throughout the material. Additionally, topography (2D effect) acts on a much more limited number of cells compared to chemical gradients induced by (too) intense calcification (3D effect).

### 3.5. In vitro testing of the osteoinductive potential of bone graft substitute biomaterials

Having established that calcification is an essential step in a number of pathological HO and in material-induced HO, it would be of interest to have an *in vitro* test to predict the ability of materials to calcify (= mineralize) *in vivo*. The ability of materials to calcify once implanted *in vivo*, termed “bioactivity”, is considered by many investigators as a necessary step for osteoconduction [294]. Numerous methods have been developed to improve the bioactivity of biomaterials. Enhancing bioactivity has not only improved osteoconduction, but in many instances also osteoinduction. Several studies have for example demonstrated that a chemical treatment renders Ti implants much more prone to calcification and accordingly to trigger an osteoinductive response once implanted ectopically [67,80,150,295]. Considering that calcium phosphates and in particular hydroxyapatite are prone to mineralization *in vivo*, numerous authors have included calcium phosphates in composites. For example, Guillaume et al. [296] printed poly(trimethylene carbonate) scaffolds for orbital repair and demonstrated that the scaffolds became osteoinductive after addition of 40% hydroxyapatite particles into the polymer. Other investigators reported that the addition of a fine calcium phosphate powder to polymer scaffolds led to an osteoinductive response [297,298].

An *in vitro* test method has been developed and approved by the International Organization for Standardization (ISO) organization (ISO 23317:2014) as the standard to characterize implant bioactivity. For the test, the material is dipped into an aqueous solution simulating the inorganic composition of serum, the so-called “simulated body fluid” (SBF). After four weeks, the surface is observed and characterized to detect the formation of hydroxyapatite. The material is considered to be “bioactive” if it is coated with hydroxyapatite. Recently, Maazouz et al. [299] proposed an improved method, which is not only quantitative but also delivers results within 24 hours. The main differences with the ISO 23317:2014 standard are the use of an unbuffered and more concentrated SBF solution, and the constant measurement of the solution pH to detect a drop of pH value, indicative of mineralization. Maazouz et al. [300] demonstrated that the test is able to distinguish between non-osteoinductive and osteoinductive calcium phosphate granules. However, similarly to the ISO 23317:2014 standard, this new bioactivity test has limitations. For example, the extent of the solution pH drop, which is used to quantify mineralization, can be influenced by the release of soluble species from the tested material. This is in particular the case for bioactive glasses, Mg alloys, and calcium phosphate cements, as discussed hereafter.

Magnesium alloys and bioactive glasses have an inherent ability to trigger calcium phosphate calcification on their surface *in vitro* and *in vivo* [172,301–303]. However, neither of these two classes of materials have been reported to induce HO when used in bulk quantities despite claims of osteogenicity [304–307]. Magnesium and bioactive glasses generate a more alkaline environment *in vivo* than calcium phosphates [308–310]. This suggests that an alkaline environment has a detrimental effect on osteoinduction. A low corrosion or dissolution rate, or a decrease of the volume fraction of these materials would likely decrease the risk of an alkaline local pH and increase the potential for osteoinduction. In fact, the addition of a small amount of Mg was reported to make Mg-HA blends more osteoinductive [306]. Specifically, a 30%-70% blend of Mg and HA beads generated more bone ectopically than either a 50-50% blend or pure HA. Several investigators reported that the addition of a small fraction of bioactive glass particles to a polymer scaffold triggers an osteoinductive response [311–313]. The use of small fractions of dispersed bioactive glass particles might therefore be a potent way to provide osteoinductivity to porous polymer scaffolds.

Most calcium phosphates are considered to be bioactive and osteoconductive materials [178,314]. However, not all calcium phosphates have been reported to trigger bone formation upon ectopic implantation. One particular example is $\alpha$-tricalcium phosphate ($\alpha$-TCP; $\alpha$-$Ca_3(PO_4)_2$) which is chemically identical to $\beta$-TCP. Once implanted, $\alpha$-TCP hydrolyzes at close to neutral pH into octacalcium phosphate (OCP; $Ca_8(H_2)(PO_4)_6{\cdot}5H_2O$) or CDHA via a dissolution-precipitation reaction [178,314,315]. In contrast to scaffolds made of CDHA and $\beta$-TCP that have been reported to induce bone ectopically [71,221,316–318], there is to the best of our knowledge no report on the osteoinductive properties of $\alpha$-TCP scaffolds. In fact, Tang et al [70] reported the following trend of osteoinductivity: BCP > $\beta$-TCP > HA >> $\alpha$-TCP. One potential explanation is an increase in local calcium and phosphate concentrations due to $\alpha$-TCP dissolution rather than the proposed decrease hypothesized to be required for calcification-triggered HO.

## 4. Cells involved in HO and HO formation timeline

This section discusses which cells may interact with calcification induced SLIHI that triggers the biological cascade leading to pathological and material-induced HO. A quantitative timeline of HO formation is proposed also proposed in this section based on a review of the available literature.

### *4.1. Cells involved in pathological and material-induced HO*

Soft tissue injury is widely recognized as being a common prerequisite to HO occurrence [319], even in the context of a disease such as FOP (muscle trauma), tuberculosis (lung tissue damage), or Covid (lung tissue damage) [320]. The phenomena that follow soft tissue injury include hemostasis, inflammation, proliferation and remodeling [321]. There is consensus regarding the inflammatory origin of HO [25,104,139,322]. The involvement of osteoprogenitors is also clearly established [323]. This section attempts to link biological observations made in the case of pathological HO and material-induced HO with an emphasis on the cells involved in this process.

Inflammatory and immune cell response:

Urist observed in his founding paper in 1965 that histiocytes were the most numerous cells in histologic slices of ectopic explants of demineralized bone matrix [84]. Histiocytes are tissue macrophages derived from circulating monocytes. They are mononuclear phagocytic cells that participate in innate immunity. Different studies have shown that macrophages accumulate at the site of soft tissue injuries in HO [132,319,324,325]. Depletion of macrophages and mast cells impaired HO in a murine model of FOP and in immune-depressed FOP patients showed attenuated HO [326]. Sorkin identified monocytes as key players in the regulation of HO in a murine tenotomy/burn model of aberrant wound healing [327]. They identified a heterogeneous population of monocytes in response to injury, commitment to aberrant chondrogenesis early in the inflammatory phase, and identified TGF-$\beta$1 as a potential therapeutic target to modulate HO [327]. In addition, they observed that neutrophils that were abundant at early phases of the injury decreased in number with the increasing number of monocytes. Tseng recently demonstrated that neurogenic HO develops independently of neutrophils and granulocyte stimulating factor (G-CSF) by triggering it in a neutropenic and G-CSF receptor deficient mouse model [328]. Tissue macrophages have also been shown to secrete a number of osteogenic proteins such as BMP-4 [319,324] in addition to TGF-$\beta$1 [327,329] following injury prior to the development of HO [330]. The transition from M1 to M2 phenotype has been demonstrated to enhance osteogenic differentiation of mesenchymal stem cells [330–333]. However, additional yet to be characterized monocyte/macrophage populations appear to be involved [327].

In the case of material-induced HO, macrophage polarization towards the M2 phenotype has been shown to be central in the formation of ectopic bone and is influenced by topography [256]. Zhao et al. identified T-cell populations and the activation of the Nf-K$\beta$ pathway as responsible for the recruitment of mesenchymal stem cells (MSCs) to the ectopic site of implantation of biphasic calcium phosphate (BCP) bioceramics [334]. They also noted that the reduced inflammation associated with minimally invasive implantation procedures decreased the occurrence of bone in the implants. B and T lymphocytes have also been observed in close proximity to ectopic bone formation in cardiac valves [144]. However no evidence was found in the literature regarding the involvement of natural killer cells.

The role topography plays to influence initial inflammatory responses in material-induced HO has been studied extensively comparing two $\beta$-TCP materials denominated TCP-B and TCP-S [76,77,170,222,256,335–339]. TCP-S induces bone formation following ectopic implantation while TCP-B does not. TCP-S and TCP-B exhibit different microstructures, the former possessing submicroscopic grains and the latter micron-size grains. This translates to a difference in micropore size and specific surface area without a significant change in total porosity. TCP-S has been shown to evoke a larger decrease of calcium than TCP-B [256] after just 1 day in cell culture medium. TCP-S also exhibited larger surface mineralization (= calcification) after 1 day of immersion in simulated body fluid (SBF) [170]. Lower gene expression and protein quantity of interleukin 6 (Il-6) and interleukin 1$\beta$ (Il-1$\beta$) was demonstrated in TCP-S explants compared to TCP-B explants after 1 day of implantation in FVB mice. Higher vascular endothelial growth factor (VEGF) gene expression and protein quantity was detected after 4 days for TCP-S implantation. Immunohistochemical staining for F4/80, indicative of the presence of macrophages, was higher following implantation of TCP-S compared to TCP-B at 1, 4 and 7 days of implantation. In contrast, C-C Motif Chemokine Receptor 7 (CCR7) immunohistochemical staining indicative of the presence of lymphocytes was more abundant at 1- and 4-days following implantation of TCP-B. Immunohistochemical staining of clusters of differentiation $206^+$ ($CD206^+$) indicative of the presence of M2 macrophages was enhanced following implantation of TCP-S but not in TCP-B at 4 and 7 days post-implantation. These results suggest that calcification which induces local extracellular calcium depletion could be responsible for the initial differences in inflammatory response leading to an increased secretion of inflammatory cytokines such as Il-6 and IL-1$\beta$ in the case of TCP-B compared to TCP-S. The upregulation of VEGF at 4 days of implantation suggests

that the stimulation of neovascularization by TCP-S follows the initial inflammatory regulation and coincides with the presence of M2 macrophages ($CD206^+$) which are then increased until day 7 post implantation.

The presence of multinucleated giant cells (MNGCs) is observed in pathological HO [25,340–344], ectopic implantation of osteoinductive calcium phosphate materials [256,336], BMP-loaded scaffolds [345] or MSC-laden constructs [346]. The body of evidence regarding MNGCs presence in pathological HO is not as large as in the case of ectopic implants, possibly due to the fact that it may be more complicated to study the onset of pathological HO than it is to explant ectopic constructs after a deliberate time. MNGCs have been observed in tissues presenting DC [347] and before bone formation in the case of ectopic osteoinductive calcium phosphates [218]. Consequently, it seems justified to postulate that the formation and presence of MNGCs, as evidenced by histomorphological appearance, coincides with the onset of ectopic bone formation in early stages of HO.

Additionally, *in vivo* data indicates an active anabolic role of MNGCs in the bone formation process [256,336,345,346]. Notwithstanding, osteoinductive calcium phosphates (TCP-S) loaded with liposomal anti-resorptive bisphosphonate (clodronate), did not form ectopic bone [336]. Runt-related transcription factor 2 (Runx2) was upregulated by TCP-S but not by TCP-B (non osteoinductive calcium phosphate) or clodronate loaded TCP-S, suggesting a paracrine effect of MNGCs on osteoblast differentiation. Clodronate may also have had a direct effect on monocyte and macrophages depletion as well as on bone formation by inhibiting calcification in a similar manner as other pyrophosphates. Interestingly, Saito et al demonstrated that the time of bisphosphonate administration is important in preventing pathological HO [348].

Elucidation of the identity of MNGCs in the process of bone formation is important from a biological perspective. Davison et al [336] noted that not all MNGCs present in an osteoinductive $\beta$-TCP scaffold were TRAP positive indicating the heterogeneity of MNGCs throughout the explant. The same authors observed that not all TRAP positive MNGCs were located next to bone suggesting that the presence of bone may not be necessary for their formation. Various authors have in fact proposed that there are non-resorbing and resorbing MNGCs [349–351]. Despite many common features, a distinct characteristic between non-resorbing and resorbing MNGCs was proposed to be the expression of $\beta 3$ integrin: whereas monocyte/macrophage precursors and foreign body giant cells are inflammatory cells expressing $\beta 2$ integrin, resorbing MNGC formation comprises a transition from $\beta 2$ to $\beta 3$ integrin expression *in vitro* [352,353] and *in vivo* [354]. Kikuta et al [355] discovered that non-resorbing MNGCs are migrating, contrary to resorbing MNGCs which are static. One central question is whether both types of MNGCs are involved in HO. Considering that scaffold resorption is generally not observed prior to (ectopic) ossification [83,350,351,356–358] and that non-resorbing MNGCs are often associated with an osteogenic action [349,359–361], one may conclude that (pro-)osteogenic MNGCs present prior to ectopic bone formation / HO are non-resorbing MNGCs. However, studies devoted to the effect of $[Ca^{2+}]_e$ on osteoclasts suggest that a decrease of $[Ca^{2+}]_e$ might increase the number, attachment, and resorptive activity of osteoclasts [253,254,265–268,272,273]. Once woven bone is formed, bone remodeling occurs to form lamellar bone, a process that requires the recruitment and resorptive action of resorbing MNGCs. This could explain why more scaffold resorption is seen in osteoinductive BGS [316,335].

Osteoprogenitors: Recruitment and transition: The origin of osteoprogenitors in HO has been the subject of considerable debate. Multiple cell types appear to have the potential to differentiate into osteoblasts. Urist [84] incorrectly associated the presence of tissue histiocytes or perivascular connective tissue cells as potential osteoprogenitors. It has been demonstrated since that immune cells, myoblasts and somite-derived cells do not form osteoprogenitors [362]. Medici and Olsen argued that endothelial to mesenchymal transition (= dedifferentiation) is a key step to heterotopic ossification [363] due to the large percentage of endothelial-derived cells [364]. Resident endothelial cells may also be osteoblast progenitors. Circulating stem cells have also been shown to participate in HO [365,366]. Smooth muscle cells are able to produce osteochondrogenic cells [367]. Muscle derived progenitor cells that are $PDGFR\alpha^+$ have been shown to induce HO and to be present in high density close to human heterotopic ossification sites [368]. Finally, pericytes which are interstitial cells found surrounding blood vessels or in the blood brain barrier of the central nervous system were in multiple studies shown to form osteoblasts using GLAST-CreER and Tie2 labelling [362]. Investigators have also demonstrated the role of vascularization on HO [159,175]. Song et al. demonstrated that MSCs from a male beagle dog injected intravenously into a female dog homed to the HO site induced by biphasic calcium phosphate granule implantation intramuscularly at 4 weeks post implantation [369]. The above results support the role of resident and circulating stem cells from a number of tissue types that are capable of differentiating into osteoblast progenitors associated with HO.

### 4.2. HO timeline

HO formation is the result of a long cascade of reactions. An attempt is made herein to describe the general timeline of HO formation and to explain why HO occurrence is a markedly slower process than BMP-induced bone formation.

HO occurs following central nervous system trauma [109,370], blunt or high energy trauma [16,19,124], burns [5,110–113,371], surgery [7,19,119] or the implantation of a material [372]. A common aspect associated with these conditions leading to HO is soft tissue damage [142] or injury. Soft tissue damage or injury is followed by a disruption of tissue perfusion due to the rupture of the blood vessels [373] and initiates an inflammatory response at the tissue damage site [371] and calcification [348]. The local hypoxia can start in the hour following the injury and was reported to last up until 4 days [374], but it is likely that it may last much longer in cases with highly compromised blood supply, e.g. in compartment syndrome. Hypoxic conditions in addition to the injury further increase cell death by necrosis or apoptosis. As described above, inflammation orchestrated by monocyte/macrophage recruitment and then by MNGCs can lead to the differentiation of local or circulating stem cells and HO. The presence of a biomaterial with the appropriate composition and topography can amplify this inflammation-mediated process.

In calcification-triggered HO, vesicles released by necrotic cells can act as nucleation sites for the precipitation of calcium phosphates from the extracellular fluid [375]. Calcification provokes a consumption of calcium and phosphate which can lead to a SLIHI towards calcium and phosphate [79]. If local homeostasis is recovered quickly, normal soft tissue healing proceeds and phagocytosis of any precipitated minerals occurs [376]. A SLIHI constitutes the basis of the permissive environment for HO to occur. It is suggested that this SLIHI modulates the inflammatory cells towards the formation of M2 macrophages. The formation of M2 macrophages is permissive to osteoclastogenesis [376]. If M1 macrophages are dominant, the osteoclastogenesis is inhibited due to their secretion of interferon gamma (IFN-$\gamma$) and IL-12. Osteoprogenitors are recruited from circulating MSCs and/or local endothelial or tissue sources owing to the secretion of insulin-like growth factors 1 (IGF-1) and BMPs by M2 macrophages [377] and osteoclasts [378]. Interestingly, osteoclasts can exert an anabolic activ-

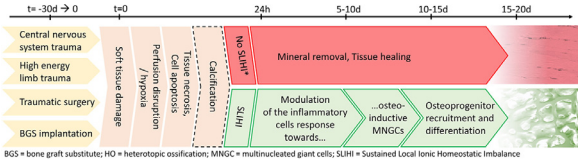


**Fig. 5.** Pathway from injury to ectopic bone / heterotopic ossification. Inflammation starts as soon as injury has occurred. In the absence of sustained local ionic homeostatic imbalance (SLIHI), soft tissue healing occurs. The timing is very approximative. Injuries to the central nervous system does not immediately lead to soft tissue injury. It may typically take a month. Bone image adapted from Lenthe et al. [472].

ity as early as 7 days by stimulating osteoprogenitors via paracrine signaling that seems to precede the catabolic activity [357]. Several studies have related the presence of TRAP/Cathepsin K multinucleated giant cells and the formation of HO [335,336,357]. Bone formation (without or with an intermediate chondrogenic stage) rarely occurs before 10 days after the process of HO is initiated.

Describing a timeline for HO formation is difficult due to the complexity of this biological phenomenon. Conclusions can be further compounded by species-dependent differences. In addition, the timing of HO initiation might not coincide with the time of injury. Soft tissue damage, which is the most common initiator of traumatic HO and which is considered by Li et al. as the unifying feature for all types of HO [12], (Fig. 5) can be a secondary phenomenon in a variety of pathologies [379]. Many investigations do not provide sufficient time points to determine the timeline of HO. Pathological HO follows mostly an endochondral pathway [4,165,166,380–382] contrary to material-induced HO where an intramembranous ossification pathway [79] is generally found (exception: [383]), perhaps due to a different tissue stiffness [384]. As a result, it can be speculated that bone appears earlier in material-induced HO compared to pathologic HO. The timeline indicated in Fig. 5 is an approximation and relies heavily on a few extensive longitudinal studies, in which the starting point of the injury and the mineralization are precisely defined. However, the time at which relevant cells can react to a SLIHI provoked by calcification is unclear. This may typically occur when an osteoinductive material such as demineralized bone or an osteoinductive calcium phosphate scaffold is implanted [85,159,390–392,218,256,379,385–389].

Bonucci and Sadun [379] noted in a rat model of trauma-induced HO that interstitial edema and scanty infiltration of inflammatory cells occurred within 12 to 24 hours after injury. A similar result was obtained by Gibson et al. [371] in a mouse model. These authors observed that abnormal muscle inflammation could last up to 2 weeks in poly injuries. Bonucci and Sadun [379] further reported that interstitial edema resolved and cellular infiltration and muscle fiber degeneration were more marked after 24 hours. Muscle calcification was only seen after at least 36 hours. Other authors have reported that tissue calcification typically starts 1-7 days after injury and/or implantation [159,393]. Bonucci and Sadun [379] observed that areas of necrosis were more conspicuous after 48 hours. Fibrous tissue proliferation was observed after 102 hours and only small areas of calcification were present. These areas of calcification were scattered throughout the muscle and were usually surrounded by macrophages and MNGCs showing marked signs of phagocytosis. Other authors reported resorption associated with presence of MNGCs after 4-7 days [389,392]. The initiation of cartilage formation was generally detected after 7-10 days [386,388,390–392,394]. This is followed by bone and bone marrow formation, typically after 10 days [386,389,391,392] and 14-15 days [391,395], respectively. This timeline may expand since Urist et al. [84,85,385,396] regularly reported bone appearance after 18-30 days. Bone marrow formation after 20-30 days has also been observed [389,394,397]. Guo et al. [218] observed a similar sequence with osteoinductive calcium phosphate scaffolds implanted at ectopic locations. They reported macrophage colonization within hours to days peaking after one day post-surgery. Macrophage polarization occurred within 7 days. Osteoclast formation was observed mainly between week 1 and 2 post-surgery. Bone formation was seen at week 4 post-surgery. In humans, HO is generally reported to be first observed at three weeks [11,110,398].

## 5. Potential approaches to control HO

Having identified a potential mechanism for HO, it is highly relevant to discuss the potential ways to prevent HO or enhance the BGS osteoinduction. Importantly, the aim here is not to address approaches to use the human body as a bioreactor to engineer large bone graft [399–405], which is an interesting topic in itself, but not the focus of the present manuscript. Fig. 5 describes the cascade of events that lead to calcification-triggered HO. When critical steps along the cascade are not fulfilled (e.g. no/insufficient MNGC anabolic action or BMP release), the process of calcification-triggered HO is arrested and normal healing resumes. Accordingly, each step (and combination of steps) can be considered as a therapeutic target, either to induce bone formation for bone regenerative purposes, or to prevent HO. Potential ways of inducing/arresting HO formation would therefore be to act (i) on the combination of inflammation and calcification-triggered SLIHI, (ii) on the monocyte/macrophage M1 to M2 transition, and cathepsin K, TRAP positive multi-nucleated giant cells (MNGCs) [359], and/or (iii) on BMPs/stem cell differentiation. It is therefore not surprising that current therapies against HO target some of these steps, mostly inflammation/MNGCs with NSAID drugs [2,30,33,35,36,153], mineralization with bisphosphonates [2,30,33,406], and stem cells with radiation therapy [2,30,34,153,407]. Reciprocally, a combined modulation of inflammation from M1 to M2 and calcification should provide a more osteoinductive BGS. In fact, a more invasive surgical approach for the implantation of an osteoinductive material, or a more bioactive bone graft substitute leads to more ectopic bone [300,408]. Some ways to increase mineralization are listed in Fig. 4. Local immune modulation could be triggered by a change of surface topography, the addition of loose inflammatory particles, or an inflammatory compound [409].

If SLIHI is the chemical trigger for HO, a potential way to modify HO occurrence is to target calcium sensing receptors, such as CaSR and G-protein-coupled receptor 6A (GPRC6A) [186]. To the best of our knowledge, the use of calcilytics and calcimimetics to enhance or respectively reduce HO has never been tried. The use of a calcilytic have been proposed against osteoporosis, but their potential to indirectly stimulate bone formation remains uncertain [410]. This could be due to the fact that CaSR is not only present in monocytes [411], macrophages [411], osteoclasts [412,413], but also in osteoblasts and osteocytes [414]. Another approach to modulate HO would be to target ion channels and pumps, such as voltage-gated calcium channels (VGCC) [266,272,415–417], Na-Ca exchange ion pump (NCX) [418–421], Plasma-membrane $Ca^{2+}$ ATPase transport protein (PMCA) [421], Transient receptor potential vanilloid cation channel (TRPV) [422,423], or the mechano-responsive PIEZO1 protein [424–426]. Since there are many ion channels and pumps [427], the problem is to decide which channel or pump should be targeted first. Tang et al. [428] observed that the only ion transporter that was significantly upregulated during BMP2-induced osteoblastic differentiation was the Na +, K + -ATPase ion pump (NKA) [429]. Tang et al. [428] demonstrated that the amount of bone formed upon ectopic implantation of a BCP scaffold could be reduced or enhanced by inhibiting, respectively activating NKA. Since a decrease of intracellu-

lar calcium happens both with the activation of NKA [473] and a decrease of extracellular calcium concentration [275], the results of Tang et al. [428] are in line with the SLIHI mechanism. Another confirmation of the importance of Ca concentrations in the context of material-induced HO was provided by Klar et al. [430] who investigated the effect of verapamil hydrochloride, a $Ca^{2+}$ channel blocker, on the osteoinductive potential of microporous coral-derived BGS. They observed a significant delay of ectopic bone formation in the presence of the channel blocker.

The next section is discussing approaches to modulate SLIHI and HO by a change of the properties of a BGS. In the following section, the conditions for materials to induce HO are then revisited.

### 5.1. Modulating SLIHI

SLIHI is the result of an imbalance between the consumption and supply of calcium and phosphate ions. The ions are consumed by calcification/mineralization on the implanted BGS surface or necrotic tissue. This section describes the consumption and supply of calcium and phosphate ions provoked by the implantation of a porous BGS. Several "rule-of-thumb" equations are presented to determine which properties of the BGS are most likely important for SLIHI excluding biological factors.

In a first approximation, one may assume that the consumption of calcium and phosphate ions by an implanted BGS material, $\Delta(Ca,P)$, is determined by chemical factors, $C_f$, and by the accessible surface area of the implant, $S_m$, on which mineralization can occur. The chemical factors, $C_f$, correspond to the three mechanisms of mineralization described by Posner [195], namely an increase of supersaturation, a reduction of the concentration of mineralization inhibitors, and the addition of a new surface. One may write:

$$\Delta(Ca,P) = C_f \times S_m \tag{1}$$

The surface of the BGS material, $S_m$, is proportional to its mass, $m$, and its specific surface area, $SSA_m$ (assuming in first approximation that calcium and phosphate ions can reach surfaces accessible to nitrogen gas as in SSA measurements):

$$S_m = m \times SSA_m \tag{2}$$

The BGS mass, $m$, is related to the theoretical density of the BGS material, $\rho_{th}$, and the volume of the BGS material, $V_m$, by the following equation:

$$m = \rho_{th} \times V_m \tag{3}$$

The volume of the BGS material, $V_m$, should not be confused with the volume of the BGS, $V_i$. The volume of the BGS corresponds to the sum of the volume of the BGS material and the volume of the pores. $V_m$ and $V_i$ are related by Eq. (4):

$$V_m = (1 - p_i) \times V_i \tag{4}$$

where $p_i$ is the implant porosity, i.e. the sum of the nano, micro and macroporosity. Combining Eqs. (1) to (4) gives:

$$\Delta(Ca,P) = C_f \times (\rho_{th} \times (1 - p_i) \times SSA_m) \times V_i \tag{5}$$

The latter equation describes the consumption of ions throughout the solid. This equation is only valid if the conditions present within the solid are identical, i.e. if the supply of ions is identical everywhere. This is likely not the case. To assess the (im)balance between consumption and supply of ions, the supply of ions must be estimated. In a first approximation, the supply of ions into the core of the BGS must be proportional to the surface of the envelope (or external surface) of the BGS, $S_E$, because all ions must cross this surface (Fig. 6). However, part of this envelope surface

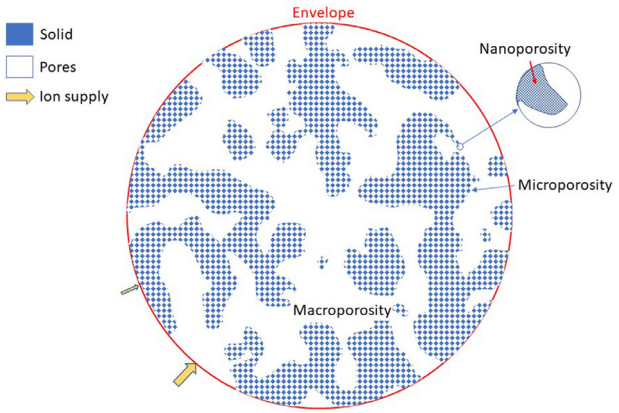


**Fig. 6.** Scheme showing the transport pathways into a porous scaffold consisting of nanopores (< 100 nm), micropores (100 nm → 10 µm) and macropores (> 10 µm). The solid is in blue, the pores in white, and the transport pathways are sketched with orange arrows. The outside envelope of the scaffold has a surface $S_E$. Ions can only be transported into the core of the scaffolds through the porous part of the outside envelope, $S_{Eo}$. $S_E$ and $S_{Eo}$ are related by the porosity pi: $S_{Eo} = S_E$ x $p_i$.

is occupied by the solid part of the material through which ion transport is null. So, only the open (= porous) external surface, $S_{Eo}$, should be considered for ion transport:

$$S_{Eo} = S_E \times p_i \tag{6}$$

The (im)balance between the consumption ("demand") of ions and the "supply" of ions can be considered as a semi-quantitative factor assessing the risk of SLIHI. It can be assessed by dividing the consumption ("demand") by the open external surface of the BGS:

$$Risk\ of\ SLIHI \propto \frac{\Delta(Ca,P)}{S_{E0}} = C_f \times \left(\frac{\rho_{th} \times (1 - p_i) \times SSA_m}{p_i}\right) \times \frac{V_i}{S_E} \tag{7}$$

An increase of this ratio is indicative of a larger imbalance between "supply" and "demand" and accordingly to a higher risk for SLIHI to occur. So, this ratio should be maximized to maximize the chances to induce HO.

Looking at Eq. (7), one may point out that the risk of SLIHI depends on three terms, one related to the local chemical environment, $C_f$, one related to the implanted material morphology or geometry, and the third one related to the volume to surface ratio of the implanted material. This means that there are multiple ways to influence SLIHI. More details are provided in the next paragraphs.

Posner [195] described three mechanisms to induce mineralization *in vivo*, namely an increase of supersaturation, a reduction of the concentration of mineralization inhibitors, and the addition of a new surface. The first mechanism, i.e. the increase of supersaturation, can be activated by an increase of calcium or phosphate ion concentration. Another approach is to increase the local pH since the solubility of hydroxyapatite decreases with an increase of pH. However, as pointed out earlier, materials whose bioactivity is due to the release of calcium or phosphate ion concentration or to an increase of pH, such as bioactive glasses, Mg alloys, or $\alpha$-TCP, have not demonstrated any osteoinductive potential when implanted in bulk amounts. The detrimental effect of the release of calcium and phosphate ions on achieving SLIHI is obvious. The effect of pH is less obvious. One may speculate that the pH increase has a detrimental effect on the formation, and/or action of MNGCs. The second mechanism proposed by Posner, i.e. the reduction of the concentration of mineralization inhibitors, can be perhaps achieved by sieving out proteins in the same way as the collagen network prevents the penetration of fetuin by size exclusion [194]. In that re-

spect, Konka et al [431] have shown that the pores of CDHA scaffolds can sieve out proteins. The third mechanism proposed by Posner is related to the ability of a foreign surface to trigger mineralization. This ability depends on the interfacial energy between the foreign surface and hydroxyapatite. If this energy is low, then the foreign surface can easily mineralize, which explains why hydroxyapatite is particularly potent at inducing bone ectopically. It also explains why an *in vitro* test may predict in certain conditions the osteoinductive potential of a BGS [300].

The second approach to modulate SLIHI is to modify the morphology of the BGS. As suggested by Eq. (7), the two main strategies are an increase of SSA and a decrease of porosity. While there are a number of studies showing a link between a high SSA and HO formation [74], there is limited experimental evidence related to a change of porosity (keeping the same pore size) [82].

The third approach to modulate SLIHI is to vary the volume-to-surface ratio of the implanted material. For a spherically-shaped implant, this ratio, ($V_i/S_E$), is equal to one sixth of the diameter of the sphere. In a flat-cylindrically-shaped implant, it is roughly equal to half of the cylinder height (when h << r).

$$\frac{V_i}{S_E} = \frac{\pi \times r^2 \times h}{(2 \times \pi \times r \times h) + \left(2 \times \pi \times r^2\right)} = \frac{r \times h}{2 \times (h + r)} \cong \frac{h}{2} \quad (8)$$

In practice, this means that HO can be promoted by an increase of the thickness of the implanted material ("dose" increase), in accordance with experimental observations [83].

The considerations made so far have not involved any temporal aspects even though SLIHI is time dependent. Since the supply of ions comes from the surface of the material (via a mixture of diffusion and convection), the risk of having SLIHI increases with the distance to the external surface of the implanted BGS, particularly if the path to reach the center is tortuous and thin. The distance from one point in a pore to the surface of the BGS varies within each pore, so local differences of SLIHI can be expected throughout the porous network. Accordingly, concavities should be more prone to SLIHI than convexities. If pores are cylindrical with a height *h* and a radius *r*, Eq. (8) applies and indicates that the risk of SLIHI increases in deeper and narrower cylindrical pores. Beside diffusion, ions might also be transported by convection, in particular if the BGS consists of an assembly of BGS granules. Convection is a function of the scaffold permeability, which is itself a function of the porous network. Using a simple approach, it can be shown that the permeability decreases with an decrease of porosity and pore size [432]. In fact, Fukuda et al [80] showed that more bone was found in prismatic pores of fixed length when the pore width was reduced.

A summary of the considerations presented in this section is provided in Table 2. Noteworthy, three parameters that are regularly mentioned as being essential for osteoinduction, namely microporosity [71,383], topography [70,71], and ion doping are not listed and have not been addressed specifically (but indirectly) herein yet. An inherent problem in assessing the effect of these parameters (or any material property) on osteoinduction is that the modification of one parameter may affect many other parameters. For example, it was stated in [71] that "*microporosity correlated to (…) bone induction* in vivo" because HA, which had a low microporosity (3.1%), did not induce bone ectopically contrary to $\beta$-TCP, which had a high microporosity (48.7%). This statement is correct but ignores the fact that the SSA value was increased from 0.1 $m^2/g$ to 1.2 $m^2/g$. Similarly, doping calcium phosphates with ions like Zn [433], Sr [434,435], or Si [436] has an effect on the physico-chemical properties of the material, and in particular on the chemical interactions between the material surface and a solution, as shown recently with Mg-doped HA [300]. Importantly, it is always possible to find exceptions to a rule. For example, Wang et al [175] observed more ectopic bone in convex than in concave porous structures. Similarly, Duan et al [170] found no bone in a high SSA bone substitute. However, the chemical analysis of the implanted materials is often very limited, which means that it is not possible to assess the potential effect of the chemistry factor, $C_f$, on SLIHI. The recent studies of Maazouz et al [299,300] have in fact shown that sintered compounds may contain enough surface impurities to markedly affect the chemical environment present within the pores of the material, where ectopic bone typically forms. In conclusion, it is postulated that the risk of SLIHI is a function of chemistry, implant material geometry and transport:

$$\textit{Risk of SLIHI} = f(\textit{chemistry},\ \textit{implant material geometry},\ \textit{transport})$$

### 5.2. Revisiting the conditions for materials to induce ectopic bone formation

In the context of material-induced HO, a material (defined by a composition and an architecture) was defined to be osteoinductive if (i) it calcifies *in vivo*; (ii) it is porous (porous scaffold or assembly of granules); (iii) the pores are large enough to allow blood vessels ingrowth and cell transport into the core of the material; and (iv) blood supply is insufficient to maintain physiological calcium and/or phosphate ion concentrations [79]. Some of these criteria should be revisited based on recent investigations. For example, Ginebra and co-workers suggest that the consumption of

**Table 2**
Expected effects of physico-chemical parameters on SLIHI (Sustained Local Ionic Homeostatic Imbalance). The table considers three different categories of aspects: (i) chemical aspects (basically the mechanisms proposed by Posner to explain the causes of *in vivo* mineralization), (ii) aspects related to the material geometry, (iii) aspects related to ion transport. An increase of a specific feature can either have a positive or a negative effect on SLIHI. For example, SLIHI risk is enhanced with an increase of smallest implant dimension. Similarly, SLIHI is decreased with an increase of pore size. "New surface" means simply a surface on which apatite crystals can form. Examples of "new surfaces" include cell debris, matrix vesicles, or an implant.

| Effect on SLIHI | Chemical aspects (Posner mechanisms) | Material implant geometry | Transport |
|---|---|---|---|
| POSITIVE | • New surface (e.g. cell debris, matrix vesicles, implant) | • SSA increase [74] | • Smallest implant dimension [83]<br>• Distance to the implant surface [74,80]<br>• Concavity [316,442]<br>• Tortuosity |
| NEGATIVE | • Supersaturation ($[Ca^{2+}]$, $[PO_4^{3-}]$, pH)<br>• Mineralization inhibitors (proteins, $[P_2O_7^{4-}]$, $[C_6H_5O_7^{3-}]$, $[Mg^{2+}]$)<br>• Interfacial energy between apatite nuclei and the new surface | • Porosity [82] | • Porosity [82]<br>• Pore size [80] |

**Table 3**
Comparison of two strategies to trigger HO using bone graft substitutes, one based on exogeneous BMP, and one based on calcification.

| | Exogenous BMP-triggered HO | Calcification-triggered HO |
|---|---|---|
| Location of ectopic bone | Partly controlled: Bone forms within and on the outside of the carrier | Controlled: Bone only forms within the bone graft mass |
| Dose dependency | No bone with low dose<br>Reliable bone formation with adapted dose | No bone with a single granule<br>Bone forms with adapted volume and material design |
| Concentration | Supraphysiological concentrations ($\approx 10^5 >$ to that present in bone) | Depletion of Ca and $PO_4$ (sub-physiological concentrations) |
| Potency | High | Low to possibly high |
| Drug / active substance release | Released in the host tissue | No release, but an uptake. |
| Mechanism of action | BMP pathway: exogenous stimulation of osteoprogenitor differentiation | Homeostatic imbalance caused by calcification modulates inflammation to trigger osteoprogenitor differentiation |
| Time to bone formation | ≈10 days | 3 weeks or more |
| Structure dependency | Protein conformation | 3D architecture of the biomaterial |

calcium ions by CDHA is not due to crystallization reactions but to maturation reactions [250,251,431,437]. So, criterion (i) should be broadened to "it consumes calcium and/or phosphate ions *in vivo*". Similarly, condition (iv) should be slightly revised since ion transport is not only assured by the vascular system. Condition (iv) is equivalent to "*ion transport is insufficient to maintain calcium and/or phosphate homeostasis*". Considering the previous considerations on the alkalinity and absence of osteoinductive potential of bulk Mg and bioactive glasses, one may also want to add a fifth condition related to the influence of the material on the local pH. An increase in pH is certainly not good, but the question is whether a local acidification, as seen during acidosis and perhaps very locally around CDHA, may be beneficial. A conservative approach would be to state for condition (v) that the material should not increase the local pH. The modified list of criteria providing osteoinductive properties to a BGS would be: (i) it consumes calcium and/or phosphate ions *in vivo*; (ii) it is or becomes porous (porous scaffold, assembly of granules, narrow gap between two plates, holes/channels in a material or at the material surface); (iii) the pores are large enough to allow blood vessels ingrowth and cell transport into the core of the material; (iv) the calcium and/or phosphate uptake rate per volume of implanted material is so high that the physiological ion transport is insufficient to maintain calcium and/or phosphate homeostasis; and (v) the material does not increase the local pH. Importantly, the implantation of such a material would produce an inflammation reaction which is as essential to HO as the material properties. Also, there is a temporal component as discussed in the section herein devoted to SLIHI.

## 6. Osteoinductive biomaterials

The osteoinductive potential of BMPs is generally tested by implanting the BMPs with a biomaterial carrier in soft tissue and assessing the speed and amount of new bone formation. Since the implantation of some BGS in soft tissues can trigger bone formation, should implantation of these BGS be considered as "material-based osteoinduction"?

The definition of "osteoinduction" has evolved over the years and is understood differently by different scientific communities. The biomaterials community defined osteoinduction as "*the process by which osteogenesis is induced*" where "*osteogenesis*" is the "*whole process of development and formation of bone*" [96]. This would mean that a material triggering bone formation ectopically should be qualified as being "*osteoinductive*". Despite overwhelming evidence that materials can trigger bone formation ectopically, an osteoinductive claim for such materials is to the best of our knowledge currently not accepted by the U.S. Food and Drug Administration. This is generally reserved to the use of exogenous BMP and DB.

One comment that is raised in the scientific community is that materials do not release any substances, and as such, materials cannot be called "*osteoinductive*". This is, however, based on the use of a very restrictive definition of the osteoinduction (s. above), which is not accepted by all communities, and on the false assumption that materials are inert. Materials interact chemically and physically with surrounding tissues. It is also based on the idea that only the release of substances can trigger a reaction, which is again not true because in the concept of homeostasis, not only an increase but also a lowering of the concentration of a chemical can induce a biological response. In fact, there are proposals to treat hyperammonemia [438,439] and hyperkalemia [440] by catching ammonia and potassium ions, respectively. In material-induced HO, there is a local decrease of chemicals (ions, but most likely also organic molecules such as proteins) due to calcification creating a SLIHI favoring the M0/M1 to M2 macrophage differentiation, MNGC formation, endogenous BMP release, and eventually osteogenesis. Even though material-induced and exogenous-BMP-induced HO both involve the action of BMPs, it could make sense to differentiate between these two ways to induce ectopic bone formation (more details about analogies and differences between HO produced by exogenous BMP and osteoinductive BGS are provided in the supplementary files and in Table 3). Currently, a number of authors use the concept of "*intrinsic osteoinductive property*" for scaffolds [441,442], but this does not tell much about the mechanism. One may relate the definition more directly to the mechanism, for example by referring to "*calcification-triggered osteoinduction*", and "*exogenous-BMP-triggered osteoinduction*".

## 7. Conclusion

The evaluation of the literature on heterotopic ossification (HO) reveals that the combination of inflammation and calcification plays a central role in triggering HO. It is postulated that the reason why calcification does not always lead to HO is related to the absence or presence of a sustained local ionic homeostatic imbalance (SLIHI). Without SLIHI, minerals formed in necrotic soft tissues are simply eliminated over time and normal soft tissue healing occurs. With SLIHI, inflammation is modulated towards an osteogenic pathway involving monocyte/macrophage M1 to M2 transition, and later on cathepsin K, TRAP positive multi-nucleated giant cells (MNGCs). These MNGCs are involved in the differentiation of osteoprogenitor cells and eventually HO. The overall cascade until bone is formed takes roughly 20 days. This analysis means that potential ways of inducing/arresting ectopic bone formation would be to act (i) on the combination of inflammation and SLIHI, (ii) on M1 to M2 expressing monocyte/macrophage and MNGCs, and/or (iii) on BMPs/stem cell differentiation. It is therefore not surprising that current therapies against HO target some of these

steps, mostly inflammation/MNGCs with NSAID drugs, mineralization with bisphosphonates, and stem cells with radiation therapy. Reciprocally, a combined increase of inflammation and calcification should provide, at least in a certain range, more osteoinductive bone graft substitutes (BGS). Finally, the evaluation presented here argues in favor of qualifying some BGSs as osteoinductive following the calcification-triggered osteoinduction pathway/cascade as opposed to the exogenous BMP-triggered osteoinduction pathway/cascade. Two obvious challenges for the near future are the demonstration of the occurrence of SLIHI *in vivo* and the enhancement of the osteoinductive potential of biomaterials by the modulation of SLIHI and inflammation.

## 8. Supplementary file

Mode of action of DB: In 1965, Urist [84] demonstrated that whereas bone pieces hardly induced bone formation ectopically, demineralized bone pieces almost always induced bone formation. He assumed that a "*freely diffusible tissue-specific chemical inducing substance*" [396] was responsible for that and proposed in 1971 that these chemicals are "*bone morphogenetic proteins*" (BMPs;[39]). Urist rejected the hypothesis that calcification is important in this matter for two main reasons [84]. First, "*areas of recalcification of dead matrix were few, and they rarely coincided with areas of osteogenesis*". Second, "*toluidine blue, which blocks carboxylic acid groups and binding of calcium ions, prevented recalcification of matrix but did not inhibit osteogenic induction*". At that time, it was a change of paradigm since some authors considered that calcification was "*likely to be replaced by bone no matter what tissue is involved*" [91]. Funnily, these statements contrasted with Urist' efforts to induce bone formation by inducing calcification [149] and some of his results demonstrating the importance of *in vivo* recalcification for ectopic bone formation [443,444]. For example, these authors had observed that DB demineralized in nitric acid did not recalcify *in vivo* and did not induce ectopic bone formation [444]. Additionally, the *in vitro* recalcification of DB (prior to implantation) decreased ectopic bone occurrence from 90% in HCl demineralized DB to a mere 9%, a value barely higher than lyophilized whole bone (2-3%) [444]. There are also a number of other elements questioning the conclusion of Urist that DB action is due to its BMP content: (i) bone formation induced by DB takes place in the core of the implanted DB [84,164,386,445] and not on the outside as seen with BMPs, suggesting a different mechanism of action of DB compared to BMP-loaded scaffolds; (ii) DB contains tiny amounts of BMPs (between 0.6 and 93ng/g depending on the BMP) compared to therapeutic doses (typically in the mg range), and BMP release from DB is very fast [446]; (iii) the purer the BMP extract from DB is, the less potent it becomes. For human applications, milligrams of BMP are used which corresponds to the implantation of the equivalent of roughly 1 kg of bone [447–449]. Based on the above, it appears likely that DB osteoinductive action is not only driven by BMPs, but also by calcification.

Calcification- vs BMP-based bone regeneration: In the context of bone regeneration, it is interesting to compare two strategies to produce ectopic bone using BGS. One is based on material associated calcification, and the other is based on BMP/carrier interactions (Table 2). In both cases, the mechanism of action is dose-dependent, structure-dependent (protein conformation, scaffold architecture), and kinetically driven. In calcification-triggered HO, there is a chemical uptake, whereas in BMP-induced ectopic bone formation, there is a release. In calcification- triggered HO, bone typically forms within the pores of the implanted biomaterial and a single granule that would have migrated away from the implantation bed would not generate any bone due to its inability to generate a SLIHI, and thus to induce the cascade of biological reactions (e.g. MNGC generation, BMP release) leading to ectopic bone. In BMP-induced HO, bone is often formed outside BMP-loaded biomaterials due to the release of BMP from the carrier. The concentrations of the active substances are slightly sub-physiological in calcification- triggered HO, and largely supraphysiological in exogenous BMP-induced ectopic bone formation. The latter is due to the inability to deliver biologically active BMPs exogenously. In calcification-triggered HO, bone is initially detected after 3 weeks, roughly 10 days later than with BMP-induced ectopic bone formation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

The authors would like to thank Willy Hofstetter (University of Bern) for fruitful discussions.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2022.03.057.

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