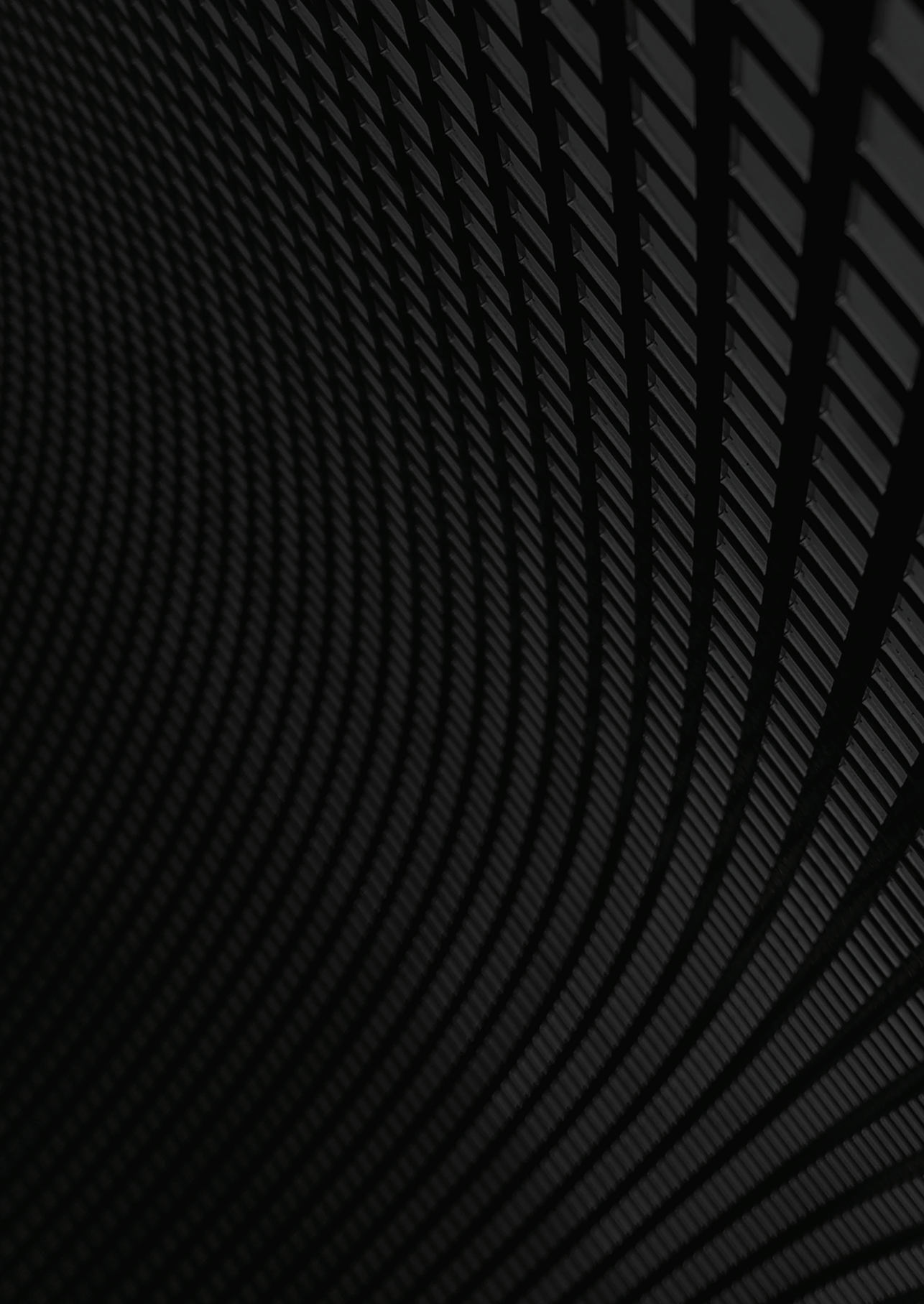


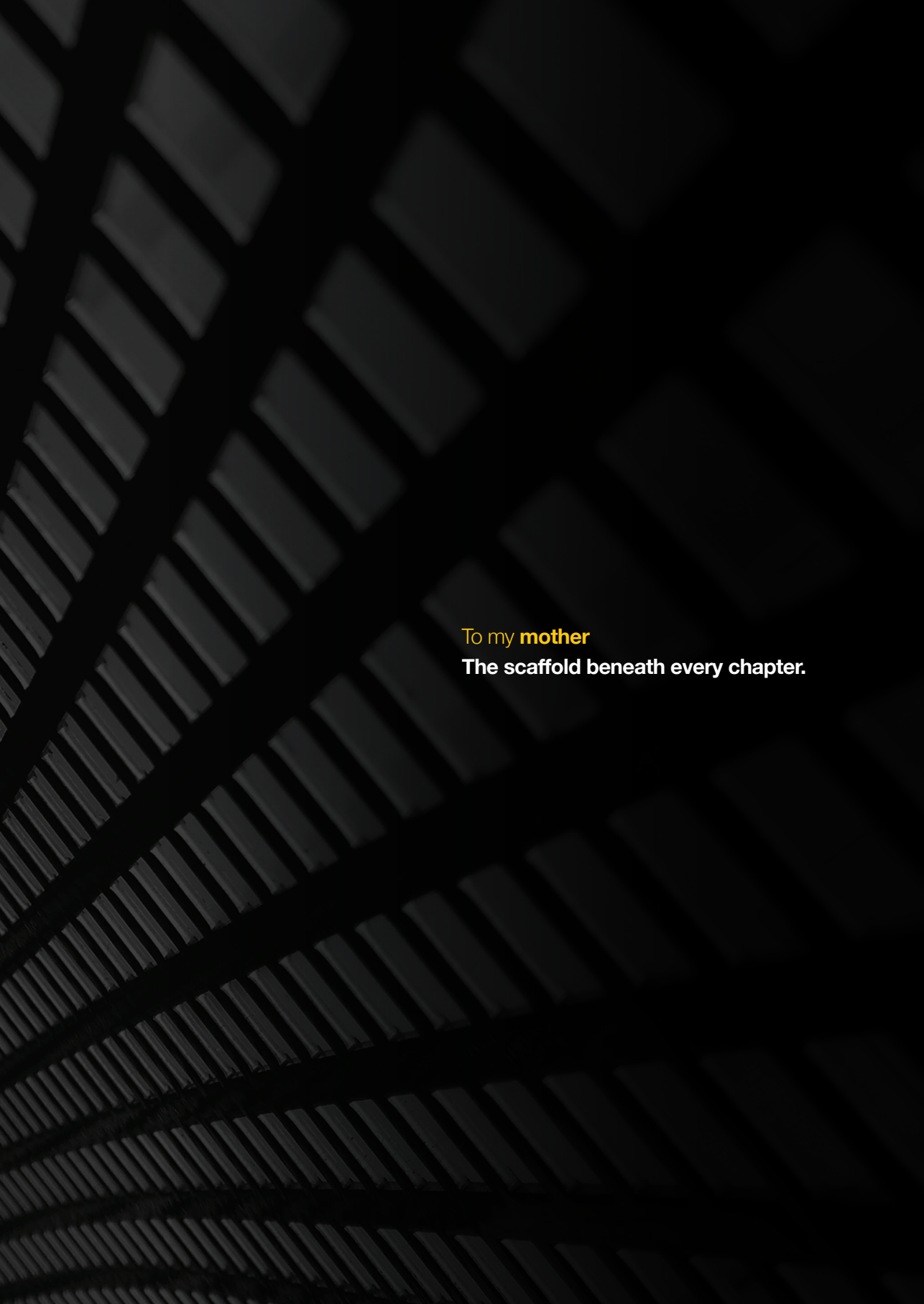
BONE-X PROJECT

INFLUENCE OF MULTILEVEL BIOMATERIAL
CHARACTERISTICS ON ORAL BONE REGENERATION

BRUNO DE CARVALHO

Thesis submitted to obtain the degree of Doctor in Dental Sciences of the University of Liège
Academic year 2026/2027





To my **mother**

The scaffold beneath every chapter.



PROMOTOR

Prof. Dr. France Lambert | University of Liège

CO-PROMOTOR

Prof. Dr. Eric Rompen | University of Liège

MEMBERS OF THE JURY

Prof. Dr. Amélie Mainjot, chair of the Jury | University of Liège

Prof. Dr. Chantal Humblet | University of Liège

Dr. Dorien Van Hede | University of Liège

Prof. Dr. Andy Temmerman | KU Leuven

Prof. Dr. Kasia Gurzawska-Comis | Aarhus University

© 2026 Bruno de Carvalho

Dental Biomaterials Research Unit (d-BRU) Institute of Dentistry, University of Liège (ULiège), Liège, Belgium.

Quai G. Kurth 45, 4020 Liège, Belgium

tel: +32 4 270 31 06

email: d-bru@uliege.be

All Rights Reserved. No part of this book may be reproduced or utilized in any form or by any means, electronic or mechanical, including photocopying, recording, or by any information storage and retrieval system, without permission in writing from the copyright owner.

Layout and graphics : www.perspectives.be

 BONE-X PROJECT

INFLUENCE OF MULTILEVEL BIOMATERIAL
CHARACTERISTICS ON ORAL BONE REGENERATION

BRUNO DE CARVALHO

Thesis submitted to obtain the degree of Doctor in Dental Sciences of the University of Liège
Academic year 2026/2027

ABSTRACT

Alveolar bone deficiency remains one of the main challenges in implant-driven oral rehabilitation. Calcium-phosphate biomaterials are widely used to restore bone volume prior to or alongside implant placement. Despite their extensive clinical use, biomaterials are still commonly evaluated according to their class, origin, or commercial brand rather than according to the physicochemical and structural characteristics that govern their biological behavior.

This thesis investigated how the physicochemical, structural, and manufacturing-related characteristics of calcium-phosphate biomaterials influence biological performance in alveolar and cranio-maxillofacial bone regeneration. Six complementary studies addressed this question through a combination of systematic evidence synthesis, controlled in vivo and in vitro investigations centered on bovine hydroxyapatite, methodological development, and analysis of emerging next-generation biomaterial concepts. The systematic synthesis demonstrated that distinct physicochemical parameters influence different regenerative outcomes, with no single parameter optimizing all outcomes simultaneously and no biomaterial category consistently outperforming all others. The experimental studies further demonstrated that biomaterials sharing the same chemistry, origin, and clinical indication may nevertheless exhibit distinct regenerative behaviors when produced through different manufacturing pathways. These differences were most pronounced during early healing and could be mechanistically linked to changes in physicochemical characteristics, protein interactions, and cellular response. In parallel, a digital-twin workflow based on intraoperative optical scanning was developed to prospectively define histomorphometric regions of interest, providing a methodology to improve spatial standardization and reduce cross-study heterogeneity in preclinical bone regeneration research. Finally, the thesis explored future directions in biomaterial development, including multiscale structural design, dynamic bioresponsive behavior, computational optimization, and patient-specific approaches.

Together, the findings of this thesis support a shift in how calcium-phosphate biomaterials are evaluated. Rather than considering biomaterials primarily according to their class or commercial designation, the results highlight the importance of the relationship between manufacturing, physicochemical characteristics and biological behavior. For clinicians, these findings suggest that differences between biomaterials within the same class may influence regenerative outcomes and should therefore be considered during material selection. For the scientific community, the work provides experimental, methodological, and conceptual foundations for a more biologically grounded approach to biomaterial evaluation and design.

RÉSUMÉ

Le déficit osseux alvéolaire demeure l'un des principaux défis de la réhabilitation orale implanto-portée. Les biomatériaux à base de phosphate de calcium sont largement utilisés pour restaurer le volume osseux avant ou lors de la pose d'implants. Malgré leur utilisation clinique étendue, les biomatériaux sont encore couramment évalués selon leur classe, leur origine ou leur marque commerciale, plutôt qu'en fonction des caractéristiques physicochimiques et structurelles qui régissent leur comportement biologique.

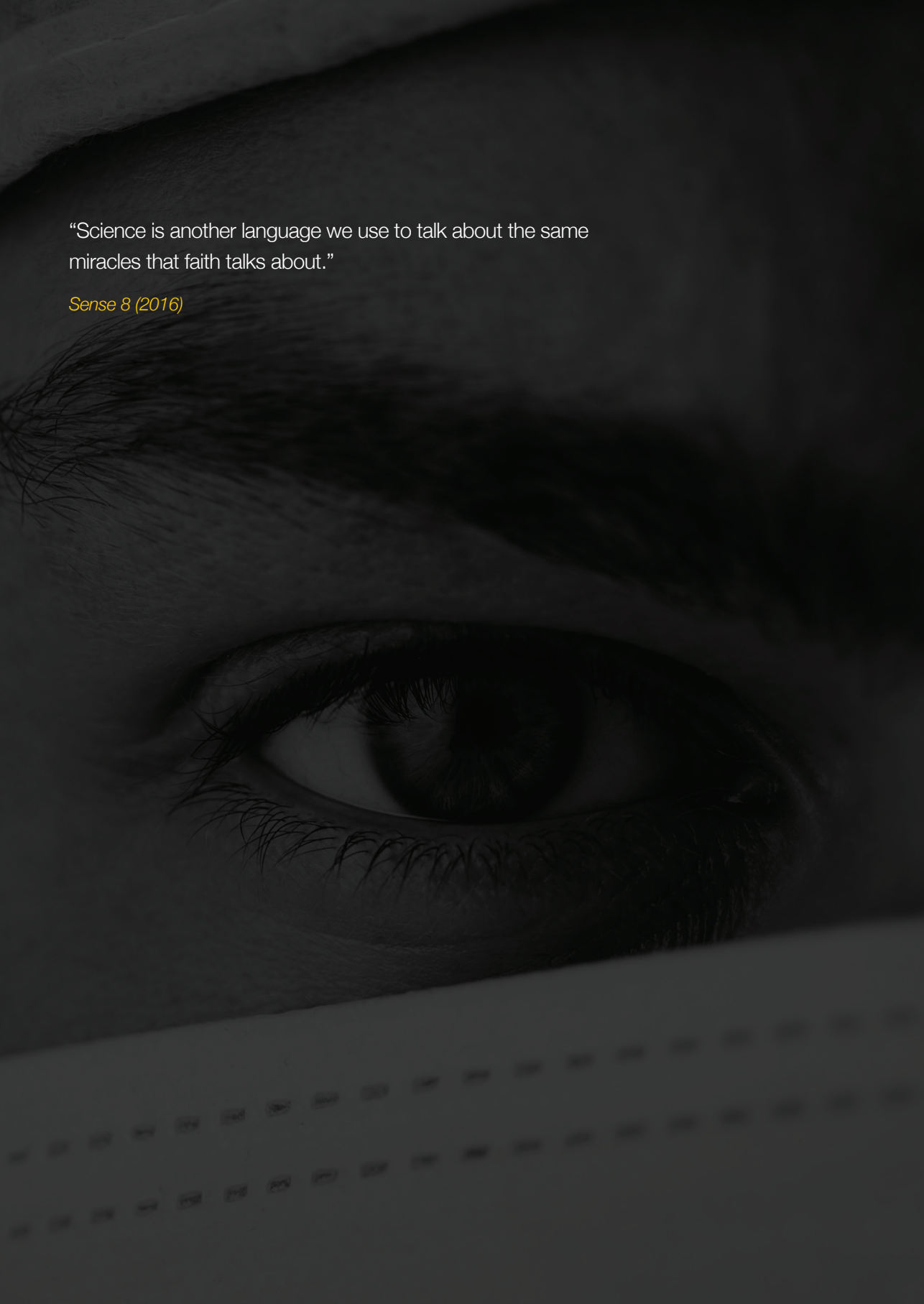
Cette thèse a étudié l'influence des caractéristiques physicochimiques, structurelles et liées aux procédés de fabrication des biomatériaux à base de phosphate de calcium sur leurs performances biologiques dans le cadre de la régénération osseuse alvéolaire. Six études complémentaires ont abordé cette question en combinant une synthèse systématique des données disponibles, des investigations contrôlées in vivo et in vitro centrées sur l'hydroxyapatite bovine, le développement d'une nouvelle approche méthodologique ainsi que l'analyse de concepts émergents relatifs aux biomatériaux de nouvelle génération.

La synthèse systématique a montré que différents paramètres physicochimiques influencent des résultats régénératifs distincts, sans qu'un seul paramètre permette d'optimiser simultanément l'ensemble de ces résultats ni qu'une catégorie de biomatériaux surpasse systématiquement toutes les autres. Les études expérimentales ont également démontré que des biomatériaux présentant une composition chimique, une origine et une indication clinique identiques peuvent néanmoins adopter des comportements régénératifs différents lorsqu'ils sont obtenus par des procédés de fabrication distincts. Ces différences étaient particulièrement marquées durant les premières phases de cicatrisation et ont pu être reliées, sur le plan mécanistique, à des variations des caractéristiques physicochimiques, des interactions protéiques et de la réponse cellulaire.

Parallèlement, un protocole fondé sur un jumeau numérique et sur la numérisation optique peropératoire a été développé afin de définir prospectivement les régions d'intérêt histomorphométriques. Cette approche méthodologique vise à améliorer la standardisation spatiale et à réduire l'hétérogénéité entre les études précliniques consacrées à la régénération osseuse. Enfin, cette thèse a exploré de futures orientations pour le développement des biomatériaux, notamment la conception structurelle multi-échelle, les comportements bio-réactifs dynamiques, l'optimisation computationnelle et les approches spécifiques au patient.

Pris dans leur ensemble, les résultats de cette thèse plaident en faveur d'une évolution de la manière dont les biomatériaux à base de phosphate de calcium sont évalués. Plutôt que de les considérer principalement selon leur classe, leur origine ou leur désignation commerciale, ces résultats soulignent l'importance des relations entre les procédés de fabrication, les

caractéristiques physicochimiques et le comportement biologique. Sur le plan clinique, ces résultats montrent que des biomatériaux appartenant à une même classe peuvent présenter des performances régénératives différentes, un facteur qui mérite d'être considéré lors de la sélection du matériau. Sur le plan scientifique, ces travaux établissent des bases expérimentales, méthodologiques et conceptuelles pour une évaluation et une conception des biomatériaux davantage guidées par leur comportement biologique.



“Science is another language we use to talk about the same miracles that faith talks about.”

Sense 8 (2016)

TABLE OF CONTENTS

TABLE OF CONTENTS

GENERAL INTRODUCTION	19
METHODOLOGICAL OVERVIEW ACROSS STUDIES	33
CHAPTER 1: FOUNDATIONS AND STATE OF THE ART	37
Influence of physicochemical characteristics of calcium-phosphate-based biomaterials in cranio-maxillofacial bone regeneration. A systematic literature review and meta-analysis of preclinical models.	
CHAPTER 2: MANUFACTURING AND PROOF OF CONCEPT	97
Effect of Sintering on In Vivo Biological Performance of Chemically Deproteinized Bovine Hydroxyapatite.	
CHAPTER 3: PRECLINICAL BIOLOGICAL VALIDATION	123
Biological performance of a novel bovine hydroxyapatite in a guided bone regeneration model: A preclinical study in a mandibular defect in dogs.	
CHAPTER 4: FURTHER UNDERSTANDING THE BIOLOGICAL OUTCOMES	153
Influence of in vitro physicochemical and biological characteristics on hydroxyapatite xenografts performance.	
CHAPTER 5: METHODOLOGICAL INNOVATION	185
Digital Twin Guided Optical Impression Analysis for Anatomically Accurate ROI Determination and Histologic Alignment in Bone Regeneration Models.	
CHAPTER 6: NEXT-GENERATION BIOMATERIAL CONCEPTS	211
Next-generation biomaterials for alveolar bone regeneration: a structurally instructive, multiscale design paradigm.	
GENERAL DISCUSSION	251
CONCLUSION	263

DECLARATION ON THE USE OF GENERATIVE ARTIFICIAL INTELLIGENCE

During the preparation of this thesis, the author used generative artificial intelligence tools to assist with language editing and structuring the connecting sections of the manuscript (general introduction, general discussion, and general conclusions). All AI-assisted content was critically reviewed, edited and verified by the author, who takes full responsibility for the content of the thesis. The experimental data and results presented in the constituent studies were not generated by artificial intelligence.

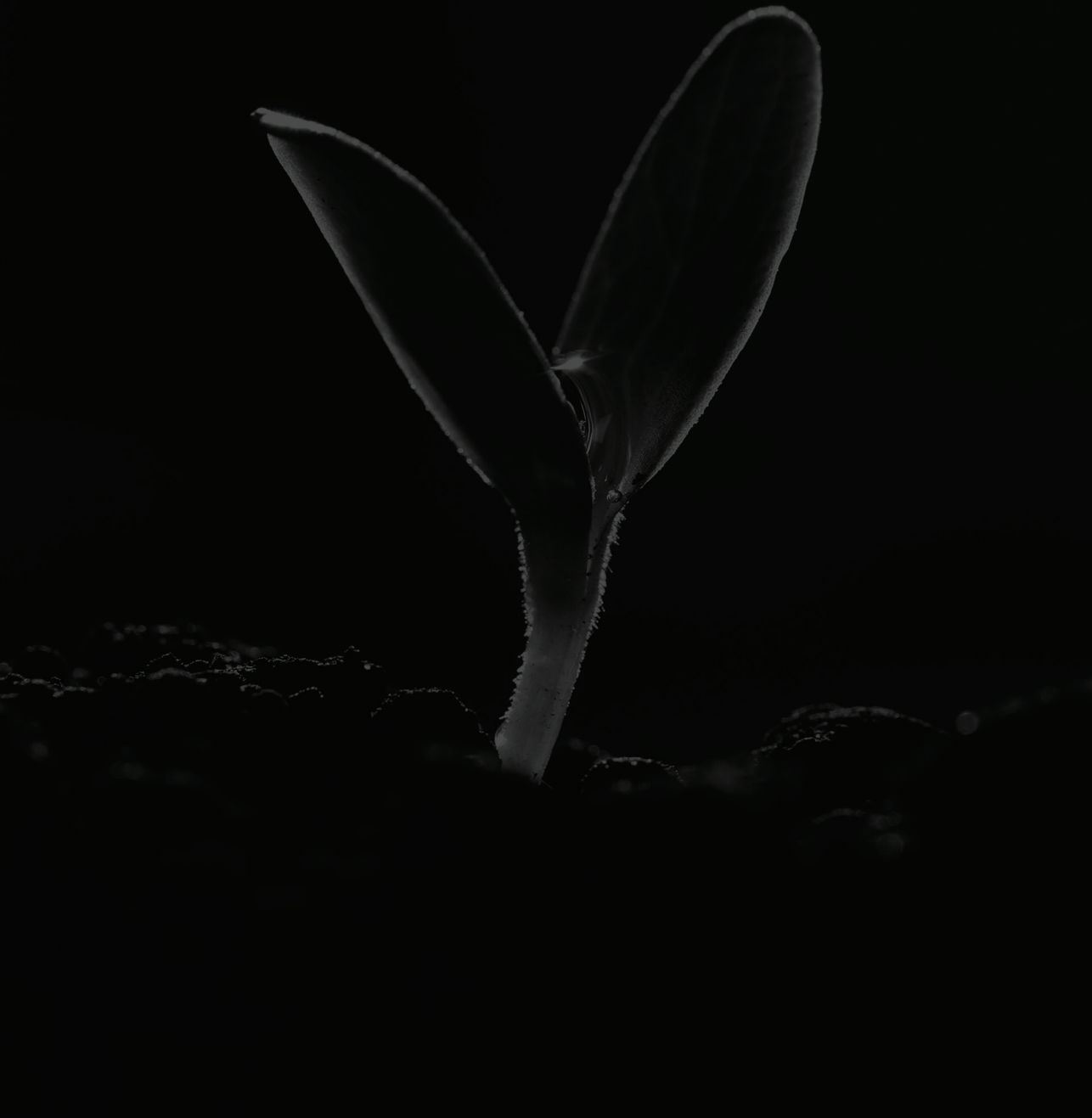
LIST OF ABBREVIATIONS

ALP :	Alkaline phosphatase
ARRIVE :	Animal Research: Reporting of In Vivo Experiments (reporting guidelines)
BCA :	Bicinchoninic acid
BCP :	Biphasic calcium-phosphate
BET :	Brunauer–Emmett–Teller
BHA :	Bovine hydroxyapatite
BJH :	Barrett–Joyner–Halenda
BMC :	Bone-to-material contact
BMP :	Bone morphogenetic protein
BO :	Bio-Oss
Ca/P :	Calcium-to-phosphorus molar ratio
CaP :	Calcium-phosphate
CaSR :	Calcium-sensing receptor
CMF :	Cranio-maxillofacial
CS :	Crystallite size
DBBM :	Deproteinized bovine bone mineral
DCP / DCPA :	Dicalcium-phosphate / dicalcium-phosphate anhydrous
DMEM :	Dulbecco's Modified Eagle's Medium (cell culture medium)
ECM :	Extracellular matrix
ESEM :	Environmental scanning electron microscopy
FBS :	Fetal bovine serum
FDA :	(United States) Food and Drug Administration
FTIR :	Fourier-transform infrared spectroscopy
GBR :	Guided bone regeneration
GLMM :	Generalized linear mixed-effects model
H&E :	Haematoxylin and eosin (histological stain)
HA / HAp :	Hydroxyapatite
HAN :	Bovine hydroxyapatite non-sintered
HA820 :	Bovine hydroxyapatite sintered at 820 °C
HA1200 :	Bovine hydroxyapatite sintered at 1200 °C
hBMSC :	Human bone marrow stromal (mesenchymal stem) cells
HMDS :	Hexamethyldisilazane (drying agent for electron-microscopy sample preparation)
ICP-MS :	Inductively coupled plasma mass spectrometry
IOS :	Intraoral optical scanning
M1 / M2 :	Pro-inflammatory (M1) anti-inflammatory (M2) macrophage phenotypes

MC3T3 :	Murine pre-osteoblast cell line
micro-CT :	Micro-computed tomography
MMA :	Methyl methacrylate (embedding resin)
MSC :	Mesenchymal stem cells
NB :	Newly formed bone
OCP :	Octacalcium-phosphate
PBS :	Phosphate-buffered saline
PCL :	Polycaprolactone
PGA :	Polyglycolic acid
PICOS :	Population, Intervention, Comparator, Outcome, Study design
PLA :	Polylactic acid
PMMA :	Polymethylmethacrylate
PROSPERO :	International Prospective Register of Systematic Reviews
RAW 264.7 :	A murine macrophage cell line
RG :	Residual graft
rhBMP-2 :	Recombinant human bone morphogenetic protein-2
RoB :	Risk of bias
ROI :	Region of interest
ROI1 :	Original defect envelope reconstructed from intraoperative scanning, prior to healing
ROI2 :	Regenerated tissue within the original defect boundaries
ROI3 :	Post-healing compartment identified histologically at the study endpoint (digital twin study)
Runx2 :	Runt-related transcription factor 2
SD :	Standard deviation
SEM :	Scanning electron microscopy
SPC :	Skeletal progenitor cells
SSA :	Specific surface area
STL :	Standard Tessellation Language; a triangulated three-dimensional surface-mesh file format
SYRCLE :	Systematic Review Centre for Laboratory Animal Experimentation (risk-of-bias tool for animal studies)
TCP :	Tricalcium-phosphate
β-TCP :	Beta-tricalcium-phosphate
TCPS :	Tissue culture polystyrene (cell-culture positive control surface)
TE :	Tissue engineering
WHA :	Wishbone Hydroxyapatite
W-GLMM :	Weighted generalized linear mixed-effects model
XRD :	X-ray diffraction

“Big things have small beginnings”

Prometheus (2012)



GENERAL INTRODUCTION

GENERAL INTRODUCTION & AIMS

The replacement of missing teeth by osteointegrated dental implants has become a foundation stone of contemporary oral rehabilitation. The demand for implant rehabilitation has continued to grow over the past decades, driven by population aging, expanded access to advanced dental care, and the well-documented functional improvements and quality of life benefits with implant-supported prostheses [1, 2]. The recurrent clinical challenge associated with this trend is the insufficient alveolar bone volume at the prospective implant site, which limits the correct three-dimensional, prosthetically driven implant placement [3, 4]. Several augmentation strategies have become routine in periodontology, oral surgery, and implantology, including socket preservation, horizontal and vertical guided bone regeneration, sinus floor elevation, and block grafting. Across these indications, the clinical purpose is to restore a sufficient volume of vital, well-vascularized and mechanically resistant bone, while maintaining its volumetry over the lifetime of the implant [5, 6]. Evidence from systematic reviews and consensus reports indicates that these procedures are predictable when appropriate techniques and materials are selected, but that the choice of grafting material remains a determinant factor in the regenerative outcome [7, 8].

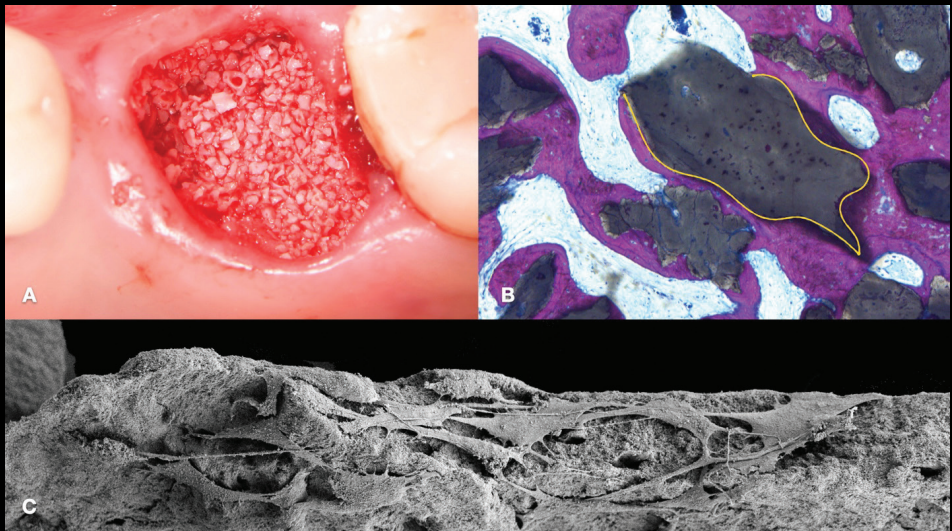
Bone is a dynamic and highly vascularized tissue that is continuously maintained through the coordinated activity of osteoblasts, osteoclasts, and osteocytes within a mineralized collagen matrix. Unlike most tissues of the human body, bone retains an important capacity for true regeneration rather than simple fibrous repair. This regenerative potential is, however, limited, and large defects or defects healing under compromised conditions often fail to regenerate spontaneously [9]. Bone healing occurs through three overlapping phases, an inflammatory, a reparative (proliferative), and a remodeling phase. Following injury, the initial inflammatory phase leads to hematoma formation and recruitment of immune cells to the defect site. It overlaps with the ensuing reparative (proliferative) phase, during which mesenchymal progenitor cells migrate, proliferate, and differentiate into osteoblasts capable of producing new bone matrix. Finally, a prolonged remodeling phase gradually replaces the initially formed woven bone with mature lamellar bone [10, 11]. The early, inflammatory phase of this cascade exerts a particular influence on the final outcome. Macrophages recruited at this stage undergo a functional polarization between pro-inflammatory and reparative phenotypes, and a balanced, well-resolved inflammatory response favors the subsequent transition to osteogenesis [12, 13]. Adequate vascularisation is a further prerequisite, supplying oxygen, nutrients, and progenitor cells to the healing defect [14].

When a bone substitute biomaterial is implanted into a defect, it interacts with this regenerative cascade. Its contribution is traditionally described through three principal mechanisms:

osteoconduction, defined as the ability to support bone growth along the material surface; osteoinduction, referring to the stimulation of progenitor cells toward osteogenic differentiation; and osteogenesis, which corresponds to direct bone formation by viable cells contained within the graft itself [15]. This interaction can be appreciated at successive levels of observation: from the clinical placement of the biomaterial within the bony defect, to the histological apposition of newly formed bone along the particle surface, down to the cellular adhesion occurring at the material interface (Figure 1).

Figure 1

The biomaterial–tissue interaction observed across length scales. (A) Clinical view of a particulate calcium-phosphate biomaterial packed within an alveolar bone defect. (B) Non-decalcified histological section illustrating osteoconduction: newly formed bone (magenta) deposited directly along the surface of a biomaterial particle (outlined in yellow), the interface at which bone-to-material contact is quantified. (C) Scanning electron micrograph showing osteogenic cells spread and adhering across the biomaterial surface. Together, the panels show that the regenerative performance of a bone substitute is governed by characteristics acting simultaneously at the clinical, tissue, and cellular levels



Calcium-phosphates are a family of bioceramics widely used as bone substitutes because of their chemical similarity to the mineral phase of bone. They differ in their calcium-to-phosphorus (Ca/P) ratio and crystallinity, properties that determine their solubility and biological behaviour. Hydroxyapatite (HA; Ca/P = 1.67) is the most stable crystalline phase and most closely

resembles biological apatite, whereas tricalcium-phosphates (TCP) are more soluble. The calcium-phosphate biomaterials investigated in this thesis, centred on bovine hydroxyapatite, primarily act as osteoconductive scaffolds. Their interaction with the regenerative environment begins immediately after implantation, when proteins from blood and interstitial fluids rapidly adsorb onto the biomaterial surface. Cells therefore interact first with this adsorbed protein layer rather than with the bare material itself, and this early interface governs subsequent processes such as cell attachment, proliferation, differentiation, and matrix deposition [16]. Consequently, the physicochemical and structural characteristics of the biomaterial surface are positioned at the very beginning of the regenerative cascade, within the early inflammatory and reparative phases and may substantially influence the overall biological response.

Over the past two decades, the development and clinical adoption of bone substitute biomaterials have progressed faster than the systematic understanding of why one material outperforms another in each clinical setting [17]. The same calcium-phosphate chemistry, when subjected to different manufacturing routes, can create products with substantially different physicochemical characteristics and biological behaviors [18]. It progressively became clear that production-related variations in surface morphology, porosity, ion exchange behavior, hydrophilicity, and crystallinity could substantially influence cell response, osteoconductivity, volumetric stability, and integration with host tissues, thereby reshaping the design principles of bone substitute biomaterials [17, 19]. The consequence of this conceptual shift is that biomaterials of the same nominal class can no longer be assumed to behave equally. Two bovine hydroxyapatites, or two β -tricalcium-phosphate scaffolds, may share chemistry, origin, and intended indication and yet differ substantially in their osteoconductivity, dissolution behavior, and integration with host tissues [20-23]. This variability has been documented across xenografts, alloplasts, and biphasic materials, and traces back to differences in the manufacturing pathway. Compounding this complexity, regenerative performance is itself multidimensional. Parameters such as newly formed bone, bone-to-material contact, residual graft content, volumetric stability, and integration with host tissues do not necessarily respond uniformly to a given biomaterial. A material may therefore perform favorably according to one regenerative endpoint while showing less favorable behavior in another [6, 24]. As a result, classifying bone substitutes solely according to their origin or commercial category obscures both the heterogeneity within each biomaterial class and the diversity of biological outcomes against which they should be evaluated. This perspective progressively shifted the focus of the field away from the biomaterial category itself and toward the structure–function relationships governing biological performance. Attention is now increasingly centered on how manufacturing-related variations in physicochemical and structural properties influence osteoconductivity, tissue integration, volumetric stability, and remodeling dynamics.

However, this evidence remained fragmented across heterogeneous experimental conditions, including different animal models, defect morphologies, biomaterial configurations, and outcome measures [25, 26]. In addition, preclinical studies are typically conducted under controlled conditions characterized by standardized defects, preserved vascularity, and young healthy animals, which may overestimate the regenerative performance achievable in clinical practice. A systematic and quantitative synthesis of this fragmented body of evidence was therefore needed.

The biological performance of a bone substitute biomaterial is strongly determined by its manufacturing pathway [27]. Sintering, in particular, is a near-universal step in the processing of calcium-phosphate biomaterials, applied to synthetic hydroxyapatite, β -tricalcium-phosphate, biphasic calcium-phosphates, and natural xenografts, with documented effects on crystallinity, surface morphology, mechanical properties, and dissolution kinetics across all these classes [28-30]. Among the calcium-phosphate biomaterials clinically used in cranio-maxillofacial regeneration, bovine hydroxyapatite occupies a particular position. It is the most widely used xenograft for alveolar bone augmentation [31], it retains, when produced under appropriate conditions, the hierarchical architecture of native bone and its production requires a deproteinization step that constitutes a major manufacturing decision point. This step is typically achieved either through high-temperature sintering above 1000 °C, which simultaneously removes organic compounds and improves mechanical properties at the cost of surface, crystalline, and morphological changes, or through chemical extraction which avoids high-temperature exposure of the apatite phase. However, the extent to which such manufacturing decisions translate into differences in newly formed bone, bone-to-material contact, and regenerative performance *in vivo* remained insufficiently characterized [32, 33]. Likewise, the mechanistic links connecting physicochemical properties, *in vitro* biological response, and *in vivo* regeneration had rarely been investigated within an integrated experimental framework.

Comparative studies evaluating manufacturing variants of the same xenogenic biomaterial are particularly limited, in clinically relevant guided bone regeneration models that approximate human GBR conditions. Moreover, these studies are rarely performed under sufficiently standardized conditions to confidently attribute the observed differences to manufacturing-related variables rather than experimental variability. Demonstrating that manufacturing variants can produce divergent regenerative outcomes *in vivo* raises a second and equally important question: through which mechanisms do these differences emerge? Early biological processes such as ion exchange, hydrophilicity, protein adsorption, cell attachment and proliferation, and immunomodulatory behavior had previously been investigated in isolated *in vitro* assays but were rarely studied in conjunction with clinically relevant *in vivo* observations. As a result, the

mechanistic pathways linking manufacturing-dependent physicochemical variations to differential regenerative performance remained incompletely integrated. The demonstration that biomaterials sharing the same nominal chemistry could nevertheless produce distinct regenerative outcomes *in vivo* therefore created the need for complementary physicochemical and cellular investigations.

Such inferences, however, are only as solid as the methods that can produce them. Across the preclinical literature, the spatial unit against which bone regeneration is measured is rarely the defect that was created, but instead, what remains of that defect after weeks or months of the healing have reshaped its geometry, narrowed its boundaries, or folded them into the regenerated tissue itself. The measurement reference and the object being measured therefore co-evolve, and outcomes depend not only on what regenerated but also on how the boundary of the analysis was retrospectively reconstructed by the observer. The implications for cross-study comparability are well recognized. Identical regenerative responses can yield different reported outcomes depending on operator, sectioning orientation, and the rules used to redraw the original defect contour [34-36]. The alternative was to create a methodology able to capture the original defect geometry before healing begins and carry it forward as a stable spatial reference for quantitative analysis. Such methodology has so far remained outside the workflow of preclinical studies.

Despite advances in structure and function understanding and biomaterial characterization, the bone substitutes currently used in alveolar bone regeneration remain largely passive materials. Most are designed to provide structural support and osteoconductive surfaces, but only partially reproduce the hierarchical organization of native bone across the pico-, nano-, micro-, and macroscales to which osteogenic cells respond. In addition, bone regeneration is a dynamic biological process evolving through inflammatory, reparative, and remodeling phases, whereas the properties of the scaffold generally remain static over time. These limitations have stimulated growing interest in a new generation of biomaterials integrating multiscale architecture, bioresponsive behavior, computationally optimized geometries, and patient-specific design strategies [37, 38]. Despite these advances, the field still lacks a unified design framework capable of integrating multiscale architecture, dynamic responsiveness, and computational personalization within the alveolar and cranio-maxillofacial context.

Together, these unresolved questions outline the scope of the present thesis, whose general aim and specific research questions are stated in the following section.

AIMS

The general aim of this thesis is to investigate how physicochemical, structural, and manufacturing-related properties of calcium-phosphate biomaterials influence their biological performance in alveolar and cranio-maxillofacial bone regeneration. To address this objective, commercially available and experimental bovine hydroxyapatites were used as models to explore the relationships between manufacturing processes, material properties, in vitro biological behavior, and in vivo regenerative outcomes. The thesis also aimed to refine preclinical methods for outcome assessment and to explore current concepts guiding the design of next-generation biomaterials. On this basis, the following research questions were formulated:

1. How do the physicochemical and structural properties of calcium-phosphate biomaterials influence bone regeneration outcomes in preclinical cranio-maxillofacial models?
2. How does the sintering temperature of chemically deproteinized bovine hydroxyapatite affect its biological performance in vivo?
3. Can bovine hydroxyapatites, produced through different manufacturing processes, achieve equivalent regenerative outcomes in guided bone regeneration?
4. How do the physicochemical and biological characteristics of differently manufactured bovine hydroxyapatites influence their regenerative performance?
5. Can intraoral optical scanning accurately reconstruct the original geometry of bone defects for histomorphometric analysis?
6. Which multi-scale structural and dynamic principles may guide the design of next-generation biomaterials for alveolar ridge bone regeneration?

By addressing these questions, the present thesis aims to provide four complementary contributions to alveolar bone regeneration: (i) a structure–function framework for calcium-phosphate biomaterials grounded in preclinical evidence; (ii) experimental and mechanistic evidence that manufacturing processes influence biomaterial behavior and regenerative performance; (iii) a methodological framework for prospective region-of-interest definition in preclinical histomorphometry; and (iv) a discussion of emerging multiscale concepts that may inform the development of next-generation biomaterials.

REFERENCES

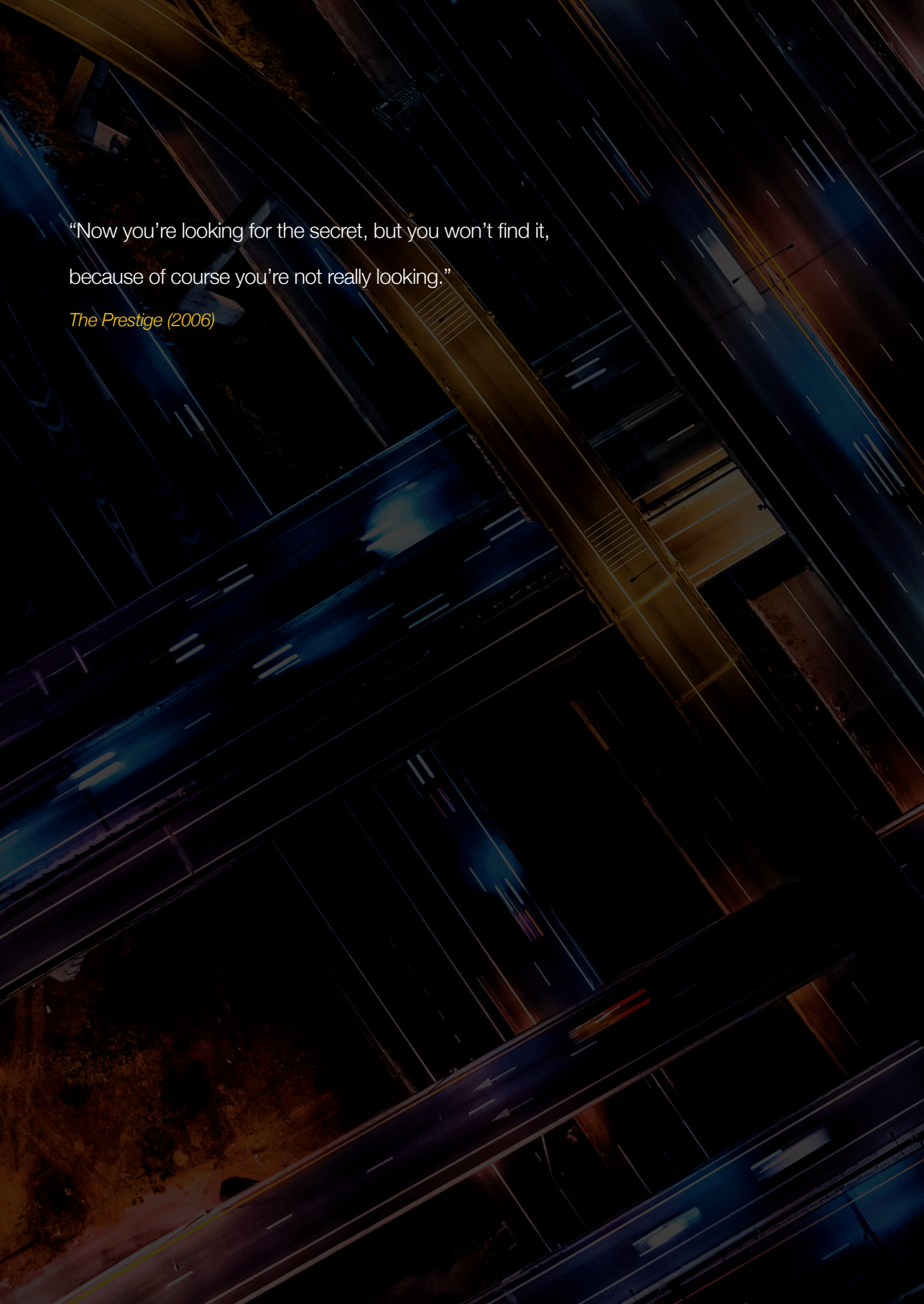
- 1 ABDELMONEIM, D., PORTER, G. C., COATES, D. E., DUNCAN, W. J., WADDELL, J. N., HAMMER, N. & LI, K. C. 2022. **The Effect of Low-Processing Temperature on the Physicochemical and Mechanical Properties of Bovine Hydroxyapatite Bone Substitutes.** *Materials* (Basel), 15.
- 2 ADHIKARA, A. G., MAHARANI, A. P., PUSPITASARI, A., NUSWANTORO, N. F., JULIADMI, D., MARAS, M. A. J., NUGROHO, D. B., SAKSONO, B. & GUNAWARMAN 2024. **Bovine hydroxyapatite for bone tissue engineering: Preparation, characterization, challenges, and future perspectives.** *European Polymer Journal*, 214, 113171.
- 3 AGHALOO, T. L., MISCH, C., LIN, G. H., IACONO, V. J. & WANG, H. L. 2016. **Bone Augmentation of the Edentulous Maxilla for Implant Placement: A Systematic Review.** *Int J Oral Maxillofac Implants*, 31 Suppl, s19–30.
- 4 ALBREKTSSON, T. & JOHANSSON, C. 2001. **Osteoinduction, osteoconduction and osseointegration.** *Eur Spine J*, 10 Suppl 2, S96–101.
- 5 BENIC, G. I. & HÄMMERLE, C. H. 2014. **Horizontal bone augmentation by means of guided bone regeneration.** *Periodontol* 2000, 66, 13–40.
- 6 BONUCCI, E., BALLANTI, P., DELLA ROCCA, C., MILANI, S., LO CASCIO, V. & IMBIMBO, B. 1990. **Technical variability of bone histomorphometric measurements.** *Bone Miner*, 11, 177–86.
- 7 BORNSTEIN, M. M., HALBRITTER, S., HARNISCH, H., WEBER, H. P. & BUSER, D. 2008. **A retrospective analysis of patients referred for implant placement to a specialty clinic: indications, surgical procedures, and early failures.** *Int J Oral Maxillofac Implants*, 23, 1109–16.
- 8 CALCIOLARI, E., CORBELLA, S., GKRANIAS, N., VIGANÓ, M., SCULEAN, A. & DONOS, N. 2023. **Efficacy of biomaterials for lateral bone augmentation performed with guided bone regeneration. A network meta-analysis.** *Periodontol* 2000, 93, 77–106.

- 9** DO DESTERRO FDE, P., SADER, M. S., SOARES, G. D. & VIDIGAL, G. M., JR. 2014. **Can inorganic bovine bone grafts present distinct properties?** *Braz Dent J*, 25, 282–8.
- 10** DONNELLY, E. 2011. **Methods for assessing bone quality: a review.** *Clin Orthop Relat Res*, 469, 2128–38.
- 11** DUONG, H. Y., ROCCUZZO, A., STÄHLI, A., SALVI, G. E., LANG, N. P. & SCULEAN, A. 2022. **Oral health-related quality of life of patients rehabilitated with fixed and removable implant-supported dental prostheses.** *Periodontol 2000*, 88, 201–237.
- 12** FERNANDEZ DE GRADO, G., KELLER, L., IDOUX-GILLET, Y., WAGNER, Q., MUSSET, A. M., BENKIRANE-JESSEL, N., BORNERT, F. & OFFNER, D. 2018. **Bone substitutes: a review of their characteristics, clinical use, and perspectives for large bone defects management.** *J Tissue Eng*, 9, 2041731418776819.
- 13** GHAYOR, C., BHATTACHARYA, I., GUERRERO, J., ÖZCAN, M. & WEBER, F. E. 2022. **3D-Printed HA-Based Scaffolds for Bone Regeneration: Microporosity, Osteoconduction and Osteoclastic Resorption.** *Materials (Basel)*, 15.
- 14** GIBON, E., LU, L. & GOODMAN, S. B. 2016. **Aging, inflammation, stem cells, and bone healing.** *Stem Cell Res Ther*, 7, 44.
- 15** HANNINK, G. & ARTS, J. J. 2011. **Bioresorbability, porosity and mechanical strength of bone substitutes: what is optimal for bone regeneration?** *Injury*, 42 Suppl 2, S22–5.
- 16** HAUGEN, H. J., LYGSTADAAS, S. P., ROSSI, F. & PERALE, G. 2019. **Bone grafts: which is the ideal biomaterial?** *J Clin Periodontol*, 46 Suppl 21, 92–102.
- 17** HE, L., YIN, J. & GAO, X. 2023. **Additive Manufacturing of Bioactive Glass and Its Polymer Composites as Bone Tissue Engineering Scaffolds: A Review.** *Bioengineering (Basel)*, 10.
- 18** IONOV, L. 2018. **4D Biofabrication: Materials, Methods, and Applications.** *Advanced Healthcare Materials*, 7, 1800412.

- 19 JEPSEN, S., SCHWARZ, F., CORDARO, L., DERKS, J., HÄMMERLE, C. H. F., HEITZ-MAYFIELD, L. J., HERNÁNDEZ-ALFARO, F., MEIJER, H. J. A., NAENNI, N., ORTIZ-VIGÓN, A., PJETURSSON, B., RAGHOEBAR, G. M., RENVERT, S., ROCCHIETTA, I., ROCCUZZO, M., SANZ-SÁNCHEZ, I., SIMION, M., TOMASI, C., TROMBELLI, L. & URBAN, I. 2019. **Regeneration of alveolar ridge defects. Consensus report of group 4 of the 15th European Workshop on Periodontology on Bone Regeneration.** J Clin Periodontol, 46 Suppl 21, 277–286.
- 20 KRISHNAN, L., WILLETT, N. J. & GULDBERG, R. E. 2014. **Vascularization strategies for bone regeneration.** Ann Biomed Eng, 42, 432–44.
- 21 LAMBERT, F., BACEVIC, M., LAYROLLE, P., SCHÜPBACH, P., DRION, P., ROMPEN, E., SCHUPBACH, P., DRION, P. & ROMPEN, E. 2017. **Impact of biomaterial micro-topography on bone regeneration: comparison of three hydroxyapatites.** Clinical Oral Implants Research, 28, e201–e207.
- 22 MARUYAMA, M., RHEE, C., UTSUNOMIYA, T., ZHANG, N., UENO, M., YAO, Z. & GOODMAN, S. B. 2020. **Modulation of the Inflammatory Response and Bone Healing.** Front Endocrinol (Lausanne), 11, 386.
- 23 MONTOYA, C., DU, Y., GIANFORCARO, A. L., ORREGO, S., YANG, M. & LELKES, P. I. 2021. **On the road to smart biomaterials for bone research: definitions, concepts, advances, and outlook.** Bone Research, 9.
- 24 NAENNI, N., LIM, H. C., PAPAGEORGIOU, S. N. & HÄMMERLE, C. H. F. 2019. **Efficacy of lateral bone augmentation prior to implant placement: A systematic review and meta-analysis.** J Clin Periodontol, 46 Suppl 21, 287–306.
- 25 NIAKAN, A., RAMESH, S., GANESAN, P., TAN, C. Y., PURBOLAKSONO, J., CHANDRAN, H., RAMESH, S. & TENG, W. D. 2015. **Sintering behaviour of natural porous hydroxyapatite derived from bovine bone.** Ceramics International, 41, 3024–3029.
- 26 PERCIVAL, K. M., PAUL, V. & HUSSEINI, G. A. 2024. **Recent Advancements in Bone Tissue Engineering: Integrating Smart Scaffold Technologies and Bio-Responsive Systems for Enhanced Regeneration.** Int J Mol Sci, 25.

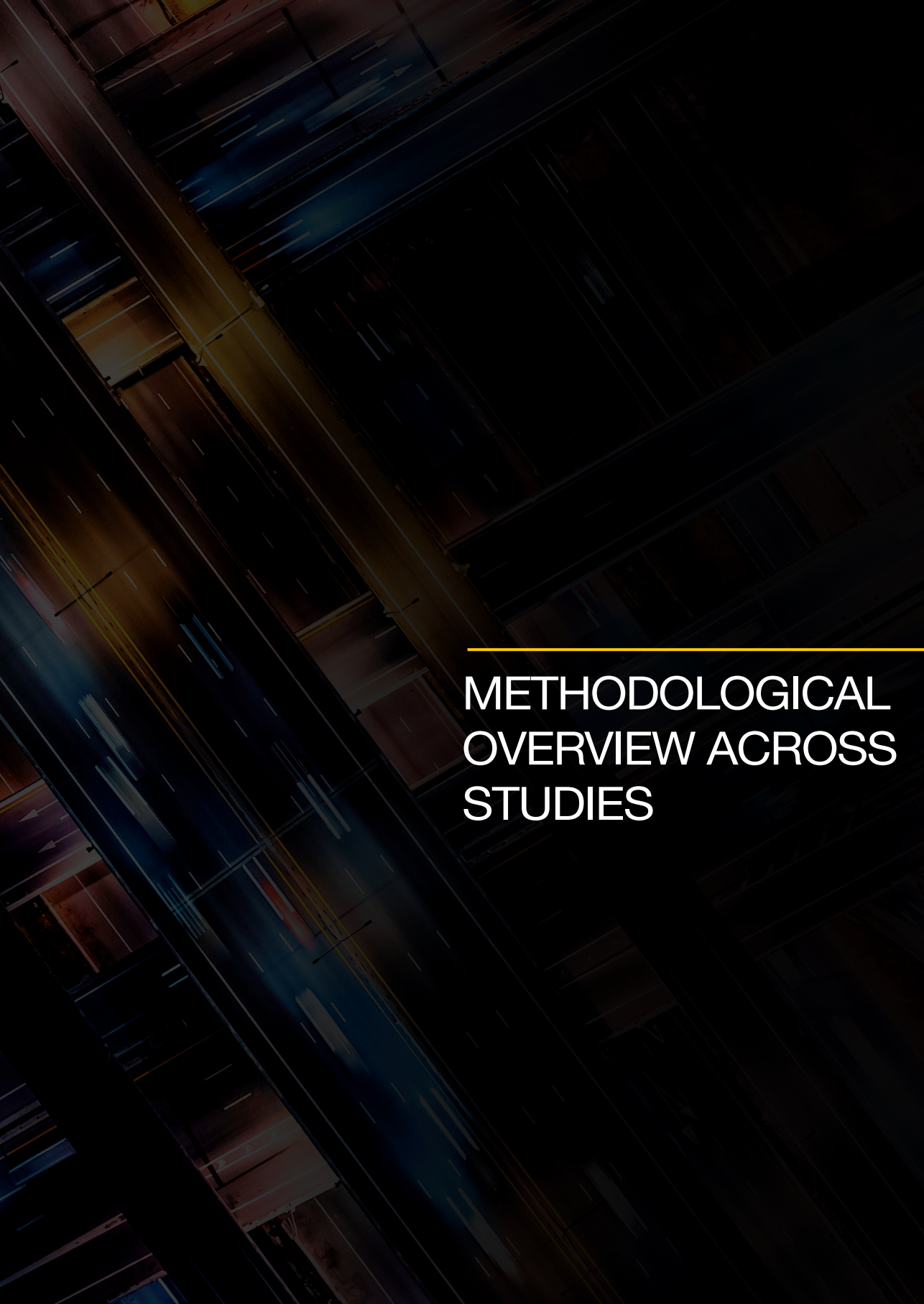
- 27 ROGOVA, V. V., PEEV, S., YOTSOVA, R., GEROVA-VATSOVA, T. & PARUSHEV, I. 2024. **Histomorphometric Assessment of Non-Decalcified Plastic-Embedded Specimens for Evaluation of Bone Regeneration Using Bone Substitute Materials-A Systematic Review.** *Materials* (Basel), 18.
- 28 SALMAN, S., GUNDUZ, O., YILMAZ, S., ÖVEÇOĞLU, M. L., SNYDER, R. L., AGATHOPOULOS, S. & OKTAR, F. N. 2009. **Sintering effect on mechanical properties of composites of natural hydroxyapatites and titanium.** *Ceramics International*, 35, 2965–2971.
- 29 SANZ, M., DAHLIN, C., APATZIDOU, D., ARTZI, Z., BOZIC, D., CALCIOLARI, E., DE BRUYN, H., DOMMISCH, H., DONOS, N., EICKHOLZ, P., ELLINGSEN, J. E., HAUGEN, H. J., HERRERA, D., LAMBERT, F., LAYROLLE, P., MONTERO, E., MUSTAFA, K., OMAR, O. & SCHLIEPHAKE, H. 2019. **Biomaterials and regenerative technologies used in bone regeneration in the craniomaxillofacial region: Consensus report of group 2 of the 15th European Workshop on Periodontology on Bone Regeneration.** *J Clin Periodontol*, 46 Suppl 21, 82–91.
- 30 SANZ-SÁNCHEZ, I., ORTIZ-VIGÓN, A., SANZ-MARTÍN, I., FIGUERO, E. & SANZ, M. 2015. **Effectiveness of Lateral Bone Augmentation on the Alveolar Crest Dimension: A Systematic Review and Meta-analysis.** *J Dent Res*, 94, 128s–42s.
- 31 SCHMIDT-BLEEK, K., SCHELL, H., SCHULZ, N., HOFF, P., PERKA, C., BUTTGEREIT, F., VOLK, H. D., LIENAU, J. & DUDA, G. N. 2012. **Inflammatory phase of bone healing initiates the regenerative healing cascade.** *Cell Tissue Res*, 347, 567–73.
- 32 SPILLER, K. L., ANFANG, R. R., SPILLER, K. J., NG, J., NAKAZAWA, K. R., DAULTON, J. W. & VUNJAK-NOVAKOVIC, G. 2014. **The role of macrophage phenotype in vascularization of tissue engineering scaffolds.** *Biomaterials*, 35, 4477–88.
- 33 STAHL, A. & YANG, Y. P. 2021. **Regenerative Approaches for the Treatment of Large Bone Defects.** *Tissue Eng Part B Rev*, 27, 539–547.

- 34** SUN, Y. X., ZHANG, J. F., LI, D. J., WU, X. M., XU, L. L., PAN, X. H. & LI, G. 2018. **Comparing the osteoconductive potential between tubular and cylindrical beta-tricalcium phosphate scaffolds: An experimental study in rats.** *J Biomed Mater Res B Appl Biomater*, 106, 1934–1940.
- 35** SUSIN, C., LEE, J., FIORINI, T., KOO, K. T., SCHÜPBACH, P., FINGER STADLER, A. & WIKESJÖ, U. M. 2022. **Screening of Hydroxyapatite Biomaterials for Alveolar Augmentation Using a Rat Calvaria Critical-Size Defect Model: Bone Formation/Maturation and Biomaterials Resolution.** *Biomolecules*, 12.
- 36** TADIC, D. & EPPLE, M. 2004. **A thorough physicochemical characterisation of 14 calcium phosphate-based bone substitution materials in comparison to natural bone.** *Biomaterials*, 25, 987–94.
- 37** TRZASKOWSKA, M., VIVCHARENKO, V. & PRZEKORA, A. 2023. **The Impact of Hydroxyapatite Sintering Temperature on Its Microstructural, Mechanical, and Biological Properties.** *Int J Mol Sci*, 24.
- 38** WILSON, C. J., CLEGG, R. E., LEAVESLEY, D. I. & PEARCY, M. J. 2005. **Mediation of biomaterial-cell interactions by adsorbed proteins: a review.** *Tissue Eng*, 11, 1–18.

An aerial, high-angle photograph of a complex highway interchange at night. The image is characterized by long, horizontal light trails from cars, creating a sense of motion. The roads are illuminated by streetlights, and the overall color palette is dominated by dark blues, greys, and the warm yellow of the lights. The perspective is looking down from a significant height, showing multiple levels of the interchange.

“Now you’re looking for the secret, but you won’t find it,
because of course you’re not really looking.”

The Prestige (2006)

An aerial, high-angle photograph of a complex highway interchange. The image is heavily blurred due to a long exposure, creating vibrant, streaky light trails from the headlights and taillights of vehicles moving through the curves and lanes of the road. The colors are predominantly dark blues, greys, and blacks, with streaks of yellow, orange, and white light. The perspective is looking down at the road, which curves and splits into multiple lanes.

METHODOLOGICAL OVERVIEW ACROSS STUDIES

Table 1

Biomaterials investigated across the experimental studies.

BIOMATERIAL	TYPE	MANUFACTURING / KEY VARIABLE	STUDIES USED IN	CHAPTER
HAN	Bovine HA xenograft	Not sintered	Sintering study	Chapter 2
HA820	Bovine HA xenograft	Sintered 820 °C, 30 min	Sintering study	Chapter 2
HA1200	Bovine HA xenograft	Sintered 1200 °C, 30 min	Sintering study	Chapter 2
WHA	Bovine HA xenograft	Chemically deproteinized (mid-range temperature)	Dog GBR + in vitro	Chapter 3, 4
BO (Bio-Oss)	Bovine HA xenograft material	Commercial reference	Dog GBR + in vitro	Chapter 3, 4

This thesis is presented as a compilation of published and submitted manuscripts. The detailed methodology is described within each respective chapter. To facilitate navigation, this section provides an overview of the biomaterials, characterization methods, and experimental models used throughout this thesis, highlighting the methodological elements shared across multiple studies before the individual manuscripts are presented.

Table 2

Physicochemical and in vitro characterization methods

METHOD	WHAT IT MEASURES	CHAPTER(S)
SEM	Surface morphology / microtopography	Chapter 2, 3, 4
BET (N ₂ adsorption)	Specific surface area (SSA)	Chapter 2, 3, 4
XRD	Crystallinity / phase composition	Chapter 2, 3
FTIR	Surface chemical composition	Chapter 4
ICP-MS	Ion release (Ca ²⁺ , Mg ²⁺)	Chapter 4
Capillarity test	Hydrophilicity / wettability	Chapter 4
Protein adsorption (total + albumin)	Surface–protein interaction	Chapter 4
Cell attachment & viability (MC3T3)	Osteoblast response	Chapter 4
Chemotaxis (RAW 264.7 macrophages)	Immune / inflammatory recruitment	Chapter 4
ALP activity	Osteogenic differentiation	Chapter 4

Table 3

In vivo and methodological models

MODEL	SPECIES / SETUP	DEFECT	TIME POINTS	CHAPTER
Sintering in vivo model	Yucatan mini pig (n = 5), maxilla	6 socket defects/animal	3 months	Chapter 2
GBR validation model	Beagle dog (n = 24), mandible	4 critical-size wall defects/animal	4, 12, 26 weeks	Chapter 3
Digital-twin / IOS workflow	Intraoperative optical scanning + GOM Inspect	ROI1 reconstruction (dog mandible)	—	Chapter 5

“The past can hurt. You can either run from it, or learn from it.”

The Lion King (1994)



1

FOUNDATIONS
AND STATE OF
THE ART

Influence of Physicochemical Characteristics of Calcium-phosphate based Biomaterials in Cranio-maxillofacial Bone Regeneration. A Systematic Literature Review and Meta-analysis of Preclinical Models

Ehsan Sadeghian Dehkord, Bruno De Carvalho, Marie Ernst, Adelin Albert, France Lambert, Liesbet Geris.

Keywords:

Calcium-phosphate - Biomaterials - Physicochemical - Cranio-maxillofacial - Intra-oral bone formation - Preclinical - Bone defect - Animal study - Bone regeneration - Bone scaffold

ABSTRACT

Objectives: Calcium-phosphate-based biomaterials (CaP) are the most widely used biomaterials to enhance bone regeneration in the treatment of alveolar bone deficiencies, cranio-maxillofacial and periodontal infrabony defects, with positive preclinical and clinical results reported. This systematic review aimed to assess the influence of the physicochemical properties of CaP biomaterials on the performance of bone regeneration in preclinical animal models.

Methods: The PubMed, EMBASE and Web of Science databases were searched to retrieve preclinical studies investigating the physicochemical characteristics of CaP biomaterials. The studies were screened for inclusion based on the intervention (physicochemical characterization and in vivo evaluation) and the reporting of measurable outcomes.

Results: A total of 1,532 articles were retrieved, and 58 studies were ultimately included in the systematic review. A wide range of physicochemical characteristics of CaP biomaterials was assessed in the included studies. Despite a high degree of heterogeneity, a meta-analysis of 39 studies demonstrated significant effects

of biomaterial characteristics on bone regeneration outcomes. Specifically, macropore size, Ca/P ratio, and compressive strength significantly influenced the formation of newly regenerated bone. Moreover, particle size, Ca/P ratio, and surface area affected bone-to-material contact during the regeneration process. Regarding biodegradability, the amount of residual graft was determined by macropore size, particle size, and compressive strength.

Conclusion: This systematic review demonstrated that the physicochemical characteristics of CaP biomaterials are key determinants of scaffold performance, emphasizing their importance in designing the next generation of bone scaffolds to achieve enhanced bone regeneration.

1. INTRODUCTION

Bone defects in the cranio-maxillofacial (CMF) region can be caused by injuries, cancerous bone resections, periodontal diseases, congenital disorders, and bone resorption following tooth extraction. They often require bone regeneration prior to or simultaneously to implant placement in order to restore deformities and the patient's functions [1–4]. Autologous bone graft procedures remain the clinical gold standard owing to the highest level of biological safety, biocompatibility, matched mechanical requirements and structural similarity in terms of growth factors and biomolecules for osteogenesis [5,6]. Nevertheless, autografts suffer multiple drawbacks such as limited availability, donor-site morbidity, high resorption rates and difficulty in shaping into desired geometries [7–10]. Tissue engineering (TE) using cell-based or growth factor strategies, usually combined with a carrier material, can be used to regenerate the defect site. The application of TE strategies is still limited in the clinic setting due to cost and tissue complexities in the CMF region. Moreover, TE strategies require cumbersome processes in laboratories [11]. Therefore, guided bone regeneration (GBR) combined with biomaterials has become an alternative used in periodontology, implantology and oral surgery for the regeneration of CMF bone defects [12–14]. This biomaterial-based strategy consists in implanting an acellular biodegradable scaffold that can recruit the necessary mesenchymal stem cells and/or osteoprogenitor cells from the surrounding tissues to regenerate the defect [15]. GBR solutions offer several potential advantages such as no restrictions on availability, reduced risk of immunoreactivity, fewer surgical complications as well as the possibility of tailoring the structure to regulate the bone formation microenvironment by manipulating the physicochemical specifications [16,17].

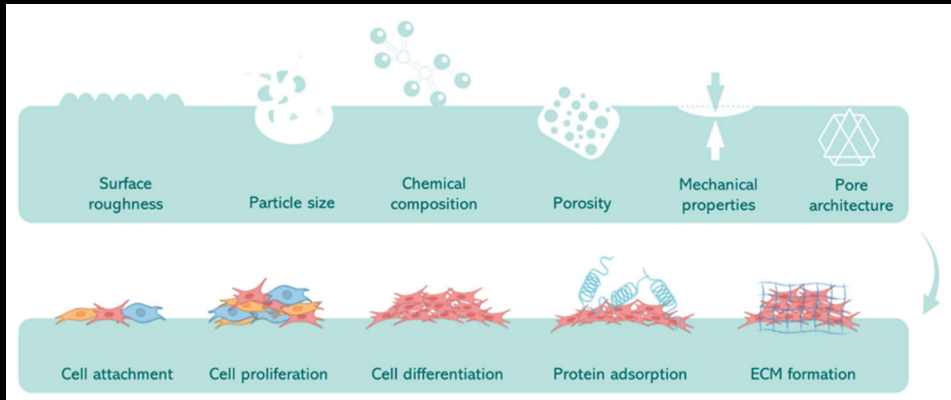
A wide variety of biomaterials have been utilized in CMF bone regeneration, belonging to different material classes and from different origins such as autografts, allografts, xenografts and alloplasts (synthetic biomaterials). Bioceramic materials comprise the majority of inorganic biomaterial

scaffolds whereas biopolymers represent the majority of organic ones. While the ideal bone graft substitute should be accessible, economical and free of ethical and immunological issues with predictable handling, the morphological and physicochemical properties of biomaterials seem to play an important role in their regenerative performance as suggested by several authors [18–20]. Polymers like polylactic acid (PLA), polyglycolic acid (PGA), polycaprolactone (PCL), and methacrylates present advantages such as customizable forms, low immunogenicity, controllable resorbability, porosity, and tunable physiochemical properties [21,22]. These characteristics make them attractive for applications requiring tailored solutions and biocompatibility. Nevertheless, concerns persist regarding the release of acidic degradation byproducts, which can alter local pH levels and potentially hinder osteoconductivity. Additionally, these polymers may exhibit poor cell adhesion capacity, limiting their utility in certain dental procedures where robust tissue integration is crucial [23]. On the other hand, Metals such as nickel-titanium and magnesium-based bone substitutes offer desirable properties like osteoconduction, robust mechanical strength, and resistance to corrosion. These qualities support their effectiveness in providing structural support and promoting bone healing. However, their use may require surgical follow-ups and pose risks such as the possibility of soft tissue dehiscence [24]. CaP ceramics have demonstrated enhanced bone formation and the potential to serve as substitutes for bone grafting, thanks to their osteoconductive characteristics, resorbability, and outstanding biocompatibility [25,26]. Nevertheless, they may suffer from low mechanical adaptability such as brittleness, rapid resorption, and degradation [24].

In this systematic review, we focus on CaP-based biomaterials for CMF bone regeneration as this application domain presents its own unique challenges for bone regeneration that are not entirely overlapping with that of applications in the appendicular skeleton, as discussed above. Furthermore, as the addition of cells, coatings or growth factors alters the mechanism of action of bone regeneration in the biomaterial, and increases the cost of the implant, we focus on studies investigating the results of biomaterials-only strategies. The majority of CaP biomaterials currently used in clinical applications are of natural (human and animal) origin because of their similarities with natural bone structures. However, there is an increasing interest in the development and use of synthetic biomaterials in the clinic to increase safety (risk of disease transmission), ethics compliance and availability. The most widely used CaP biomaterials in CMF bone regeneration are based on hydroxyapatite (HAp), α and β -tricalcium-phosphate (TCP) and/or biphasic calcium phosphate (BCP) [27]. However, synthetic biomaterials also present some limitations such as a lack of osteoconductivity or high resorption rates that might compromise the volumetric dimensions of the regenerated bone [28,29]. Given all this, producing optimized synthetic CaP biomaterials is crucially dependent on the fundamental understanding of the influence of the physicochemical biomaterial properties on the bone healing mechanisms [30] (Fig. 1).

Figure 1

An illustration of the key physicochemical properties influencing biological events. Biomaterials can manipulate molecular and cellular responses through their structural characteristics and composition (ECM: extracellular matrix).



This systematic review aimed to provide a comprehensive overview of the effect of the physicochemical properties of the CaP biomaterials on the biological performance of bone graft substitutes used for CMF bone regeneration or intra-oral bone augmentation in animal models. Following a well-documented approach, the current state of the art was assessed from reports published in the literature, and a meta-analysis was performed on the data extracted from these reports. The presented information can be used as input for the optimization of the structure and composition of future CaP biomaterials developments.

2. MATERIAL AND METHODS

2.1. Study design

The present study was designed as a systematic literature review on the influence of physicochemical properties of CaP biomaterials implanted in cranio-maxillofacial bone defects. All studies involving the physicochemical characterization of CaP biomaterials and their in vivo evaluation in a cranio-maxillofacial animal model were assessed. The protocol for this review was registered with the international prospective register of systematic reviews (PROSPERO) with registration number CRD42019121604.

2.2. Focused question and search strategy

The systematic review was performed according to SYRCLE's (SYstematic Review Centre for Laboratory animal Experimentation) guidelines [31], and the focus question was: "What is the influence of different physicochemical characteristics of CaP biomaterials in the preclinical cranio-maxillofacial bone regeneration process?". An electronic search of the literature was run on November 14, 2023, in the following databases: PubMed (MEDLINE), EMBASE and Web of Science (all databases). Only articles in the English language were included without restrictions on the geographical area of the study. A detailed search strategy including all search terms and relationships between them (Table 1) was developed for PubMed and then adopted appropriately for EMBASE and Web of Science.

Eligibility was initially determined by reading the title and abstracts identified in each search. For this purpose, all references were imported into an Endnote X9 database. After eliminating the duplicates [32] and non-relevant ones, they were exported to an online webtool (Rayyan QCRI) to perform the screening. An Excel sheet with all references was generated from the database. The list of unique titles and abstracts was screened by two independent reviewers (ESD and BDC) to determine the eligibility of each study based on the inclusion and exclusion criteria described below. The two lists of selected references were then compared in the webtool, and all disagreements were solved by discussion, or if persistent, by a third reviewer (FL). For the potentially relevant publications, the full-text paper publication was collected and screened to check if all specified inclusion criteria were indeed met.

2.3. Inclusion/exclusion criteria

Studies were selected according to the inclusion and exclusion criteria specified in Table 2.

2.4. Data extraction

The data extraction started once the selection process was validated. One reviewer (ESD) extracted the data from the included studies. The second reviewer (BDC) revised the extraction results to check the quality. Studies meeting the criteria for inclusion were read carefully to identify the following information: authors, title, year of publication, scaffold used in the study, scaffold's physical form, characterization methods, animal characteristics (species and numbers), bone defect model, duration of implantation, scaffold's physicochemical characteristics (primary outcomes), and tissue regeneration responses (secondary outcomes). The data were collected in an excel file. In case the information was not reported, the table entry was indicated as N/P (Not Provided).

Table 1

The search terms used in PubMed. [MeSH]: medical subject headings; [Supplementary concept]: terms in supplementary concept records (a thesaurus distinct from MeSH); [TIAB]: title/abstract terms.

NUMBER	SEARCH TERMS AND COMBINATIONS
#1	"calcium-phosphate, dibasic, anhydrous" [Supplementary Concept] OR calcium-phosphate[TIAB] OR hydroxyapatite[TIAB] OR "Hydroxyapatites» [MeSH] OR "tricalcium-phosphate" [Supplementary Concept] OR "beta-tricalcium-phosphate" [Supplementary Concept] OR "hydroxyapatite-beta tricalcium-phosphate" [Supplementary Concept] OR tricalcium-phosphate[TIAB]
#2	"Periodontal Atrophy»[MeSH] OR "guided tissue regeneration, periodontal» [MeSH] OR "alveolar ridge augmentation»[MeSH] OR "Parietal Bone/surgery» [MeSH] OR "Skull/drug effects»[MeSH] OR "Skull/injuries»[MeSH] OR "Skull Fractures»[MeSH] OR "Skull/surgery»[MeSH] OR maxillofacial injury[MeSH] OR periodontal-resorption[TIAB] OR Periodontal-Atrophy[TIAB] OR periodontal-guided-tissue-regeneration[TIAB] OR guided-bone-regeneration[TIAB] OR alveolar-bone*[TIAB] OR maxillofacial-bone-defect[TIAB] OR mandibledefect [TIAB] OR mandibular-bone-defect[TIAB] OR maxilla-defect [TIAB] OR maxillary-bone-defect[TIAB] OR maxillary-sinus-lift[TIAB] OR sinus-lift-model[TIAB] OR sinus-augmentation[TIAB] OR alveolarridge-augmentation[TIAB] OR skull-defect [TIAB] OR jaw-defect[TIAB] OR parietal-defect[TIAB] OR cranial-defect[TIAB] OR tooth-defect* [TIAB] OR calvarial-defect[TIAB] OR calvarial-defect[TIAB] OR calvarium-defect[TIAB] OR socket-preservation[TIAB] OR socketgrafting[TIAB] OR dental-socket*[TIAB]
#3	"Microscopy, Electron, Scanning»[MeSH] OR «X-Ray Diffraction»[MeSH] OR "Spectroscopy, Fourier Transform Infrared»[MeSH] OR "Microscopy, Scanning Tunneling»[MeSH] OR "Microscopy, Electron, Transmission»[MeSH] OR "Microscopy, Atomic Force»[MeSH] OR Brunauer–Emmett–Teller[TIAB] OR profilometr*[TIAB] OR X-Ray[TIAB] OR XRD[TIAB] OR ATM[TIAB] OR atomic-force-microscop*[TIAB] OR STM[TIAB] OR scanning-tunneling-microscop* [TIAB] OR scanning-tunnelling-microscop*[TIAB] OR FT-IR[TIAB] OR FTIR [TIAB] OR infrared-microscop*[TIAB] OR electron-microscop*[TIAB] OR electron-scanning-microscop*[TIAB] OR TEM[TIAB] OR SEM[TIAB] OR surface-roughness [TIAB] OR surface-propert*[TIAB] OR chemicalcharact*[TIAB] OR chemical-propert*[TIAB] OR physical-charact* [TIAB] OR physical-propert*[TIAB] OR morphological-charact*[TIAB] OR morphological-propert*[TIAB] OR mechanical-charact*[TIAB] OR mechanical-propert*[TIAB]
#4	(#1) AND (#2) AND (#3)

Table 2

*Preliminary inclusion and exclusion criteria used in this systematic review.
PICOS is abbreviation of population, intervention, comparator, outcome, and study design.*

PICOS	INCLUSION CRITERIA	EXCLUSION CRITERIA
Population	All animals receiving a pure CaP biomaterial implant in periodontal and craniomaxillofacial intra-bone defects induced by researchers	Human subjects, in vitro research, animals receiving implants in tissues other than cranio-maxillofacial bones, animals receiving implants that are not purely CaP biomaterials, animals receiving implants for bone defects that are not experimentally induced
Intervention	CaP biomaterial implants with distinct microstructural properties (particle size, porosity, pore size, mechanical properties, chemical composition, surface properties) in any physical form without tissue engineering or regenerative medicine approach (cell/drug/growth factor loaded)	All implants that are not CaP biomaterials, all implants that are not the only treatment in the defect site and have interference with other treatments, CaP biomaterial implants in the non-scaffold applications like coating, film, carrier, membrane etc.
Comparator	Not applicable	Not applicable
Outcome	Tissue regeneration responses (one or more) including newly formed bone (NB), bone-to-material contact (BMC), residual graft (RG)	None
Study design	In vivo experimental animal studies	Clinical trials, in vitro studies, reviews, case reports, observational research, uncontrolled studies

NB refers to the bone tissue that grows and integrates into the surface of the implanted bone biomaterial; BMC corresponds to the physical interaction between the host bone tissue and the implanted biomaterial; RG pertains to the remaining portion of the graft material that has not integrated with the surrounding bone tissue.

2.5. Quality assessment and risk of bias

The methodological quality of the selected articles was assessed using the criteria outlined in the SYRCLE's Risk of Bias (RoB) tool which is specifically developed for animal studies [33]. The tool contains 10 entries that are related to selection bias, performance bias, detection bias, attrition bias, reporting bias and other biases. Following the signaling questions proposed by Hooijmans et al. (2014), the items in the RoB tool were scored as low risk, high risk or "unclear", the latter indicating that the item was not reported and, therefore, the risk of bias was unknown. Supplementary Table S1 presents the risks and how they were scored for RoB assessment. As animal studies are known for their poor reporting quality in comparison with randomized controlled trials, it is likely that many items of the RoB tool were not reported or poorly reported (Hooijmans et al. 2014). Therefore, in addition to the RoB assessment, four indicators of methodological quality were checked. One overall study quality indicator (Q11 in Table S1) scored whether any randomization was reported for any level of the experiment. Likewise, we included two overall study quality indicators to acquire additional information on the reporting quality of the studies (Q12 & Q13 in Table S1). Specifically, the number of interventions for the animals and the number of animals to be analyzed for each time point were retrieved. In addition, we included a final study quality indicator related to how the outcomes were reported in different studies: quantitative numeric format versus qualitative (Q14 in Table S1). When study results were only available graphically, the item was scored "unclear". To extract data from graphs, WebPlotDigitizer was used (A. Rohatgi, <https://automeris.io/WebPlotDigitizer>, v4.7, 2024) was used. One reviewer (ESD) conducted the RoB and methodological quality assessment, and in case of doubt, a second reviewer (BDC) was consulted.

Prior to the meta-analysis, a descriptive review of the study characteristics was carried out. The meta-analysis was performed on a dataset including all covariates (quantitative physicochemical properties of biomaterials used in the studies) and outcome variables (NB, BMC, and RG).

2.6. Statistical methods

Each outcome variable Y (NB, BMC, or RG) was analyzed with respect to covariates available from N experimental samples (meta statistical units), each sample resulting from a clearly identified experiment conducted on a number (n) of animals over a certain time, whether published in the same paper or in different papers, whether involving the same animals or not. Results were expressed as mean, standard deviation (SD) and range for quantitative variables, while frequency tables (number and percent for each category) were used for categorical findings. In some cases, to avoid the influential effect of outliers or extreme values, the mean was replaced by the median and the standard deviation by a robust version $SD = 0.74 \times (P75 - P25)$, with P25

and P75, the 25th and 75th percentiles of the sample data, respectively. The Spearman correlation coefficient was used to quantify the association between the physicochemical characteristics of biomaterials. Agreement between reviewers was assessed by the Cohen kappa coefficient (κ). A log-transform was applied to some covariates to normalize their skewed distribution. Outcomes being expressed in percent, a logit transform was applied to normalize their distribution, specifically $\text{Logit}(Y) = \log[Y/(100 - Y)]$. Furthermore, outcome data being obtained from a varying number (n) of animals, a weight (w) was associated with each value as the inverse of their sampling variance ($SE^2 = SD^2/n$), provided SD and n were available; if not, w was estimated by regression and imputed from existing data. Lastly, since outcome data were not all statistically independent (some were correlated because repeated on the same animals), meta weighted generalized linear mixed effects models (W-GLMM) were used to estimate outcomes and assess the potential effect of covariates like time or type of biomaterial. All meta regression coefficient estimates were presented with their standard error and p-value. Results were considered significant at the 5 % critical level ($p < 0.05$). Calculations were performed using SAS (Version 9.4; Analytics Software and Solution, SAS Institute, Cary, North Carolina) and R (Version 3.6.1; R Foundation for Statistical Computing, Vienna).

3. RESULTS

3.1. Systematic review and study data description

The initial literature search yielded 2103 potentially eligible articles. After removing the duplicates and the ones not in English, 1532 articles were screened in the subsequent process. Of this number, 1342 were excluded based on inclusion and exclusion criteria. The full text of 190 articles were assessed after which another 132 were excluded, resulting in 58 articles meeting the final inclusion/exclusion criteria. The interreviewer agreement was $\kappa = 0.92$ for titles and abstract evaluation and $\kappa = 0.89$ for full-text evaluation. The search strategy and retrieved articles are summarized in Fig. 2 and Table 3, the latter providing a summary of the study characteristics of the included publications. In 25 out of the 58 final included studies, HAp scaffolds were characterized and implanted; TCP scaffolds were used in 15 studies, BCP scaffolds in 24 studies, octacalcium-phosphate (OCP)/amorphous calcium-phosphate (ACP) and their combination in 2 studies, dicalcium-phosphate (DCP) in one study and other types of CaPs in 2 studies (eggshell CaPs, respectively non-defined CaP). The scaffolds were implanted in the form of 3D rigid structures (disc, block, cylinder, cube, sponge, and tube) in 24 studies, non-rigid structures (granules, particles, spheres, and powder) in 32 studies, and aqueous

structures (paste and injectable cement) in 2 studies. The actual physical form is provided in Table 3. In terms of animal models, there was substantial heterogeneity between studies with rats used in 23 out of 58 studies (for a total number of animals $n > 608$), rabbits in 20 studies ($n > 208$), dogs in 9 ($n > 67$), mini-pigs in 3 ($n = 19$), sheep in one ($n = 6$), mice in one ($n = 5$) and baboons in one ($n = 4$). Bone defect models were the typical defects used in CMF bone regeneration studies: calvarial defects (32 studies), mandibular defects (9), parietal defects (4), alveolar defects (4), sinus augmentation (2), skull defects (3), bilateral (maxilla and mandibular) defects (1), cranial defects (2) and mastoid obliteration (1). Outcomes were assessed over observation periods (time) ranging from 30 days to 40 weeks (Table 3).

Figure 2

Flow diagram for the review and selection process of studies included in the systematic review.

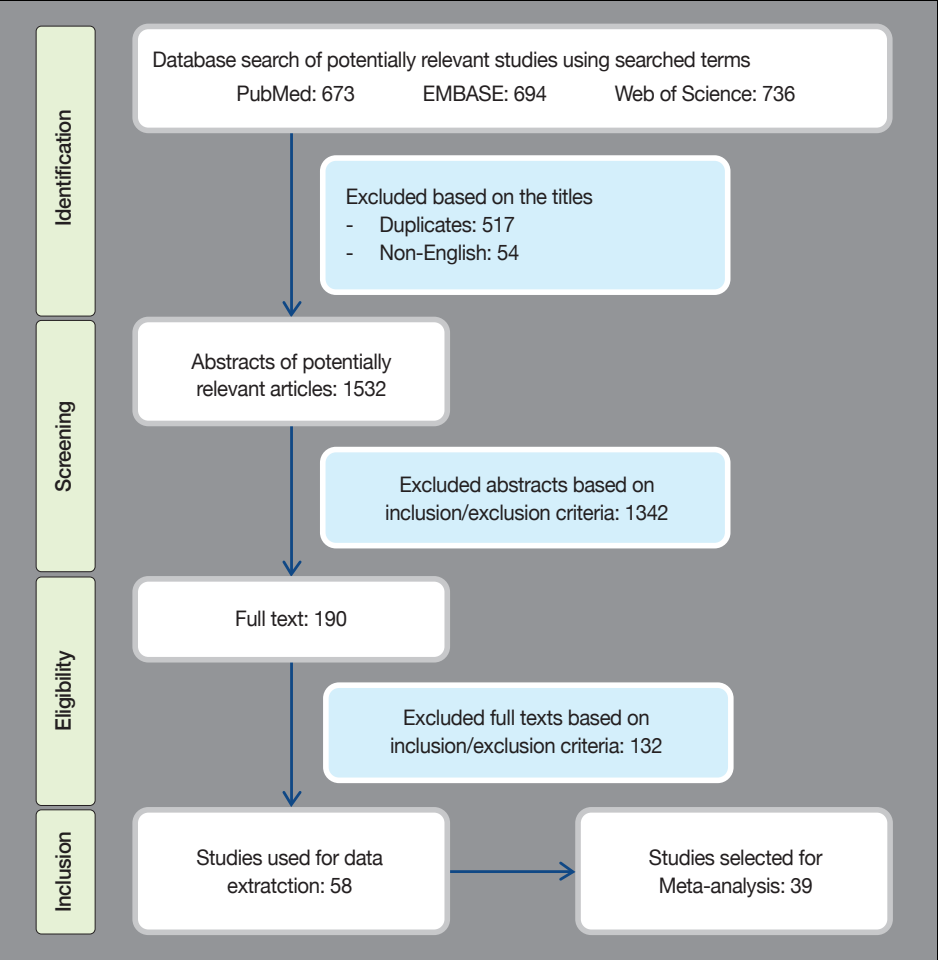


Table 3

Summary characteristics of the papers included in the systematic review. Papers with qualitative outcomes were not included in the meta-analysis. XRD: X-ray diffraction; SEM: scanning electron microscopy; AES: auger electron microscopy; IRRS: infrared reflection spectroscopy; FT-IR: Fourier-transform infrared spectroscopy; OM: optical microscopy;

PAPER ID	BIOMATERIAL(S)	PHYSICAL FORM	CHARACTERIZATION METHODS	CHARACTERISTICS
Abdel-Fattah et al. (1994) [34]	VHAP	Block	XRD	Diffraction pattern
Denissen et al. (1995) [35]	HAp	Tube	SEM, AES, XRD, IRRS	Particle diameter, diffraction pattern, absorbance spectra, surface area, Ca/P ratio
Roy et al (2003) [36]	HAp-Ch. HAp-No	Disk	Mercury porosimetry, XRD	Particle size, diffraction pattern, surface area, Ca/P ratio
Fleckenstein et al. (2006) [37]	HAp/TCP	macroporous disk microporous disk granules	SEM	micro-pore size
Suzuki et al. (2006) [38]	OCP Ca-deficient HAp (HL6h) Ca-deficient HAp (HL48h)	Granules	XRD, FT-IR	Diffraction pattern, absorbance spectra, surface area, chemical composition, Ca/P ratio
Simon et al. (2007) [39]	HAp(DW250S) HAp(DW250 M) HAp(DW250L) HAp(DW400S) HAp(DW400 M) HAp(DW400L)	Disc	OM, SEM	Void volume
Park et al. (2008) [40]	BioOss Egg-shell(ES) ES-CaP-1 ES-CaP-2 ES-CaP-3	Granules	SEM, EDXA, XRD, FT-IR	Diffraction pattern, absorbance spectra, Ca/P ratio

EDXA: energy dispersive X-ray analysis; FESEM: field-emission scanning electron microscopy; Mech: mechanical characterization; ICP-AES: Inductively coupled plasma atomic emission spectroscopy; BET: Brunauer–Emmett–Teller.

ANIMAL	QTY.	BONE DEFECT	DURATION	OBSERVATION	OUTCOME
Rabbit	6	Mandibular defect	3 months	Radiography SEM Infra-red spectral analysis	Bone healing Bone-implant integration Carbonate and phosphate resorption
Dog	2	Mandibular defect	6 months	Radiography Histology	Interfaces observation Bone ingrowth
New Zealand white rabbits	12	Mandibular	6 months	Histology Histomorphometry	New bone area(%) Linear ingrowth(%) Mineral apposition rate (um/day) continuity index
Rat	33	calvarial defect	10 weeks	Histology Histomorphometry	New bone formation (%)
Rat	30	Calvaria defect	12 weeks	Histology Histomorphometry Radiography XRD	Newly formed bone(%) Structural changes
New Zealand white rabbits	16	Calvarial defect	16 weeks	microCT radiography	Bone ingrowth volume (mm ³) Normalized bone ingrowth(%)
Rat	30	Calvarial defect	8 weeks	Histology Histomorphometry	Newly formed bone area(%) Remaining bone graft particle area(%) BMC(%)

Table 3 (continued)

PAPER ID	BIOMATERIAL(S)	PHYSICAL FORM	CHARACTERIZATION METHODS	CHARACTERISTICS
Ripamont et al. (2008) [41]	HAp/ β -TCP(19/81) HAp/ β -TCP(4/96)	Disc	SEM, XRD, FT-IR	Macro and micro-pore size, diffraction pattern, absorbance spectra
Xu et al. (2008) [42]	β -TCP	Bulk	FESEM, XRD, Mech.	Porosity, Macro-pore size, diffraction pattern, compressive strength
Appleford et al. (2009) [43]	Micro and Nano-HAp	Cylinder	SEM, XRD	Porosity
Hirota et al. (2009) [44]	β -TCP (OSferion®)	Granules	SEM	Particle size, porosity, Macropore size
Takahashi et al. (2009) [45]	TCP	Sponge	SEM	Macro-pore size
Wang et al. (2009) [46]	β -TCP	Cube	SEM	Porosity, Macro-pore size
Yao et al. (2009) [47]	BCP	Cylinder	SEM, XRD	Porosity, Macro and micropore, size, chemical composition, mechanical properties
Park et al. (2010) [48]	CaP	Evacuated and filled microspheres	XRD, SEM	Particle size, diffraction pattern, wall thickness, evacuated area
Park et al. (2010) [49]	n-BCP-1 n-BCP-2 MBCP Osteon Cerasorb Bio-Oss	Granules	SEM	Particle size, chemical composition

ANIMAL	QTY.	BONE DEFECT	DURATION	OBSERVATION	OUTCOME
Baboon	4	Calvarial defect	365 days	Histology Histomorphometry	Bone volume fraction(%) Matrix volume fraction (%) FVA(%)
Rabbit	12	Calvarial defect	16 weeks	Micro-CT Histology Histomorphometry	New bone(%) Residual material(%)
Dog	10	Mandibular defect	12 weeks	Micro-CT Histology Histomorphometry	Mineralized bone formation(%) Area of Total Tissue(%) Area of Mineralized Bone (%)BV/TV, BS/TV, BS/BV, Tb.N, Tb.Sb, Tb.Th
Rat	10	Mandibular defect	5 weeks	Histology Histomorphometry	Bone formation rate(%) Amount of material(%)
Dog	10	Mandibular defect	8 weeks	Histopathology	Average bone mass (center and top of cavity) (mm ²)
Dog	4	Mandibular defect	24 weeks	Histomorphometry Radiography Sequential fluorescent labeling	Newly formed bone(%) Height and thickness of alveolar ridge bone (mm) Mineralization level(%)
Dog	10	Mandibular defect	8 weeks	99mTc-MDP SPECT Mech. Pro analysis Histology	New bone filled
New Zealand white rabbits	N/P	Calvarial defect	6 weeks	Histology	Bone formation
New Zealand white rabbits	20	Calvarial defect	8 weeks	Histology Histomorphometry	New bone(%)

Table 3 (continued)

PAPER ID	BIOMATERIAL(S)	PHYSICAL FORM	CHARACTERIZATION METHODS	CHARACTERISTICS
Hung et al. (2011) [50]	PC-HAp/ β -TCP, MBCP	Granules	SEM, XRD	Particle size, chemical composition, crystallite size
De Oliveira Lomelino et al. (2012) [51]	BCP	Granules	SEM, XRD, FT-IR	Particle size, diffraction pattern, absorbance spectra, chemical composition
Klijn et al. (2012) [52]	CPC-IP	injectable cement	SEM, μ CT, Gillmore test	Porosity, Chemical composition
Lee et al. (2012) [53]	synthetic hydroxyapatite (sHA) eggshell hydroxyapatite (eHA)	Granules	FT-IR, XRD, SEM	Granule size, diffraction pattern, absorbance spectra
Cho et al. (2013) [54]	HAp	Granules	FESEM, XRD, EPMA, FTIR, ICP-AES, ion chromatography	Particle size, diffraction pattern, absorbance spectra
Lee et al. (2013) [55]	60 TCP40HA	Granules	XRD, SEM	Porosity, Macro-pore size, diffraction pattern, chemical composition
Lee et al. (2013) [56]	HAp, β -TCP, BCP	Granules	XRD, SEM, FE-SEM, Micro-CT	Granule size, porosity, Macro-pore size, diffraction pattern, chemical composition, interconnectivity
Jang et al. (2014) [57]	HApS	Granules	SEM	Granule size
Kobayashi et al. (2014) [58]	OCP ACPOCP/ACP	Granules	FT-IR, XRD, SEM	Granule size, diffraction pattern, absorbance spectra, chemical composition, Degree of Supersaturation
Lee et al. (2014) [59]	sHA eHA	Powder	FT-IR, XRD, SEM	Diffraction pattern, absorbance spectra

ANIMAL	QTY.	BONE DEFECT	DURATION	OBSERVATION	OUTCOME
Dog	4	Bilateral maxilla and mandible defect	16 weeks	Histology Histomorphometry	New bone(%) Ratio of new bone formation
Rat	10	Calvarial defect	45 days	Histology Histomorphometry	New bone tissue(%)
Rat	12	Skull defect	12 weeks	Histology Histomorphometry	Newly formed bone(%) Appositional bone height(μ m)
New Zealand white rabbits	16	Calvarial defect	8 weeks	Histomorphometry	Total new bone(%) Residual graft(%)
New Zealand white rabbits	8	Calvarial defect	4 weeks	Histomorphometry	Newly formed bone(%) Implanted granule(%) Soft tissue(%)
Rat	52	Calvarial defect	8 weeks	Histology Micro-CT	BV/TV, BS/BV, Tb.Pf, SMI, Tb.Th, Tb.N, Tb.Sp, DA
Rat	130	Calvarial defect	8 weeks	Histology Micro-CT	BV/TV, BS/BV, Tb.Pf, SMI, Tb.Th, Tb.N, Tb.Sp, DA
Rat	10	Mastoid obliteration	12 weeks	Fluorescent labeling Histology Micro-CT	Osteoconduction Ca deposition Resorption
Rat	N/P	Calvarial defect	12 weeks	Radiography XRD Histology Histomorphometry	Radiopacity(%) New bone(%)
Rat	30	Parietal defect	8 weeks	Histomorphometry	Total new bone(%) Residual graft(%)

Table 3 (continued)

PAPER ID	BIOMATERIAL(S)	PHYSICAL FORM	CHARACTERIZATION METHODS	CHARACTERISTICS
Xia et al. (2014) [60]	macroporous HAp	Block	SEM	Rod diameter, Macropore size and interconnected pore size
Yang et al. (2014) [61]	β -TCP (Cerasorb), BCPs	Granules	SEM, XRD	Particle size, porosity, Macro and micro-pore size, diffraction pattern, chemical composition
Calasans-Maia et al. (2015) [62]	HAp and carbonated HAp	Spheres	SEM	SEM image
Calvo-Guirado et al. (2015) [63]	BCP (4Bone [®])	Granules	SEM	Granule size, Porosity, Macro and micro-pore size, chemical composition
Khan et al. (2015) [64]	BCP	Granules	helium pycnometry, Acoustic & electro-acoustic spectrometer	Particle size distribution, Chemical composition, Surface area, Density
Lim et al. (2015) [65]	BCPs	Block	XRD, SEM, Mech.	Porosity, Macro-pore size, Diffraction pattern, chemical composition, compressive strength, Crystalline phase (%)
Manchon et al. (2015) [66]	β -TCP	Granules	SEM, XRD, BET	Granule size, Porosity, pores fraction, pore size, diffraction pattern, surface area
Manchon et al. (2015) [67]	β -TCP	Granules	SEM, XRD, BET	Porosity, pore size, pores fraction, diffraction pattern, surface area, blend composition, lattice parameters

ANIMAL	QTY.	BONE DEFECT	DURATION	OBSERVATION	OUTCOME
Rat	6	Calvarial defect	8 weeks	Micro-CT Histology Histomorphometry	BMD(mgHA/cm) ³ Tb.Th New bone area(%) New vessel area(%)
New Zealand white rabbits	10	Calvarial defect	8 weeks	Histology Histomorphometry	Area of augmentation (mm ²) New bone are (mm ² ,%) Residual particle area (mm ² ,%)
Rat	15	Alveolar defect	42 days	Histomorphometry	BV/TV(%) BiomatV/TV (%) RANKL(pg/mL) OPG(pg/mL)
New Zealand white rabbits	7	Parietal defect	12 weeks	Histomorphometry	Cortical Defect Closure (%) New bone(%) Connective tissue(%) Residual material(%)
New Zealand white rabbits	12	Parietal defect	8 weeks	Histomorphometry	Bone growth(%) Graft(%) Soft tissue(%)
New Zealand white rabbits	12	Calvarial defect	8 weeks	Histology Histomorphometry	Newly formed bone(%) Residual material(%)
New Zealand white rabbits	4	Calvarial defect	12 weeks	Histology Histomorphometry	New bone(%) Residual graft material (%) Fibrous tissue(%)
New Zealand white rabbits	4	Parietal defect	12 weeks	Histology Histomorphometry	New bone(%) Residual graft material (%) Fibrous tissue(%)

Table 3 (continued)

PAPER ID	BIOMATERIAL(S)	PHYSICAL FORM	CHARACTERIZATION METHODS	CHARACTERISTICS
Mangano et al. (2015) [68]	BCP	Block	SEM, XRD	Rod diameter, porosity, Macro and micro-pore size,
Lee et al. (2016) [69]	nano-sized β -TCP	Granules	SEM, XRD, FT-IR, BET, μ CT, particle size analyzer, Mech.	Porosity, Macro-pore size, diffraction pattern, absorbance spectra, surface area, compressive strength
Sheikh et al. (2016) [70]	dicalcium phosphate	Disc	SEM, XRD	Porosity, diffraction pattern, surface area, Ca/P ratio, Density, Compressive strength
Lambert et al. (2017) [71] SHA (Osbone)	BHA (Bio-Oss) CBHA (Endobon)	Granules	SEM, BET	Particle size, surface area white
Diao et al. (2018) [72]	3D Plotted β -TCP	Paste	μ CT, SEM, XRD	Porosity, Macro-pore size, Diffraction pattern, Connectivity
Fan et al. (2018) [73]	ABBM, BioOss	Granules	SEM, BET, XPS, XRD, FTIR	Macro-pore size, Diffraction pattern, absorbance spectra, XPS spectrum, surface area, average pore volume
Yao et al. (2018) [74]	BCPs	Block	SEM, XRD	Porosity, Macro-pore size, Diffraction pattern, Chemical composition
Madhumathi et al. (2018) [75]	CDHA β -TCP CDHA/ β -TCP	Granules	XRD, FT-IR, TEM	Diffraction pattern, surface area

ANIMAL	QTY.	BONE DEFECT	DURATION	OBSERVATION	OUTCOME
Sheep	6	Sinus	90 days	Micro-CT Histology Histomorphometry	Newly deposited bone area/field area(%) peripheral and central
Dog	18	Mandibular defect	12 weeks	Histology	Newly formed bone(%) Residual quantity(%) Resorption(%)
New Zealand white rabbits	5	Calvarial defect	12 weeks	Histology Histomorphometry SEM XRD	Bone volume(%) Remaining graft(%)
New Zealand rabbits	24	Calvarial defect	12 weeks	Histology Histomorphometry BMC(%)	Newly formed bone(%) Biomaterial(%)
SD Rats	N/P	Calvarial defect	12 weeks	Micro-CT Histology Biomechanical	New bone(%) BMD(g.cm-3) BV(mm ³) Max load(N) Stiffness(N/mm)
New Zealand white rabbits	16	Calvarial defect	12 weeks	Micro-CT Histology Histomorphometry	Newly formed bone(%) remnant graft(%)
Dog	9	Bilateral alveolar ridges	12 weeks	Fluorescent dye labeling Histology Histomorphometry Spiral CT	New bone formation(%) Graft volume
Rat	12	Cranial defect	12 weeks	Micro-CT Histology Immunohisto-chemistry	total healing score

(continued on next page →)

Table 3 (continued)

PAPER ID	BIOMATERIAL(S)	PHYSICAL FORM	CHARACTERIZATION METHODS	CHARACTERISTICS
da Silva Brum et al. (2019) [76]	BCP	nano-particle	SEM, XRD, mercury intrusion porosimetry, helium gas pycnometry	Porosity, Macro-pore size, Diffraction pattern, Density
De Carvalho et al. (2019) [77]	Non-sintered HAP HAp sintered at 820 °C HAp sintered at 1200 °C	Granules	SEM, XRD, BET	Macro and micro-pore size, Diffraction pattern, Surface area, Pore volume
Park et al. (2019) [78]	β -TCPs(a,b,c,d)	Block	SEM	Porosity, Macro and micropore size
Zhang et al. (2019) [79]	HAp α -TCP β -TCP BCP (30 % β -TCP) BCP (70 % β -TCP)	Disc	SEM, XRD, Mech.	Diffraction pattern, Ca/P ratio, Compressive strength, Solubility
Hung et al. (2019) [80]	BCP	Granules	SEM	Porosity, Macro-pore size, Chemical composition, Compressive strength, Density
Chi et al. (2020) [81]	3D-HAp	Cylinder	SEM, Mech.	Pore size, Compressive modulus
Jensen et al. (2020) [82]	TCP	Cylinder	XRD, Mech.	Pore size, Diffraction pattern, Compressive forc
Intapibool et al. (2020) [83]	BCP(30 % TCP70 %HAp) BCP(70 % TCP30 %HAp)	Granules	SEM, XRD	Granules size, Diffraction pattern, Micro-pore size, Macro-pore size, Chemical composition

ANIMAL	QTY.	BONE DEFECT	DURATION	OBSERVATION	OUTCOME
Rat	48	Calvarial defect	4 weeks	Histology Histomorphometry	Graft remaining particles area(%) Graft remaining particles number(%) New tissue area(%)
mini-pig	5	Alveolar defects	3 months	Histology Histomorphometry	Regenerated area Regeneration(%) Newly formed bone(%) Biomaterial(%) Soft tissue(%) Bone-to-material contact(%)
Rat	48	Calvarial defect	4 weeks	Micro-CT Histology Immunohistochemistry	New bone formation Osteoblastic differentiation Type I collagen
Dog	N/P	Skull defect	8 months	Histology	New bone regeneration Vascularization Bone resorption
mini-pig	6	Sinus augmentation	12 weeks	Micro-CT Histometry	New bone(%) Connective tissue(%) Residual particles(%) Bone density variables (BA/TA and BS/TV) Bone architecture variables (Tb.Th and Tb.N) Bone spacing variable (Po.(OP))
Rat	6	Skull defect	12 weeks	Micro-CT Histometry	BV/TV(%), Tb.N, Tb.Sp, Tb.Th,
Mouse	5	Calvarial defect	8 weeks	Luciferase scanning histology	Implant surface with bone(%)
Pig	8	Calvarial defect	16 weeks	Histology Histomorphometry	New bone(%) Residual graft material (%) Bone-to-material contact(%)

Table 3 (continued)

PAPER ID	BIOMATERIAL(S)	PHYSICAL FORM	CHARACTERIZATION METHODS	CHARACTERISTICS
Kiyochi et al. (2020) [84]	BCP	Disc	SEM, XRD, Mech., Micro-hardness	Diffraction pattern, Particle size, Density, Porosity, Vickers hardness, Compressive strength
De Oliveira Junior et al. (2021) [85]	BioOss, BCPs	Granules	SEM, BET	Pore volume, specific surface area
Seo et al. (2021) [86]	BCPs	Granules	SEM	Pore diameter, porosity, chemical composition
Wang et al. (2021) [87]	HAp fiber, BioOss	Fiber, Granules	FE-SEM, XRD	Diffraction pattern, Particle diameter, Pore size
Ghayor et al. (2022) [88]	HAp1100 HAp1200 HAp1300 HAp1400	Cube	SEM, Mech, Microporosity, BET	Grain size, surface area Microporosity, Compressive strength
Da Silva et al. (2023) [89]	BCP	Cylinder	XRD, SEM/EDS	Diffraction pattern, Chemical composition
Wu et al. (2023) [90]	BCPs	Disc	SEM, FT-IR, Mech.	Porosity
Youseflee et al. (2023) [91]	nHAp	Disc	FE-SEM, XRD	Diffraction pattern

ANIMAL	QTY.	BONE DEFECT	DURATION	OBSERVATION	OUTCOME
Rat	35	Calvarial defect	60 days	SEM Histology	Bone mineralization Presence of blood vessels
Rat	45	Calvarial defect	90 days	Histology Histomorphometry	Bone neoformation (μm^2)
Rabbit	6	Calvarial defect	8 weeks	Micro-CT Histometry	New bone formation (mm^3) Residual graft(mm^3)
Rabbit	8	Cranial defect	8 weeks	Micro-CT Histometry	BV/TV(%) New bone area(mm^2) Residual graft(%)
Rabbit	N/P	Calvarial defect	8 weeks	Histomorphometry	Bone regeneration(%)
Rat	25	Alveolar defect	120 days	Histology	Alveolar regeneration
Rat	6	Calvarial defect	12 weeks	Micro-CT Histology	New bone area(%)BV/TV(%)
Rat	5	Calvarial defect	45 days	Histopathology histomorphometry, Immunohisto-chemistry	Fibrous connective tissue(%) New bone formation(%) Angiogenesis(%)

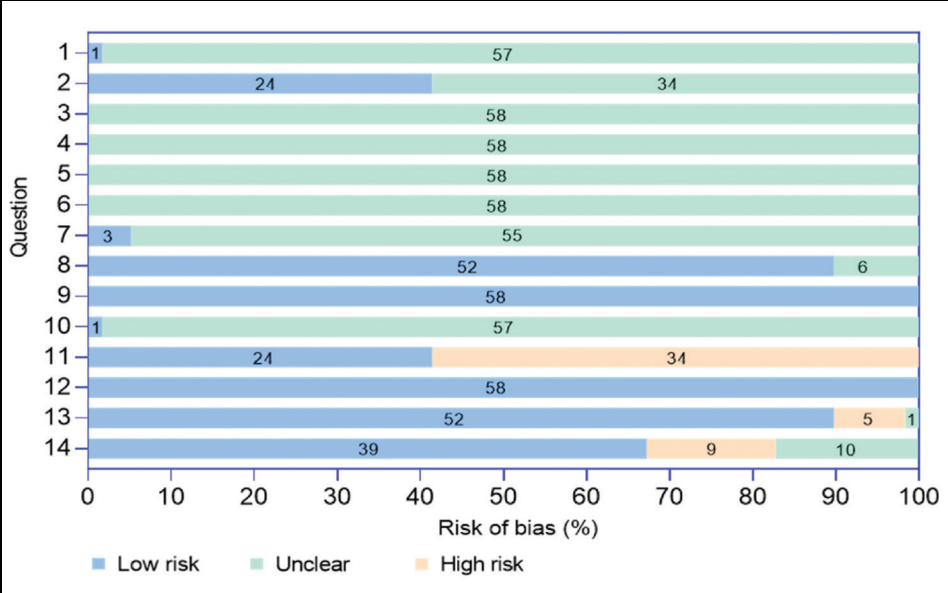
Various types of physicochemical characteristics were recorded from various studies. Pore size and porosity were assessed in 29 studies (N 98 experimental samples) and 24 studies (N = 84), respectively. Particle and granule sizes were reported in 23 studies (N = 79); 20 studies (N = 53) reported the composition of CaP used for their *in vivo* tests. XRD patterns were reported in 34 studies (N = 105) and FT-IR spectra in 12 studies (N = 40), confirming the chemical composition and phases of biomaterials. Surface properties were characterized in 12 studies (N = 42) and mechanical properties in 11 studies (N = 30). Ca/P ratio was reported in 6 studies (N = 18) and some other characteristics were reported less frequently: density (5 studies, N = 9), pore or void volume (4 studies), interconnectivity (2 studies), crystallinity (2 studies) and wall thickness (1 study). Thirty-nine studies reported bone regeneration responses in a quantitative way and the remaining 19 studies reported the responses qualitatively. Consequently, the meta-analysis was conducted on the 39 studies with quantitative outcomes. The outcome variables reported in these studies were distributed as follows: newly formed bone (38 studies, N = 163), residual graft (21 studies, N = 80) and bone-to-material contact (4 studies, N = 31). All the outcomes reported in the included articles are detailed in Table 3.

3.2. Quality assessment and risk of bias

Due to the insufficient number of reported items, the RoB and methodological quality assessments yielded many “unclear” scores, representing an unknown risk of bias (Fig. 3). The individual scores of the RoB tool and the methodological quality indicators of each included study are provided in Supplementary Table S2. Regarding selection bias (Fig. 3; Q1–Q3), the sequence generation process was reported in only one study (2 %; Q1), mentioning the use of software for group randomization (Calvo-Guirado et al., 2015). The randomization method was unclear in all other studies, however, many of them mentioned that the animals were randomly assigned to exposure groups. Baseline similarities were reported more often (41 %; Q2), whereas information about allocation concealment was not reported at all (Q3). Likewise, no study reported on random housing and blinding of caregivers (Q4 and Q5, respectively). As a result, performance bias could not be judged. With respect to detection bias (Q6 and Q7), no study described the random selection of animals for outcome assessment (Q6) and in only three studies (5 %; Q7) the outcome assessor for the histological assessment was reported to be blinded (Yang et al., 2014; Lim et al., 2015 and Mangano et al., 2015). Incomplete outcome data were adequately addressed in most of the included studies (89 %; Q8), resulting in a low attrition risk of bias for these studies. In the assessment of reporting bias (Q9), no study was found to have a high risk of bias. Other potential sources of bias were identified in one study (2 %; Q10) because of simultaneous additional implantations (Ripamonti et al., 2008). In

Figure 3

Results of the risk of bias and methodological quality indicators for all included studies. The items in SYRCLE's Risk of Bias assessment; Questions Q1–Q10 were scored indicating a low or high risk of bias or were scored “unclear” when the item was not reported, resulting in an unknown risk of bias [33]. Questions Q11–Q14 are additional study quality indicators. For Q14, “low risk” indicates a numeric quantitative value was provided in the study, and a “high risk” indicates only qualitative results were reported. The label “unclear” was used for studies showing indicators only in graphical format. For additional information on the questions and indicators, see the methods section and Hooijmans et al. (2014).



addition to the risk of bias, four study quality indicators were used to assess the methodological quality of the studies. In 41 % of the studies, randomization at any level of the experiment was reported (Q11). In all studies, the number of interventions on the animals was reported (Q12) and most of them reported the number of animals to be analyzed at each time point (89 %; Q13). Assessment of the outcomes revealed that 68 % of the studies reported the outcome in a numeric quantitative format, 15 % of them reported qualitatively and the remaining 17 % showed their results using graphs only (Q14).

3.3. Meta-analysis

The meta-analysis was based on N = 164 experimental samples, resulting from 73 animal experiments from 39 scientific articles, each experimental sample yielding at least one outcome

value. NB was missing for one experimental sample (da Silva Brum et al., 2019), while BMC and RG were available for 31 and 80 experimental samples, respectively. Overall brute force mean $\pm$ SD (range) for NB values was 29.4 ± 22.6 % (1.6–95.5 %), for BMC values 45.3 ± 23.8 % (6.2–78.8 %), and for RG values 34.2 ± 17.3 % (4.6–91.5 %). Data for each outcome were widely dispersed which may be explained by the many factors characterizing the in vivo experiments discussed below. The distributions of all study characteristics employed in the meta-analysis are provided in Supplemental Table S3.

3.3.1. ANALYSIS OF NEW BONE (NB)

Mean NB values were weighed by the inverse of their sampling variance, specifically by $w = n/SD^2$. Weights could not be calculated for 32 experimental samples (SD and/or n missing). Instead, they were estimated by regressing $\log(w)$ on $\text{logit}(NB)$, namely $w = \exp\{-2.52 - 0.95 \log[NB/(100-NB)]\}$, as pictured in Supplemental Fig. S1. Indeed, a highly significant relationship was found between the two variables ($r = 0.65$, $p < 0.0001$). Five NB values (all < 5 %) had extremely high weights ($w > 15$) compared to the other w -values (median $\pm$ robust SD of weights equal to 0.24 ± 0.41). These highly influential NB observations, respectively $w = 49.9$ and 15.9 (Xia et al., 2014), 15.9 (Yang et al., 2014), 33.3 and 30.8 (Intapibool et al., 2020), were ultimately discarded (see Supplemental Table S5), leaving 158 data points for the metaanalysis. A weighted generalized linear mixed model (W-GLMM) fitted to NB values alone (without covariates) yielded an estimated NB average of 11.4 %. Thus, when taking into consideration the statistical precision of NB values, the global mean was much lower than the one computed bluntly from all observations.

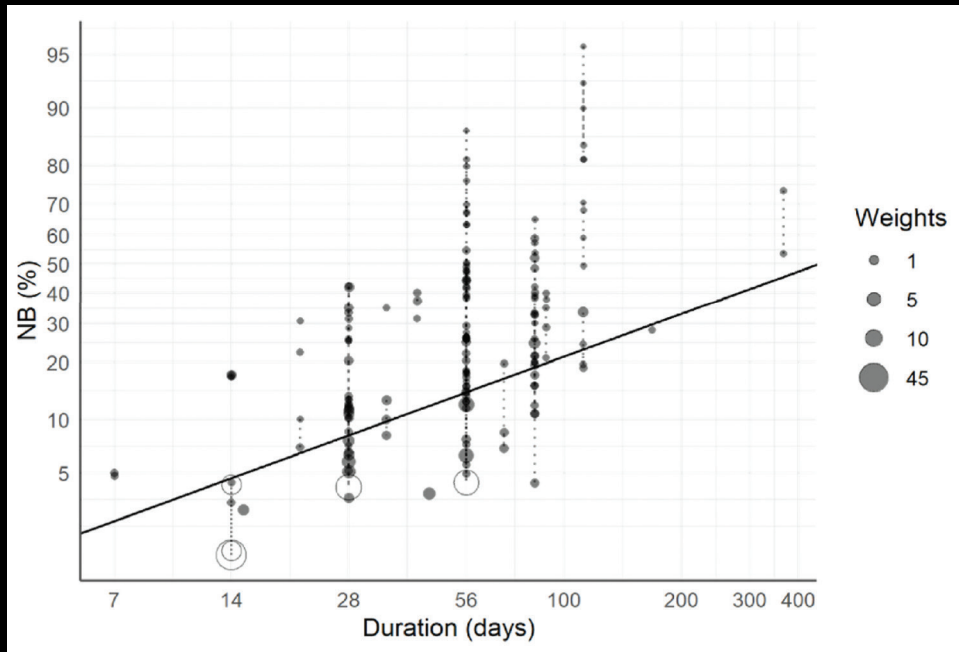
3.3.1.1. Effect of time. As displayed in Fig. 4, a highly significant relationship ($p < 0.0001$) was found between NB and time (i.e. the duration of the experiment expressed in days), and the estimated regression equation was $\text{Logit}(NB) = -5.31 + 0.87 \log(\text{time})$. Thus, for an experiment duration of e.g. 50 days, the expected NB would be 12.9 %.

3.3.1.2. Biomaterials. The W-GLMM model evidenced significant differences of NB between biomaterials ($p = 0.0021$). The estimated NB values were low for HAp, TCP and BCP (8.6 - 13 %) and much higher for OCP-ACP-OA, DCP and CaP (25.7 - 39 %), but the latter were based on smaller sample sizes. Multiple comparisons showed significant difference between TCP and CaP ($p = 0.016$). When restricting the comparison of biomaterials to the first three types (HAp, TCP and BCP), significant differences were still detected ($p = 0.027$), the largest difference being between TCP and BCP (Supplemental Table S4). No interaction was found between biomaterials and time.

3.3.1.3. Experimental features. No significant association was found between NB and the

Figure 4

Relationship between new bone NB (%) and duration of the animal experiment (log-scale) derived by weighted generalized linear mixed modeling. Five NB values with outlying weights (big hollow dots at days 14; 28 and 56) were eliminated from the analysis.



number of implantations ($p = 0.56$), nor between NB and the physical form of the biomaterial ($p = 0.35$). By contrast, NB varied with the animal species used in the experiment ($p = 0.0025$), being 51.7 % for baboons, 10.6 % for dogs, 19.9 % for mini-pigs, 12.4 % for rabbits and 8.0 % for rats. The two latter species were also among the most used in the studies reported. NB values also differed significantly according to type of defect ($p < 0.0001$), being 9.6 % for calvarial defect (the most frequent, $N = 131$) and higher elsewhere but based on lower sample sizes: mandibular ($N = 11$, 10.4 %), alveolar ($N = 6$, 10.8 %), skull ($N = 1$, 10.8 %), sinus augmentation ($N = 2$, 25.4 %), cranial ($N = 4$, 41.1 %), parietal ($N = 4$, 47.2 %), and bilateral maxillary and mandibular defect ($N = 4$, 61.2 %). Estimated NB values (mean $\pm$ SE) with respect to the type of biomaterial, animal species and types of defects are displayed in Supplemental Fig. S2.

3.3.1.4. Biomaterial characteristics. When fitting W-GLMM to NB data according to each biomaterial characteristic, significant effects were observed for macropore size ($N = 71$, slope = -0.0016 ± 0.00031 , $p < 0.0001$), and to a lesser extent to Ca/P ratio ($N = 18$, slope = -5.31 ± 2.33 , $p = 0.041$) and to compressive strength ($N = 20$, slope = -0.11 ± 0.035 , $p = 0.020$) (Fig. 5). The two first effects remained significant when including time in the regression analysis. Of note, no effect was observed for particle size ($N = 79$, $p = 0.85$), porosity ($N = 74$, $p = 0.13$), log-transformed micropore size ($N = 27$, $p = 0.64$), surface area ($N = 32$, $p = 0.18$), and density ($N = 6$, $p = 0.40$).

3.3.2. ANALYSIS OF BONE-TO-MATERIAL CONTACT (BMC)

BMC (%) was reported in only 4 papers involving 9 animal studies yielding a total of $N = 31$ experimental units. Weights could be calculated for all 31 BMC values, but one observation (Intapibool et al., 2020) was discarded due to an abnormally high weight ($w = 20.8$ compared to a median $\pm$ robust SD weight of 0.053 ± 1.74) (see Supplemental Table S5). Thus, 30 BMC values were used in the analysis. A W-GLMM was fitted to BMC values alone (without covariates) yielding an estimated BMC average of 34.1 %, lower than the value reported in Supplemental Table S3 based on all observations.

3.3.2.1. Effect of time. A time effect was evidenced by W-GLMM analysis (slope for log-transformed time equal to 1.53 ± 0.38 , $p = 0.0004$) indicating that BMC values reported in the literature increased with respect to experiment duration (Fig. 6). The regression equation was: $\text{Logit (BMC)} = -6.88 + 1.53 \log(\text{duration})$. Thus, for an experiment duration of e.g. 50 days, the expected BMC would be 29 %.

3.3.2.2. Biomaterials. No difference was found in BMC values according to the three biomaterials utilized ($p = 0.58$), namely HAp ($N = 11$, 12.2 %), BCP ($N = 12$, 32.8 %) and CaP ($N = 8$, 68.6 %), as displayed in Fig. 7a.

Experimental features. A significant association was found between BMC and number of implantations ($p < 0.0001$), especially between 2 and 4 implantations (see Fig. 7b). By contrast, all BMC values were associated with non-rigid structures, and no significant association was detected between BMC and animal species ($p = 0.97$, mini-pig, rabbits, or rats), or between BMC and type of defect ($p = 0.81$ for alveolar or calvarial defects).

3.3.2.3. Biomaterial characteristics. When fitting W-GLMM to BMC data according to each biomaterial characteristic, significant effects were observed for the particle size ($p < 0.0001$, especially between 750 and 1500 μm), the Ca/P ratio ($N = 8$, slope = -12.3 ± 1.27 , $p < 0.0001$)

Figure 5

Relationship between new bone (NB) and biomaterial characteristics (macropore size, Ca/P ratio and compressive strength respectively) derived by weighted generalized linear mixed modelling. NB values with outlying weights (big hollow dots) were eliminated from the analysis. Experimental samples data from the same animals are joined by dashed lines.

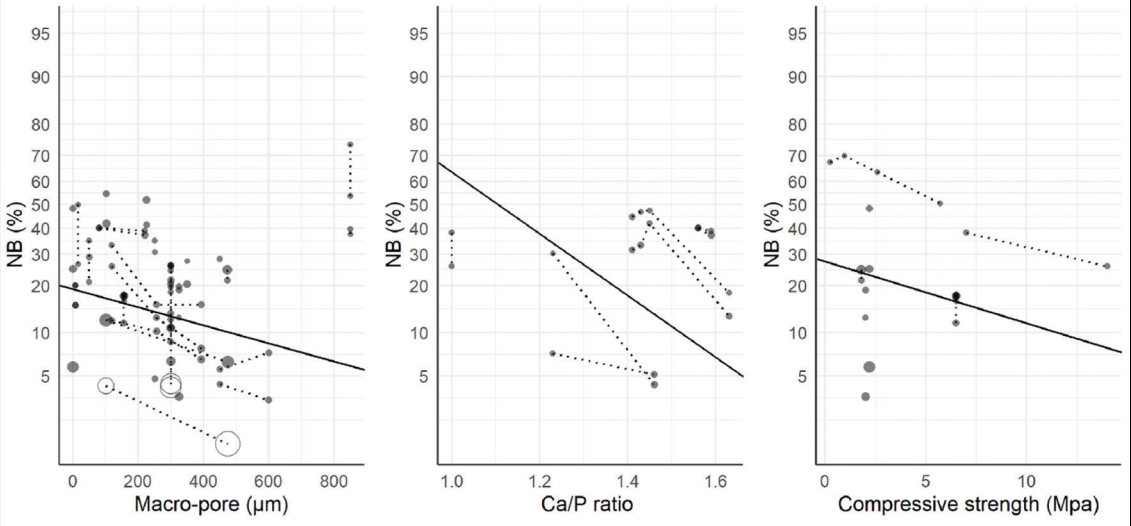
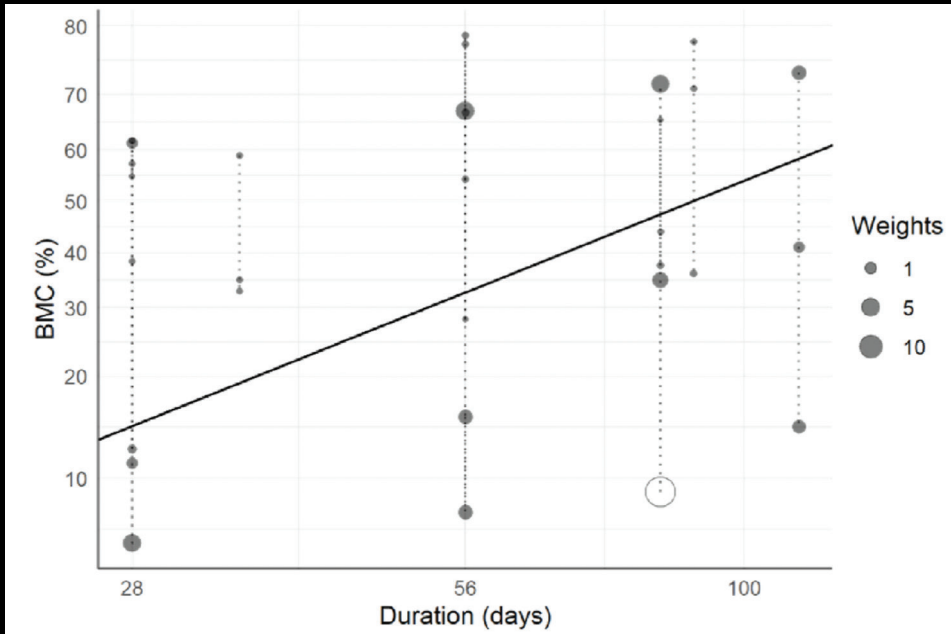


Figure 6

Relationship between BMC (%) and duration of the animal experiment (log scale) derived by weighted generalized linear mixed modelling. One BMC value with outlying weight (big hollow dot at day 84) was eliminated from the analysis.



and to a lesser extent to surface area ($N = 9$, slope = 0.017 ± 0.0063 , $p = 0.042$). Those three effects are illustrated in Fig. 8. Of note, no effect was observed for macropore size ($N = 10$, $p = 0.86$) nor for log-transformed micropore size ($N = 10$, $p = 0.73$); porosity was identical for all observations ($N = 8$, all 80 %) and there were no data on compressive strength nor density.

3.3.3. ANALYSIS OF RESIDUAL GRAFT (RG)

RG (%) was reported in 21 papers involving 43 animal studies yielding a total of $N = 80$ experimental units. Weights could be calculated for all RG values, but two observations (Yang et al., 2014 and Da Silva Brum et al., 2019) (Supplemental Table S5) had to be discarded because of an abnormally high weight ($w = 11.5$ and $w = 84.9$ compared to a median $\pm$ robust SD weight of 0.20 ± 0.41). Thus, 78 RG values were used in the meta-analysis. A weighted generalized linear mixed model (W-GLMM) was fitted to RG values alone (without covariates) yielding an estimated RG average of 20.1 %, much lower than the value reported before based on all observations.

3.3.3.1. Effect of time. No time effect could be evidenced by W-GLMM analysis (slope for log-transformed time is 0.37 ± 0.20 , $p = 0.067$) indicating that RG values were relatively stable with respect to experiment duration.

3.3.3.2. Biomaterials. By contrast (Fig. 9a), significant RG differences were seen between biomaterials ($p = 0.039$); namely HAp ($N = 22$, 27.2 %), TCP ($N = 15$, 28.0 %), BCP ($N = 33$, 15.8 %), DCP ($N = 2$, 50.2 %) and CaP ($N = 8$, 24.7 %). When combining time and biomaterials, both effects became non-significant ($p = 0.050$ for biomaterial and $p = 0.11$ for time).

3.3.3.3. Experimental features. RG differed significantly according to several experimental features: physical form ($p = 0.039$) being 43.8 % for 3D rigid structures ($N = 15$) and 18.9 % for non-rigid structures ($N = 65$) (Fig. 9b); animal species ($p = 0.022$) being 40.9 % in dogs ($N = 3$) compared to 15.6 % in rabbits ($N = 42$), 18.3 % in rats ($N = 17$), 21.4 % in mini-pigs ($N = 14$) and 25.5 % in baboons ($N = 4$) (Fig. 9c). Interestingly, no significant association was detected between RG and the number of implantations ($p = 0.080$) neither between RG nor bone defects ($p = 0.33$).

3.3.3.4. Biomaterial characteristics. For biomaterial characteristics, only macropore size ($N = 44$, slope = -0.0025 ± 0.00060 , $p = 0.0001$), compressive strength ($N = 16$, slope = 0.13 ± 0.024 , $p = 0.0030$) and particle size ($N = 42$, slope = 0.00089 ± 0.00034 , $p = 0.016$) were significantly associated with RG values.

Figure 7

Estimated bone-to-material contact (BMC) values (mean $\pm$ SE), globally (horizontal dotted lines) and with respect to (a) the biomaterials and (b) the number of implantations, as derived by weighted generalized linear mixed modelling.

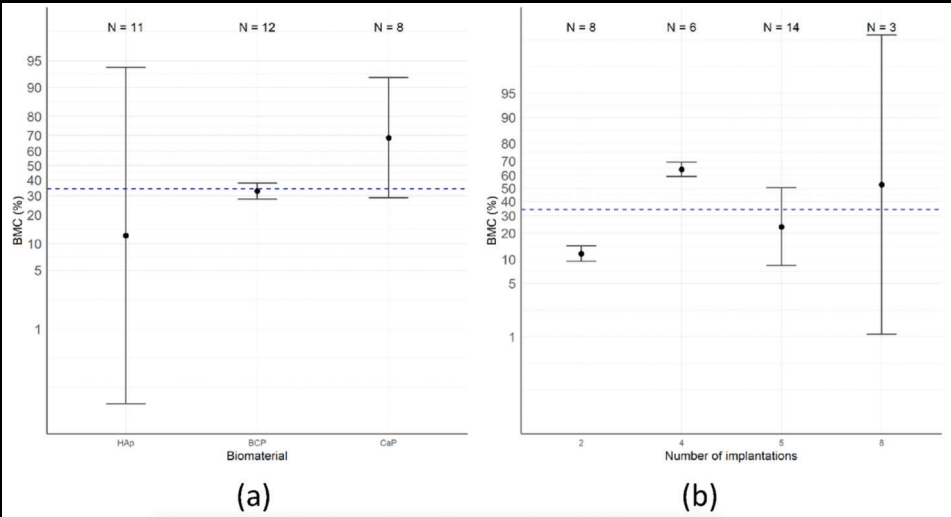


Figure 8

Relationship between bone-material-contact (BMC) and characteristics (particle size, surface area and Ca/P ratio respectively) derived by weighted generalized linear mixed modelling. Experimental samples data from the same animals are joined by dashed lines.

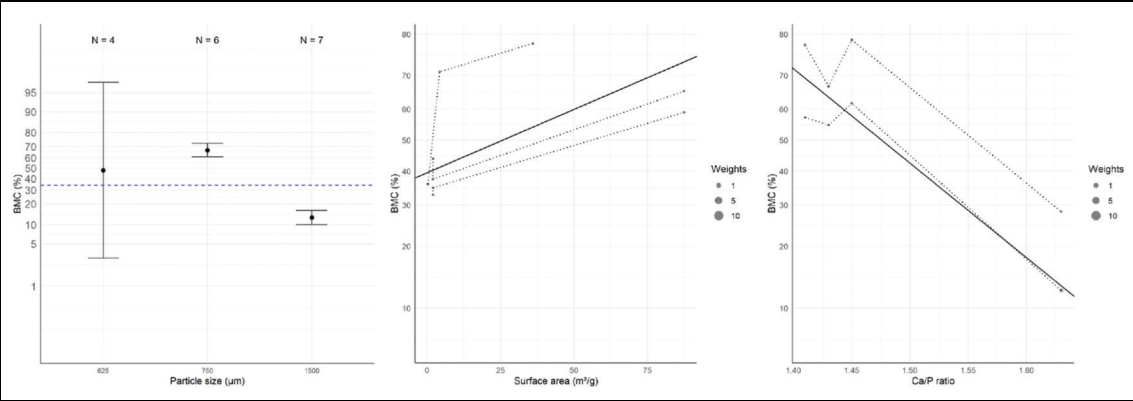
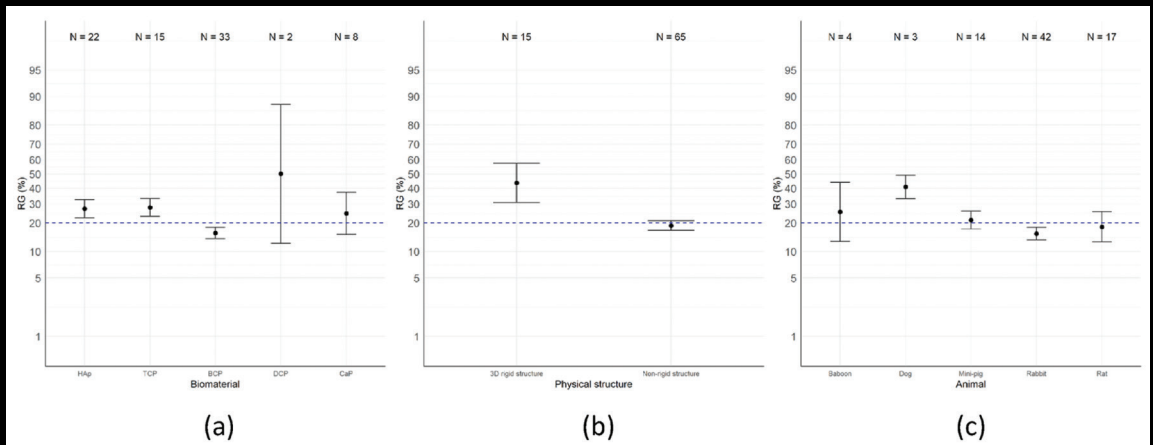


Figure 9

Estimated residual graft (RG) values (mean $\pm$ SE), globally (horizontal dotted lines) and concerning (a) biomaterials, (b) physical structure and (c) animal species, as derived by weighted generalized linear mixed modeling



3.4. Physicochemical characteristics of biomaterials

Spearman correlation analysis of the physicochemical characteristics of biomaterials exhibited perfect correlations between some of the reported characteristics such as macropore size and Ca/P ratio ($r = +1$, $p < 0.0001$), macropore size and density ($r = -1$, $p < 0.0001$), and particle size and compressive strength ($r = +1$, $p < 0.0001$). The surface area was shown to have significant correlations with particle size ($r = +0.58$, $p = 0.028$), porosity ($r = -0.86$, $p = 0.0033$), macropore size ($r = +0.61$, $p = 0.022$), and Ca/P ratio ($r = +0.85$, $p = 0.0020$). A negative correlation was also found between macropore size and compressive strength ($r = -0.61$, $p = 0.022$). It should be noted that correlations were computed on small sample sizes and did not consider repeated observations. Correlations between all characteristics with an indication of the corresponding sample size are provided in Supplemental Table S6.

4. DISCUSSION

The demand for technologies capable of enhancing the biomaterials used to treat CMF bone defects has been on the rise. This quest, accompanied by the advent of functionally tailored, biocompatible, and biodegradable materials, has motivated an enormous research interest in bone TE. As a result, different materials and fabrication methods have been investigated towards this end, leading to a deeper understanding of the geometrical, mechanical, and biological requirements associated with bone scaffolds. CaPs are a highly used scaffold material for bone regeneration because they actively promote osteogenesis. Structural control and optimization of CaPs in multi-scaled level may lead to significant developments in bone TE. The aim of this study was to systematically review the preclinical *in vivo* evidence for the influence of biomaterial characteristics on CMF bone regeneration. Overall, the results of this systematic review and meta-analysis demonstrated that physicochemical characteristics of biomaterials influence bone regenerative responses; moreover, experimental features affect both the percentage of newly formed bone and the volume of the residual graft.

In some prior systematic reviews, the performance of bone tissue engineering scaffolds in *in vivo* animal models has been studied. In 2016, de Misquita et al. concluded that in the studies with calvarial bone defects, CaPs had important osteoinductive effects which increased when combined with other classes of biomaterials [92]. Their study aimed to determine which class of materials had achieved a higher rate of bone neoformation, so no correlation between the materials' properties and their regenerative capacity was developed. Additionally, in 2017, Shanbhag et al. conducted a systematic review of animal models to study how cell-based bone TE strategies enhance bone regeneration and/or biomaterial osteointegration in experimental peri-implant defects, compared to grafting with autogenous bone or only biomaterial scaffolds. They observed that bone regeneration and osseointegration in peri-implant defects were enhanced by the addition of osteogenic cells to biomaterial scaffolds [93].

To the best of our knowledge, the current study is the only systematic review and meta-analysis that has evaluated the effect of physicochemical characteristics of CaP biomaterials on CMF bone regeneration. To do so, we employed a comprehensive search and robust data assimilation procedure. For the 58 studies that were finally selected, a database was built based on the physicochemical characteristics of scaffolds and their bone regeneration capacity. The risk of bias (RoB) was assessed using SYRCLE's RoB tool. Each study was subjected to 10 questions related to the general risk of bias and 4 questions related to the quality of randomization and study item reporting. Meta-analysis was only performed for the 39 studies reporting quantitative outcomes. Our analysis of data from these studies demonstrates the impact of structural characteristics along with the experimental features on CMF bone regeneration.

4.1. Characteristics of animal models

Guidelines for designing preclinical animal models in bone TE have indicated some essential criteria as follows: (1) the models should match the clinical and biological environment and material formulation to the greatest extent possible; (2) they should allow the use of quantifiable parameters to evaluate success and functional performance of regenerated tissues; and (3) they should detect and predict clinically relevant differences in biological performance between the regenerative therapies assessed [94]. In the present study, all these experimental features were attentively observed and their effect on the regeneration outcomes was analyzed. The average time of implant placement within included studies was 83.6 ± 53.5 days. Although some studies with relatively long evaluation times (Ripamonti et al., 2008 and Zhang et al., 2019) were present among the included studies, additional long-term in vivo assessments are needed to ensure preclinical safety. In the present meta-analysis, a significant effect of time on new bone tissue formation was observed. Regarding the animal models, seven different species were used across the included studies and small animals composed most tested animals (66 % rats and 22.7 % rabbits). The remaining 11.3 % were large animals (dog, mini-pig, mouse, sheep and baboon). In general, large animals resemble the clinical conditions in bone TE better than small ones. This is due to similarities to the human bone in terms of composition and density [95]. Nevertheless, rodents are more frequently used for the assessment of bone biomaterials for CMF indications (and in general) as they are less costly and easier in housing. However, they are primarily being used for preliminary screenings followed by verification in large animals that are considered for the last phase of validating a new intervention [95,96]. Moreover, small rodents have a more controlled and clearer genetic background with less variation among individual animals requiring a lower number of experimental values to achieve statistically valid data [96,97]. Rabbits also have the advantages of small size and easy handling while achieving their skeletal maturity by 6 months of age allowing higher volumes of bone tissue to be formed in set-ups for testing periodontal and CMF reconstruction therapies. They also report higher reliability in terms of critical size defects [98].

4.2. Types of bone defects and biomaterials

Various types of defects, both acute and chronic, are studied in the CMF region. In this systematic review, 70.6 % of the included studies assessed cranial defects (calvarial, parietal and skull) and 29.4 % of them tested maxillo-facial ones (mandibular, alveolar, bilateral maxillo-mandibular and sinus augmentation). It should be remembered that the focus point in this review is the impact of physicochemical characteristics on the regeneration process. Therefore, only acute surgically created defects were studied here by excluding the defects with any factors

interfering with bone regeneration. Likewise, for the biomaterial type, only pure CaP-based biomaterials free of any bioactive substances (cells, drugs, growth factors etc.) were included. This gives a better indication of how the physicochemical cues can affect the regeneration phase. There are some systematic reviews in which the implementation of cell-based approaches was studied in pre-clinical periodontal animal models [99–101]. A wide variety of biomaterial physical forms has been used in these bone defects. To avoid confusion due to the different terms used, they were grouped into three main categories. In total, 65.9 % of the biomaterials applied in the meta-analysis were non-rigid structures including granules, particles, spheres, and powder; 28 % were in 3D rigid forms including disc, block, cylinder, cube, sponge and tube; and 6.1 % were putties. Generally, the latter materials consist of a mixture of granules and an aqueous solution. They harden after in situ implantation or injection. The mechanical stability in these biomaterials is provided by the physical entanglement of CaP crystals. Their good handling and injectability extend their field of application, for instance for the treatment of bone fractures by minimally invasive techniques [102].

4.3. Outcome measures

Bone regenerative outcomes in this systematic review showed differences across all experimental circumstances mentioned above. Both NB and BMC increased significantly over time. NB also differed notably among animal species and defect types. BMC was influenced by the number of implantations and physical form of biomaterials. RG was influenced significantly by biomaterial type, animal species and physical form. On the biomaterial type, the NB value for TCP was lower than for HAp and BCP while presenting the lowest RG in the meantime. Undoubtedly, the experimental variables documented across the studies included (such as biomaterial type, implantation time, number of implantations, animal species, defect type and biomaterial's physical form) have demonstrated varying degrees of influence on the regenerative outcomes. It should be noted that BMC analysis allows the evaluation of the osteointegration and osteoconductive behavior of the bone substitutes and can be correlated to surface topography and/or to sintering temperatures used in the fabrication process. The manufacturing method of bone fillers allows for the preservation of a certain surface roughness favoring cell colonization, osteoconductivity and better bone regeneration [71,77]. High osteoconductive properties are important from a regenerative point of view since a tight network between bone and biomaterial plays a key role in implant primary stability and implant survival rates [71,103,104].

4.4. Influence of physicochemical characteristics on regenerative responses

A series of structural properties of CaPs have been analyzed within the regeneration process of included studies. Macropore size exhibited a significant effect on both NB and RG. The pore architecture at the macro and micro level affects the capability of the surrounding tissue to promote cell infiltration, migration, vascularization, and nutrient and oxygen flows [105]. The distribution of macropores in the included studies ranged from 0.4 μm to 850 μm and the ones of the micropores from 4 nm to 150 μm . These ranges are wider than what is usually suggested in the literature for successful bone regeneration (100–600 μm for macropores and micropores bigger than 20 nm) [105,106]. The significant effect of macro and micropore size on both NB and RG in the models developed here emphasizes the crucial role of porosity in the biomaterial's design regardless of the size. However, the issue remains for the optimal architecture of the pores in the structure. The presence of macropores favors osteointegration and angiogenesis while micropores increase the surface area available for protein adsorption [106]. It has been shown that the distribution of micropores on the walls of macropores could play a positive role in favoring the adsorption of proteins [107]. The pore size distribution was shown to influence the degradation performance of the scaffold, as shown here in RG values and, therefore, the biodegradation kinetics could be modulated by varying the pore architecture [108]. Another parameter that has mostly been dealt with in the included studies on non-rigid CaPs is particle size. This quantity varied from 0.5 μm to 1500 μm in the included articles and showed a significant effect on BMC and RG. Particle size was shown to be a highly determinant feature for the bioactivity of granulated CaPs that could be influenced by altering available surface area, roughness, mechanical performance and the resorbability of scaffolds [105].

Among the surface properties within the included studies, extensive attention has been directed toward the surface area, with ten studies spanning a wide range from 0.24 to 87.5 (m^2/g). This parameter plays a vital role in cellular attachment, offering ample anchoring sites for cell expansion and proliferation [109]. High surface areas exhibit a remarkable capability to absorb large quantities of biomolecules, thereby promoting extracellular responses [110]. The meta-analysis conducted herein elucidated a significant effect of surface area on BMC, emphasizing its crucial role in influencing cellular behaviors and material performance.

Mechanical properties were barely investigated in the included studies, and this is mainly because the CMF defects studied here were low or non-load bearing and did not require biomaterials matching the natural mechanical properties. Of note, the compressive modulus of sections of bone from the skull containing both cortical and cancellous regions is on the order of 0.36–5.6 GPa, depending on the direction of the load. These mechanical characteristics can be difficult to achieve with porous materials. CaPs may more easily approach these characteristics

(though with lower toughness than native bone); however, it seems that such a high degree of mechanical competence is not necessarily needed (or even desirable) for bone repair [111]. Compressive strength is the most reported mechanical property in the literature as it is the dominant type of loading on BTE scaffolds in the body [112]. The compressive strength of various CaPs in the included studies varies from 1.8 MPa to 14 MPa and showed a significant effect on both NB and RG values. Nonetheless, mechanical properties can come with concerns in the CMF area when the implanted biomaterial does not fit the defect space. In the stiffer biomaterials, stress-shielding at the bone-material interface can cause greater bone loss and in the softer biomaterials, instability and limited motion can cause further damage [111]. One approach to overcome this issue is to employ in situ implantable CaPs that harden within the defect space [113,114].

Another physicochemical parameter that exhibited an impact on the regenerative responses of CaP biomaterials in the included studies is the Ca/P ratio. Overall, this ratio for CaP biomaterials used in biological applications can vary, depending on stoichiometry, from 1 to 1.67 for dicalcium phosphate (DCPA) and pure HAp, respectively. The values of almost the whole of this range have been extracted from the studies and then fed to the meta-analysis. The analysis showed a significant effect of the Ca/P ratio on NB and, to a lesser extent, on BMC. CaP biomaterials offer fast or slow degradation rates depending on their Ca/P molar ratio. β -TCP with a Ca/P ratio of 1.5 has been classified as a resorbable material while sintered HAp with a Ca/P ratio of 1.67 may show slower resorbability. Nevertheless, HAp becomes more resorbable in the presence of certain impurities, such as water-soluble binders and biodegradable polymers [115,116]. The other approach to increase HAp's resorbability is to reduce its grain size to nano-scale [117]. Hence, Ca/P ratio is a compositional parameter that is quite tunable for various functions. In some situations, fast resorbing materials with low mechanical properties are needed, therefore a lower ratio of Ca/P such as TCP is preferred. In other cases, materials in a higher ratio of Ca/P with more stability and slower resorption characteristics (e.g., HAp) or a proper combination of both (TCP and HAp) are recommended.

Although the meta-analysis is able to extract quantified conclusions from the reviewed literature, the results do not permit going beyond establishing the general trends of influence of specific physicochemical parameters. The range of tested parameters are constrained by the choices made in the individual studies and often do not cover the entire parameter space. Furthermore, finding optimal properties also depends on the context of use for the biomaterial, requiring dedicated studies for each condition (cfr section on clinical relevance). For example, in a previously published study, we analyzed a series of in-house experiments performed in a rabbit sinus augmentation model, allowing us to use a different empirical model linking physicochemical biomaterial characteristics to intra-oral bone formation [118].

4.5. Limitations

One of the limitations of this systematic review is that only four studies have reported BMC. Additional data on this aspect would provide a more robust indication of how the experimental features and material characteristics interact with BMC. Likewise, some physicochemical characterizations were only scarcely investigated in the included studies, which limits interpreting their impact on the regeneration process. Mechanical properties were less thoroughly considered in the included studies, even though facial bones such as maxillary and mandible act as load-bearing bones for the dental region [119]. The mechanics of biomaterials are highly important for CMF defect treatments and future biomaterial developments should focus on this. These considerations should not only include the stiffness difference between biomaterial and bone (potentially leading to stress shielding) but also the fixation of the biomaterial (avoiding movement) as well as ease of application. Other characteristics mostly neglected in the studies were surface energy, crystallinity and surface roughness. Surface roughness has a key role in protein adsorption [106], requiring further investigation in particular when the CaP biomaterials are implanted with biomacromolecules. Though there are studies that study these factors in detail, in this review we only selected studies that include an *in vivo* assessment. As a consequence, many of the selected studies had a focus that was not necessarily on the quantification of all the aforementioned physicochemical characteristics that have been shown to be. Finally, the meta-analysis was limited to the studies reporting the responses in a quantitative manner, so the studies using qualitative outputs were excluded from the analysis.

Despite searching for relevant studies in major electronic databases, we cannot rule out the possibility of missing studies. It should be noted as well that there was substantial heterogeneity across studies for all outcomes assessed, hence the results should be interpreted with caution. The study of applications combining scaffolds with cells, drugs or growth factors would be useful to elucidate how the structural elements should be tailored in their presence.

4.6. Clinical relevance

Systematic reviews and meta-analyses of animal studies can be useful for designing future clinical trials, capturing the underlying heterogeneity between studies and treatment effects, and improving the methodological quality of future studies [33]. The biomaterial design and selection for clinically successful CMF bone regeneration is fully dependent on the treatment strategy's requirements, emphasizing the role of biomaterial composition and physicochemical properties [120]. To test the efficacy and predictability of bone substitute materials, preclinical *in vivo* studies in clinically relevant animal models are a fundamental step in translational research [97,98,121]. Multiple experimental and preclinical studies demonstrate impressive results on bone

neoformation in CaP scaffolds with a wide variety of characteristics [122,123], however, not all of them have exhibited similar efficacy in humans [124]. Developing statistical models for the meta-analysis of such studies, as done here for the preclinical models, will provide an indication of the most influencing elements to be considered in the early phases of biomaterial design. Future prospective randomized clinical trials should then be performed to identify clear indications in humans and to demonstrate clinical outcomes. The inherent limitation of preclinical modeling should always be considered while interpreting the results of the meta-analyses. In the context of *in vivo* experiments, the defects are usually surgically created and well controlled, with intact surrounding soft tissues and generally uncompromised blood supply, and most often in young and healthy animals. These conditions are often not present in clinical scenarios which may lead to the overestimation of clinical performance [94]. Therefore, the experimental defect models should be standardized in future preclinical investigations to better represent the clinical settings [125]. Moreover, in synthesizing the preclinical data encompassing regions from the cranium to the midface, mandibular bone, and the dento-alveolus, it is crucial to recognize inherent physiological, anatomical, and biomechanical distinctions within the human body. While the animal studies incorporated in this review predominantly focused on evaluating the regenerative potential of substitute materials, they did not sufficiently address the specific pre requisite characteristics crucial to each distinct region (e.g., the use of animal cranial defect for human mandibular bone applications). Consequently, the outcomes were combined in a unified meta-analysis in the present study. Moving forward, it is imperative for future clinical studies to acknowledge and address these differences. The selection of biomaterials and their requisite characteristics should be tailored to meet the unique specifications of each region. Therefore, a strategic approach involving separate sub-analyses is warranted, ensuring a more refined understanding of the regenerative outcomes and paving the way for more targeted and effective clinical interventions.

5. CONCLUSION

This systematic review provides tangible evidence in support of the influence of fundamental and structural properties on the bone regenerative capacity of CaP biomaterials. Our findings from the included studies showed that macropore size, Ca/P ratio and compressive strength are influencing factors for newly formed bone in the regeneration process. Additionally, the contact between biomaterials and their surrounding tissue was notably influenced by particle size, Ca/P ratio and surface area. Regarding biodegradability, macropore size and compressive strength seemed to determine the amount of residual graft. Furthermore, the experimental

setting is strongly determining the scaffold's performance. These observations may be useful in designing the next generation of bone scaffolds to target higher rates of regeneration. Additional investigations of CaP scaffolds in standardized preclinical studies could provide more insight into their fundamental features, promoting their application on a more regular basis and improving clinical outcomes.

CRedit authorship contribution statement

Ehsan Sadeghian Dehkord: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization.

Bruno De Carvalho: Writing – review & editing, Investigation, Data curation.

Marie Ernst: Writing – review & editing, Methodology, Investigation, Formal analysis.

Adelin Albert: Writing – review & editing, Software, Methodology, Investigation, Formal analysis.

France Lambert: Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Liesbet Geris reports financial support was provided by European Research Council under the European Union's Horizon Europe programme/ERC Consolidator Grant No. 101088919. Liesbet Geris and France Lambert report financial support was provided by Walloon Region via the BIOWIN-BIOPTOS and Win2Wal-B2Bone projects. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

This is a systematic review. All data is in the public domain and appropriately referenced.

Acknowledgments

This project received funding from the European Research Council under the European Union's Horizon Europe programme/ERC Consolidator Grant No. 101088919, and from the Walloon Region via the BIOWIN-BIOPTOS and Win2Wal-B2Bone project. We are grateful to Dr. Bert Avau (Centre for Evidence-Based Practice (CEBaP)) for his skillful support and assistance. We also acknowledge the kind support from Dr. Krizia Tuand and Dr. Kristel Paque (KU Leuven Bibliotheken).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mtbio.2024.101100>.

REFERENCES

- 1 Y. Kinoshita, H. Maeda, **Recent developments of functional scaffolds for cranio-maxillofacial bone tissue engineering applications**, Sci. World J. 2013 (2013) 1–21, <https://doi.org/10.1155/2013/863157>.
- 2 V. Fitzpatrick, Z. Martín-Moldes, A. Deck, R. Torres-Sanchez, A. Valat, D. Cairns, C. Li, D.L. Kaplan, **Functionalized 3D-printed silk-hydroxyapatite scaffolds for enhanced bone regeneration with innervation and vascularization**, Biomaterials 276 (2021) 120995, <https://doi.org/10.1016/j.biomaterials.2021.120995>.
- 3 T.L. Aghaloo, P.K. Moy, **Which hard tissue augmentation techniques are the most successful in furnishing bony support for implant placement?** Int. J. Oral Maxillofac. Implants 22 (Suppl) (2007) 49–70.
- 4 S. Jepsen, F. Schwarz, L. Cordaro, J. Derks, C.H.F. Hammerle, L.J. Heitz-Mayfield, F. Hernández-Alfaro, H.J.A. Meijer, N. Naenni, A. Ortiz-Vigón, B. Pjetursson, G. M. Raghoobar, S. Renvert, I. Rocchietta, M. Roccuzzo, I. Sanz-Sánchez, M. Simion, C. Tomasi, L. Trombelli, I. Urban, **Regeneration of alveolar ridge defects. Consensus report of group 4 of the 15th European workshop on periodontology on bone regeneration**, J. Clin. Periodontol. (2019), <https://doi.org/10.1111/jcpe.13121>.
- 5 M. Sanz, C. Dahlin, D. Apatzidou, Z. Artzi, D. Bozic, E. Calciolari, H. De Bruyn, H. Dommisch, N. Donos, P. Eickholz, J.E. Ellingsen, H.J. Haugen, D. Herrera, F. Lambert, P. Layrolle, E. Montero, K. Mustafa, O. Omar, H. Schliephake, **Biomaterials and regenerative technologies used in bone regeneration in the craniomaxillofacial region: consensus report of group 2 of the 15th European Workshop on Periodontology on Bone Regeneration**, J. Clin. Periodontol. 46 (2019) 82–91, <https://doi.org/10.1111/jcpe.13123>.
- 6 V. Chappuis, L. Rahman, R. Buser, S.F.M. Janner, U.C. Belser, D. Buser, **Effectiveness of contour augmentation with guided bone regeneration: 10-year results**, J. Dent. Res. 97 (2018) 266–274, <https://doi.org/10.1177/0022034517737755>.

- 7 G.F. Rogers, A.K. Greene, **Autogenous bone graft: basic science and clinical implications**, *J. Craniofac. Surg.* 23(2012)323–327, <https://doi.org/10.1097/SCS.0b013e318241dcba>.
- 8 A. Sakkas, F. Wilde, M. Heufelder, K. Winter, A. Schramm, **Autogenous bone grafts in oral implantology—is it still a “gold standard”? A consecutive review of 279 patients with 456 clinical procedures**, *Int. J. Implant Dent.* 3 (2017) 23, <https://doi.org/10.1186/s40729-017-0084-4>.
- 9 A. Ho-Shui-Ling, J. Bolander, L.E. Rustom, A.W. Johnson, F.P. Luyten, C. Picart, **Bone regeneration strategies: engineered scaffolds, bioactive molecules and stem cells current stage and future perspectives**, *Biomaterials* 180 (2018) 143–162, <https://doi.org/10.1016/j.biomaterials.2018.07.017>.
- 10 M. Esposito, M.G. Grusovin, J. Rees, D. Karasoulos, P. Felice, R. Alissa, H. V. Worthington, P. Coulthard, **Interventions for replacing missing teeth: augmentation procedures of the maxillary sinus**, in: *The Cochrane Collaboration (Ed.)*, Cochrane Database Syst. Rev., John Wiley & Sons, Ltd, Chichester, UK, 2010, p. CD008397, <https://doi.org/10.1002/14651858.CD008397>.
- 11 L.N. Melek, **Tissue engineering in oral and maxillofacial reconstruction**, *Tanta Dent. J.* 12 (2015) 211–223, <https://doi.org/10.1016/j.tdj.2015.05.003>.
- 12 A. Turri, I. Elgali, F. Vazirisani, A. Johansson, L. Emanuelsson, C. Dahlin, P. Thomsen, O. Omar, **Guided bone regeneration is promoted by the molecular events in the membrane compartment**, *Biomaterials* 84 (2016) 167–183, <https://doi.org/10.1016/j.biomaterials.2016.01.034>.
- 13 B. Trajkovski, M. Jaunich, W.-D. Müller, F. Beuer, G.-G. Zafiropoulos, A. Houshmand, **Hydrophilicity, viscoelastic, and physicochemical properties variations in dental bone grafting substitutes**, *Materials* 11 (2018) 215, <https://doi.org/10.3390/ma11020215>.
- 14 A. Kolk, J. Handschel, W. Drescher, D. Rothamel, F. Kloss, M. Blessmann, M. Heiland, K.-D. Wolff, R. Smeets, **Current trends and future perspectives of bone substitute materials – from space holders to innovative biomaterials**, *J. Cranio-Maxillofac. Surgery (St Louis)* 40 (2012) 706–718, <https://doi.org/10.1016/j.jcms.2012.01.002>.

- 15 M.Á. Brennan, D.S. Monahan, B. Brulin, S. Gallinetti, P. Humbert, C. Tringides, C. Canal, M.P. Ginebra, P. Layrolle, **Biomimetic versus sintered macroporous calcium phosphate scaffolds enhanced bone regeneration and human mesenchymal stromal cell engraftment in calvarial defects**, *Acta Biomater.* 135 (2021) 689–704, <https://doi.org/10.1016/j.actbio.2021.09.007>.
- 16 G. Thirivikraman, A. Athirasala, C. Twohig, S.K. Boda, L.E. Bertassoni, **Biomaterials for craniofacial bone regeneration**, *Dent. Clin.* 61 (2017) 835–856, <https://doi.org/10.1016/j.cden.2017.06.003>.
- 17 J. Yun, J. Lee, C.W. Ha, S.J. Park, S. Kim, K. Koo, Y. Seol, Y. Lee, **The effect of 3-D printed polylactic acid scaffold with and without hyaluronic acid on bone regeneration**, *J. Periodontol.* 93 (2022) 1072–1082, <https://doi.org/10.1002/JPER.21-0428>.
- 18 D.B. Bhuiyan, J.C. Middleton, R. Tannenbaum, T.M. Wick, **Mechanical properties and osteogenic potential of hydroxyapatite-PLGA-collagen biomaterial for bone regeneration**, *J. Biomater. Sci. Polym. Ed.* 27 (2016) 1139–1154, <https://doi.org/10.1080/09205063.2016.1184121>.
- 19 X. Chen, H. Fan, X. Deng, L. Wu, T. Yi, L. Gu, C. Zhou, Y. Fan, X. Zhang, **Scaffold structural microenvironmental cues to guide tissue regeneration in bone tissue applications**, *Nanomaterials* 8 (2018) 960, <https://doi.org/10.3390/nano8110960>.
- 20 H. Zhang, H. Zhang, Y. Xiong, L. Dong, X. Li, **Development of hierarchical porous bioceramic scaffolds with controlled micro/nano surface topography for accelerating bone regeneration**, *Mater. Sci. Eng. C* 130 (2021) 112437, <https://doi.org/10.1016/j.msec.2021.112437>.
- 21 H.J. Haugen, S.P. Lyngstadaas, F. Rossi, G. Perale, **Bone grafts: which is the ideal biomaterial?** *J. Clin. Periodontol.* 46 (2019) 92–102, <https://doi.org/10.1111/jcpe.13058>.
- 22 B. Sigusch, S. Kranz, A.C. Von Hohenberg, S. Wehle, A. Guellmar, D. Steen, A. Berg, U. Rabe, M. Heyder, M. Reise, **Histological and histomorphometric evaluation of implanted photodynamic active biomaterials for periodontal bone regeneration in an animal study**, *Int. J. Mol. Sci.* 24 (2023) 6200, <https://doi.org/10.3390/ijms24076200>.

- 23 S.P. Pilipchuk, A.B. Plonka, A. Monje, A.D. Taut, A. Lanis, B. Kang, W. V. Giannobile, **Tissue engineering for bone regeneration and osseointegration in the oral cavity**, *Dent. Mater.* 31 (2015) 317–338, <https://doi.org/10.1016/j.dental.2015.01.006>.
- 24 R. Zhao, R. Yang, P.R. Cooper, Z. Khurshid, A. Shavandi, J. Ratnayake, **Bone grafts and substitutes in dentistry: a review of current trends and developments**, *Molecules* 26 (2021) 3007, <https://doi.org/10.3390/molecules26103007>.
- 25 R.A. Alsaifi, H.A. Mitwalli, A.A. Balhaddad, M.D. Weir, H.H.K. Xu, M.A.S. Melo, **Regenerating craniofacial dental defects with calcium phosphate cement scaffolds: current status and innovative scope review**, *Front. Dent. Med.* 2 (2021) 743065, <https://doi.org/10.3389/fdmed.2021.743065>.
- 26 X. Gui, B. Zhang, Z. Su, Z. Zhou, Z. Dong, P. Feng, C. Fan, M. Liu, Q. Kong, C. Zhou, Y. Fan, X. Zhang, **3D-printed degradable hydroxyapatite bioactive ceramics for skull regeneration**, *MedComm – Biomater. Appl.* 2 (2023) e41, <https://doi.org/10.1002/mba2.41>.
- 27 W. Wang, K.W.K. Yeung, **Bone grafts and biomaterials substitutes for bone defect repair: a review**, *Bioact. Mater.* 2 (2017) 224–247, <https://doi.org/10.1016/j.bioactmat.2017.05.007>.
- 28 A. Bruyas, F. Lou, A.M. Stahl, M. Gardner, W. Maloney, S. Goodman, Y.P. Yang, **Systematic characterization of 3D-printed PCL/ β -TCP scaffolds for biomedical devices and bone tissue engineering: influence of composition and porosity**, *J. Mater. Res.* 33 (2018) 1948–1959, <https://doi.org/10.1557/jmr.2018.112>.
- 29 R.S. Valtanen, Y.P. Yang, G.C. Gurtner, W.J. Maloney, D.W. Lowenberg, **Synthetic and Bone tissue engineering graft substitutes: what is the future?** *Injury* 52 (2021) S72–S77, <https://doi.org/10.1016/j.injury.2020.07.040>.
- 30 M. Rahmati, E.A. Silva, J.E. Reseland, C.A. Heyward, H.J. Haugen, **Biological responses to physicochemical properties of biomaterial surface**, *Chem. Soc. Rev.* 49 (2020) 5178–5224, <https://doi.org/10.1039/D0CS00103A>.

- 31 C.R. Hooijmans, K.E. Wever, R.B. de Vries, **SYRCLE's starting guide for systematic reviews of preclinical animal interventions studies**, <https://www.radboudumc.nl/getmedia/4b1cbcb8-d9b6-45d5-b9fe-c92e43ab1dd4/SYRCLE-starting-guide-tool.aspx>, 2016.
- 32 W.M. Bramer, D. Giustini, G.B. De Jonge, L. Holland, T. Bekhuis, **De-duplication of database search results for systematic reviews in EndNote**, *J. Med. Libr. Assoc.* 104 (2016), <https://doi.org/10.5195/jmla.2016.24>.
- 33 C.R. Hooijmans, M.M. Rovers, R.B. de Vries, M. Leenaars, M. Ritskes-Hoitinga, M. W. Langendam, **SYRCLE's risk of bias tool for animal studies**, *BMC Med. Res. Methodol.* 14 (2014) 43, <https://doi.org/10.1186/1471-2288-14-43>.
- 34 W.I. Abdel-Fattah, W.G. Osiris, S.S. Mohamed, M.R. Khalil, **Reconstruction of resected mandibles using a hydroxyapatite veterinary bone graft**, *Biomaterials* 15 (1994) 609–614, [https://doi.org/10.1016/0142-9612\(94\)90211-9](https://doi.org/10.1016/0142-9612(94)90211-9).
- 35 H. Denissen, W. Kalk, E. Van Beek, C. Löwik, S. Papapoulos, A. Van Den Hooff, **Composites of hydroxyapatite and bisphosphonate: properties and alveolar bone response**, *J. Mater. Sci. Mater. Med.* 6 (1995) 35–40, <https://doi.org/10.1007/BF00121245>.
- 36 T. Dutta Roy, J.L. Simon, J.L. Ricci, E.D. Rekow, V.P. Thompson, J.R. Parsons, **Performance of hydroxyapatite bone repair scaffolds created via threedimensional fabrication techniques**, *J. Biomed. Mater. Res.* 67A (2003) 1228–1237, <https://doi.org/10.1002/jbm.a.20034>.
- 37 K.B. Fleckenstein, M.F. Cuenin, M.E. Peacock, M.A. Billman, G.D. Swiec, T. B. Buxton, B.B. Singh, J.C. McPherson, **Effect of a hydroxyapatite tricalcium phosphate alloplast on osseous repair in the rat calvarium**, *J. Periodontol.* 77 (2006) 39–45, <https://doi.org/10.1902/jop.2006.77.1.39>.
- 38 O. Suzuki, S. Kamakura, T. Katagiri, M. Nakamura, B. Zhao, Y. Honda, R. Kamijo, **Bone formation enhanced by implanted octacalcium phosphate involving conversion into Ca-deficient hydroxyapatite**, *Biomaterials* 27 (2006) 2671–2681, <https://doi.org/10.1016/j.biomaterials.2005.12.004>.

- 39 J.L. Simon, S. Michna, J.A. Lewis, E.D. Rekow, V.P. Thompson, J.E. Smay, A. Yampolsky, J.R. Parsons, J.L. Ricci, **In vivo bone response to 3D periodic hydroxyapatite scaffolds assembled by direct ink writing**, *J. Biomed. Mater. Res.* 83A (2007) 747–758, <https://doi.org/10.1002/jbm.a.31329>.
- 40 J.-W. Park, S.-R. Bae, J.-Y. Suh, D.-H. Lee, S.-H. Kim, H. Kim, C.-S. Lee, **Evaluation of bone healing with eggshell-derived bone graft substitutes in rat calvaria: a pilot study**, *J. Biomed. Mater. Res.* 87A (2008) 203–214, <https://doi.org/10.1002/jbm.a.31768>.
- 41 U. Ripamonti, P.W. Richter, R.W.N. Nilen, L. Renton, **The induction of bone formation by smart biphasic hydroxyapatite tricalcium phosphate biomimetic matrices in the non-human primate *Papio ursinus***, *J. Cell Mol. Med.* 12 (2008) 2609–2621, <https://doi.org/10.1111/j.1582-4934.2008.00312.x>.
- 42 S. Xu, K. Lin, Z. Wang, J. Chang, L. Wang, J. Lu, C. Ning, **Reconstruction of calvarial defect of rabbits using porous calcium silicate bioactive ceramics**, *Biomaterials* 29 (2008) 2588–2596, <https://doi.org/10.1016/j.biomaterials.2008.03.013>.
- 43 M.R. Appleford, S. Oh, N. Oh, J.L. Ong, **In vivo study on hydroxyapatite scaffolds with trabecular architecture for bone repair**, *J. Biomed. Mater. Res.* 89A (2009) 1019–1027, <https://doi.org/10.1002/jbm.a.32049>.
- 44 M. Hirota, Y. Matsui, N. Mizuki, T. Kishi, K. Watanuki, T. Ozawa, T. Fukui, S. Shoji, M. Adachi, Y. Monden, T. Iwai, I. Tohnai, **Combination with allogenic bone reduces early absorption of .BETA.-tricalcium phosphate (.BETA.-TCP) and enhances the role as a bone regeneration scaffold. Experimental animal study in rat mandibular bone defects**, *Dent. Mater. J.* 28 (2009) 153–161, <https://doi.org/10.4012/dmj.28.153>.
- 45 K. Takahashi, **Effect of new bone substitute materials consisting of collagen and tricalcium phosphate**, *Bull. Tokyo Dent. Coll.* 50 (2009) 1–11, <https://doi.org/10.2209/tdcpublication.50.1>.
- 46 S. Wang, Z. Zhang, J. Zhao, X. Zhang, X. Sun, L. Xia, Q. Chang, D. Ye, X. Jiang, **Vertical alveolar ridge augmentation with β -tricalcium phosphate and autologous osteoblasts in canine mandible**, *Biomaterials* 30 (2009) 2489–2498, <https://doi.org/10.1016/j.biomaterials.2008.12.067>.

- 47 J. Yao, X. Li, C. Bao, H. Fan, X. Zhang, Z. Chen, **A novel technique to reconstruct a boxlike bone defect in the mandible and support dental implants with In vivo tissue-engineered bone**, J. Biomed. Mater. Res. B Appl. Biomater. 91B (2009) 805–812, <https://doi.org/10.1002/jbm.b.31459>.
- 48 J.-S. Park, S.-J. Hong, H.-Y. Kim, H.-S. Yu, Y.I. Lee, C.-H. Kim, S.-J. Kwak, J.-H. Jang, J.K. Hyun, H.-W. Kim, **Evacuated calcium phosphate spherical microcarriers for bone regeneration**, Tissue Eng. 16 (2010) 1681–1691, <https://doi.org/10.1089/ten.tea.2009.0624>.
- 49 J.-W. Park, E.-S. Kim, J.-H. Jang, J.-Y. Suh, K.-B. Park, T. Hanawa, **Healing of rabbit calvarial bone defects using biphasic calcium phosphate ceramics made of submicron-sized grains with a hierarchical pore structure**, Clin. Oral Implants Res. 21 (2010) 268–276, <https://doi.org/10.1111/j.1600-0501.2009.01846.x>.
- 50 C.-L. Hung, J.-C. Yang, W.-J. Chang, C.-Y. Hu, Y.-H. Lin, C.-H. Huang, C.-C. Chen, S.-Y. Lee, N.-C. Teng, **In vivo graft performance of an improved bone substitute composed of poor crystalline hydroxyapatite based biphasic calcium phosphate**, Dent. Mater. J. 30 (2011) 21–28, <https://doi.org/10.4012/dmj.2010-060>.
- 51 R. de Oliveira Lomelino, I.I. Castro-Silva, A.B.R. Linhares, G.G. Alves, S.R. de Albuquerque Santos, V.S. Gameiro, A.M. Rossi, J.M. Granjeiro, **The association of human primary bone cells with biphasic calcium phosphate (β TCP/HA 70:30) granules increases bone repair**, J. Mater. Sci. Mater. Med. 23 (2012) 781–788, <https://doi.org/10.1007/s10856-011-4530-1>.
- 52 R.J. Klijn, J.J.J.P. van den Beucken, R.P. Felix Lanoo, G. Veldhuis, S.C. Leeuwenburgh, J.G.C. Wolke, G.J. Meijer, J.A. Jansen, **Three different strategies to obtain porous calcium phosphate cements: comparison of performance in a rat skull bone augmentation model**, Tissue Eng. 18 (2012) 1171–1182, <https://doi.org/10.1089/ten.tea.2011.0444>.
- 53 S.-W. Lee, S.-G. Kim, C. Balázs, W.-S. Chae, H.-O. Lee, **Comparative study of hydroxyapatite from eggshells and synthetic hydroxyapatite for bone regeneration**, Oral Surg. Oral Med. Oral Pathol. Oral Radiol. 113 (2012) 348–355, <https://doi.org/10.1016/j.tripleo.2011.03.033>.

- 54 J.S. Cho, H.-S. Kim, S.-H. Um, S.-H. Rhee, **Preparation of a novel anorganic bovine bone xenograft with enhanced bioactivity and osteoconductivity**, J. Biomed. Mater. Res. B Appl. Biomater. 101B (2013) 855–869, <https://doi.org/10.1002/jbm.b.32890>.
- 55 J.H. Lee, M.Y. Ryu, H.-R. Baek, K.M. Lee, J.-H. Seo, H.-K. Lee, **Fabrication and evaluation of porous beta-tricalcium phosphate/hydroxyapatite (60/40) composite as a bone graft extender using rat calvarial bone defect model**, Sci. World J. 2013 (2013) 1–9, <https://doi.org/10.1155/2013/481789>.
- 56 J.H. Lee, M.Y. Ryu, H.-R. Baek, K.M. Lee, J.-H. Seo, H.-K. Lee, H.-S. Ryu, **Effects of porous beta-tricalcium phosphate-based ceramics used as an E. coli-derived rhBMP-2 carrier for bone regeneration**, J. Mater. Sci. Mater. Med. 24 (2013) 2117–2127, <https://doi.org/10.1007/s10856-013-4967-5>.
- 57 C.H. Jang, Y.B. Cho, C.H. Choi, Y.S. Jang, W.-K. Jung, J.K. Lee, **Comparision of osteoconductivity of biologic and artificial synthetic hydroxyapatite in experimental mastoid obliteration**, Acta Otolaryngol. 134 (2014) 255–259, <https://doi.org/10.3109/00016489.2013.859397>.
- 58 K. Kobayashi, T. Anada, T. Handa, N. Kanda, M. Yoshinari, T. Takahashi, O. Suzuki, **Osteoconductive property of a mechanical mixture of octacalcium phosphate and amorphous calcium phosphate**, ACS Appl. Mater. Interfaces 6 (2014) 22602–22611, <https://doi.org/10.1021/am5067139>.
- 59 S.-W. Lee, C. Bal#azsi, K. Balázsi, D. Seo, H.S. Kim, C.-H. Kim, S.-G. Kim, **Comparative Study of hydroxyapatite prepared from seashells and eggshells as a bone graft material**, Tissue Eng. Regen. Med. 11 (2014) 113–120, <https://doi.org/10.1007/s13770-014-0056-1>.
- 60 L. Xia, K. Lin, X. Jiang, B. Fang, Y. Xu, J. Liu, D. Zeng, M. Zhang, X. Zhang, J. Chang, Z. Zhang, **Effect of nano-structured bioceramic surface on osteogenic differentiation of adipose derived stem cells**, Biomaterials 35 (2014) 8514–8527, <https://doi.org/10.1016/j.biomaterials.2014.06.028>.

- 61 C. Yang, O. Unursaikhan, J.-S. Lee, U.-W. Jung, C.-S. Kim, S.-H. Choi, **Osteoconductivity and biodegradation of synthetic bone substitutes with different tricalcium phosphate contents in rabbits: bone Regeneration of Synthetic Bone Graft Material**, J. Biomed. Mater. Res. B Appl. Biomater. 102 (2014) 80–88, <https://doi.org/10.1002/jbm.b.32984>.
- 62 M.D. Calasans-Maia, B.R. de Melo, A.T.N.N. Alves, R.F. de B. Resende, R.S. Louro, S.C. Sartoretto, J.M. Granjeiro, G.G. Alves, **Cytocompatibility and biocompatibility of nanostructured carbonated hydroxyapatite spheres for bone repair**, J. Appl. Oral Sci. 23 (2015) 599–608, <https://doi.org/10.1590/1678-775720150122>.
- 63 J.L. Calvo-Guirado, M. Garces, R.A. Delgado-Ruiz, M.P. Ramirez Fernandez, E. Ferres-Amat, G.E. Romanos, **Biphasic β -TCP mixed with silicon increases bone formation in critical site defects in rabbit calvaria**, Clin. Oral Implants Res. 26 (2015) 891–897, <https://doi.org/10.1111/clr.12413>.
- 64 R. Khan, L. Witek, M. Breit, D. Colon, N. Tovar, M.N. Janal, R. Jimbo, P. G. Coelho, **Bone regenerative potential of modified biphasic graft materials**, Implant Dent. (2015), <https://doi.org/10.1097/ID.0000000000000220>. Publish Ahead of Print.
- 65 H.-C. Lim, K.-H. Song, H. You, J.-S. Lee, U.-W. Jung, S.-Y. Kim, S.-H. Choi, **Effectiveness of biphasic calcium phosphate block bone substitutes processed using a modified extrusion method in rabbit calvarial defects**, J. Periodontal Implant Sci. 45 (2015) 46, <https://doi.org/10.5051/jpis.2015.45.2.46>.
- 66 A. Manchón, M. Alkhraisat, C. Rueda-Rodriguez, J. Torres, J.C. Prados-Frutos, A. Ewald, U. Gbureck, J. Cabrejos-Azama, A. Rodriguez-González, E. López-Cabarcos, **Silicon calcium phosphate ceramic as novel biomaterial to simulate the bone regenerative properties of autologous bone: silicon Calcium Phosphate Ceramic as Novel Biomaterial**, J. Biomed. Mater. Res. 103 (2015) 479–488, <https://doi.org/10.1002/jbm.a.35196>.
- 67 A. Manchón, M. Hamdan Alkhraisat, C. Rueda-Rodriguez, J.C. Prados-Frutos, J. Torres, J. Lucas-Aparicio, A. Ewald, U. Gbureck, E. López-Cabarcos, **A new iron calcium phosphate material to improve the osteoconductive properties of a biodegradable ceramic: a study in rabbit calvaria**, Biomed. Mater. 10 (2015) 055012, <https://doi.org/10.1088/1748-6041/10/5/055012>.

- 68 C. Mangano, B. Barboni, L. Valbonetti, P. Berardinelli, A. Martelli, A. Muttini, R. Bedini, S. Tetè, A. Piattelli, M. Mattioli, **In vivo behavior of a custom-made 3D synthetic bone substitute in sinus augmentation procedures in sheep**, *J. Oral Implantol.* 41 (2015) 240–250, <https://doi.org/10.1563/AAID-JOI-D-13-00053>.
- 69 D. Lee, Y. Pai, S. Chang, D. Kim, **Microstructure, physical properties, and bone regeneration effect of the nano-sized β -tricalcium phosphate granules**, *Mater. Sci. Eng., C* 58 (2016) 971–976, <https://doi.org/10.1016/j.msec.2015.09.047>.
- 70 Z. Sheikh, J. Drager, Y.L. Zhang, M.-N. Abdallah, F. Tamimi, J. Barralet, **Controlling bone graft substitute microstructure to improve bone augmentation**, *Adv. Healthcare Mater.* 5 (2016) 1646–1655, <https://doi.org/10.1002/adhm.201600052>.
- 71 F. Lambert, M. Bacevic, P. Layrolle, P. Schüpbach, P. Drion, E. Rompen, **Impact of biomaterial microtopography on bone regeneration: comparison of three hydroxyapatites**, *Clin. Oral Implants Res.* 28 (2017) e201–e207, <https://doi.org/10.1111/clr.12986>.
- 72 J. Diao, J. OuYang, T. Deng, X. Liu, Y. Feng, N. Zhao, C. Mao, Y. Wang, **3D-Plotted beta-tricalcium phosphate scaffolds with smaller pore sizes improve in vivo bone regeneration and biomechanical properties in a critical-sized calvarial defect rat model**, *Adv. Healthcare Mater.* 7 (2018) 1800441, <https://doi.org/10.1002/adhm.201800441>.
- 73 Y.-P. Fan, J.-F. Lu, A.-T. Xu, F.-M. He, **Physiochemical characterization and biological effect of anorganic bovine bone matrix and organic-containing bovine bone matrix in comparison with Bio-Oss in rabbits**, *J. Biomater. Appl.* 33 (2018) 566–575, <https://doi.org/10.1177/0885328218804926>.
- 74 J. Yao, H. Chen, Q. Gao, Z. Liang, **Evaluation of osteoinductive calcium phosphate ceramics repairing alveolar cleft defects in dog model**, *Bio Med. Mater. Eng.* 29 (2018) 229–240, <https://doi.org/10.3233/BME-171725>.
- 75 K. Madhumathi, Y. Rubaiya, M. Doble, R. Venkateswari, T.S. Sampath Kumar, **Antibacterial, anti-inflammatory, and bone-regenerative dual-drug-loaded calcium phosphate nanocarriers—in vitro and in vivo studies**, *Drug Deliv. Transl. Res.* 8 (2018) 1066–1077, <https://doi.org/10.1007/s13346-018-0532-6>.

- 76 I. da Silva Brum, J.J. de Carvalho, J.L. da Silva Pires, M.A.A. de Carvalho, L.B. F. dos Santos, C.N. Elias, **Nanosized hydroxyapatite and β -tricalcium phosphate composite: physico-chemical, cytotoxicity, morphological properties and in vivo trial**, *Sci. Rep.* 9 (2019) 19602, <https://doi.org/10.1038/s41598-019-56124-4>.
- 77 B. De Carvalho, E. Rompen, G. Lecloux, P. Schupbach, E. Dory, J.-F. Art, F. Lambert, **Effect of sintering on in vivo biological performance of chemically deproteinized bovine hydroxyapatite**, *Materials* 12 (2019) 3946, <https://doi.org/10.3390/ma12233946>.
- 78 M. Park, G. Lee, K. Ryu, W. Lim, **Improvement of bone formation in rats with calvarial defects by modulating the pore size of tricalcium phosphate scaffolds**, *Biotechnol. Bioproc. Eng.* 24 (2019) 885–892, <https://doi.org/10.1007/s12257-019-0248-6>.
- 79 B. Zhang, H. Sun, L. Wu, L. Ma, F. Xing, Q. Kong, Y. Fan, C. Zhou, X. Zhang, **3D printing of calcium phosphate bioceramic with tailored biodegradation rate for skull bone tissue reconstruction**, *Bio-Des. Man (Lond.)* 2 (2019) 161–171, <https://doi.org/10.1007/s42242-019-00046-7>.
- 80 C. Hung, E. Fu, H. Chiu, H. Liang, **Bone formation following sinus grafting with an alloplastic biphasic calcium phosphate in Lanyu Taiwanese mini-pigs**, *J. Periodontol.* 91 (2020) 93–101, <https://doi.org/10.1002/JPER.17-0748>.
- 81 H. Chi, G. Chen, Y. He, G. Chen, H. Tu, X. Liu, J. Yan, X. Wang, **3D-HA scaffold functionalized by extracellular matrix of stem cells promotes bone repair**, *Int. J. NANOMEDICINE* 15 (2020) 5825–5838, <https://doi.org/10.2147/IJN.S259678>.
- 82 M.B. Jensen, C. Slots, N. Ditzel, S. Kolstrup, M. Kassem, T. Thygesen, M. Ø. Andersen, **Treating mouse skull defects with 3D-printed fatty acid and tricalcium phosphate implants**, *J. Tissue Eng. Regen. Med.* 14 (2020) 1858–1868, <https://doi.org/10.1002/term.3146>.
- 83 P. Intapibool, N. Monmaturapoj, K. Nampuksa, K. Thongkorn, P. Khongkhunthian, **Bone regeneration of a polymeric sponge technique-Alloplastic bone substitute materials compared with a commercial synthetic bone material (MBCP + TM technology): a histomorphometric study in porcine skull**, *Clin. Exp. Dent. Res.* 7 (2021) 726–738, <https://doi.org/10.1002/cre2.394>.

- 84 H. de J. Kiyochi Junior, A.G. Candido, T.G.M. Bonadio, J.A. da Cruz, M.L. Baesso, W.R. Weinand, L. Hernandez, **In vivo evaluation of interactions between biphasic calcium phosphate (BCP)-niobium pentoxide (Nb₂O₅) nanocomposite and tissues using a rat critical-size calvarial defect model**, J. Mater. Sci. Mater. Med. 31 (2020) 71, <https://doi.org/10.1007/s10856-020-06414-5>.
- 85 J.M. de Oliveira Junior, P.G. Montagner, R.C. Carrijo, E.F. Martinez, **Physical characterization of biphasic bioceramic materials with different granulation sizes and their influence on bone repair and inflammation in rat calvaria**, Sci. Rep. 11 (2021) 4484, <https://doi.org/10.1038/s41598-021-84033-y>.
- 86 S.-J. Seo, Y.-G. Kim, **Improved bone regeneration using collagen-coated biphasic calcium phosphate with high porosity in a rabbit calvarial model**, Biomed. Mater. Bristol Engl. 16 (2020) 015012, <https://doi.org/10.1088/1748-605X/abb1fc>.
- 87 F. Wang, H. Nakata, X. Sun, W.M. Maung, M. Sato, K. Kon, K. Ozeki, R. Ikumi, S. Kasugai, S. Kuroda, **A novel hydroxyapatite fiber material for the regeneration of critical-sized rabbit calvaria defects**, Dent. Mater. J. 40 (2021) 964–971, <https://doi.org/10.4012/dmj.2020-327>.
- 88 C. Ghayor, I. Bhattacharya, J. Guerrero, M. Oezcan, F. Weber, **3D-Printed HA-based scaffolds for bone regeneration: microporosity, osteoconduction and osteoclastic resorption**, Materials 15 (2022), <https://doi.org/10.3390/ma15041433>.
- 89 C. da Silva, D. Scatolim, A. Queiroz, F. de Almeida, E. Volnistem, M. Baesso, W. Weinand, L. Hernandez, **Alveolar regeneration induced by calcium phosphate ceramics after dental avulsion: study in young rats**, Mater. Chem. Phys. 295 (2023), <https://doi.org/10.1016/j.matchemphys.2022.127082>.
- 90 Y. Wu, Q. Cao, Y. Wang, Y. Liu, X. Xu, P. Liu, X. Li, X. Zhu, X. Zhang, **Optimized fabrication of DLP-based 3D printing calcium phosphate ceramics with highprecision and low-defect to induce calvarial defect regeneration**, Mater. Des. 233 (2023), <https://doi.org/10.1016/j.matdes.2023.112230>.

- 91 P. Youseflee, F.E. Ranjbar, M. Bahraminasab, A. Ghanbari, D.R. Faradonbeh, S. Arab, A. Alizadeh, V.T. Nooshabadi, **Exosome loaded hydroxyapatite (HA) scaffold promotes bone regeneration in calvarial defect: an in vivo study**, *Cell Tissue Bank.* 24 (2023) 389–400, <https://doi.org/10.1007/s10561-022-10042-4>.
- 92 M.R.D.O.F. de Misquita, R. Bentini, F. Goncalves, **The performance of bone tissue engineering scaffolds in in vivo animal models: a systematic review**, *J. Biomater. Appl.* 31 (2016) 625–636, <https://doi.org/10.1177/0885328216656476>.
- 93 S. Shanbhag, N. Pandis, K. Mustafa, J.R. Nyengaard, A. Stavropoulos, **Alveolar bone tissue engineering in critical-size defects of experimental animal models: a systematic review and meta-analysis: alveolar bone tissue engineering in criticalsize defects**, *J. Tissue Eng. Regen. Med.* 11 (2017) 2935–2949, <https://doi.org/10.1002/term.2198>.
- 94 G.F. Muschler, V.P. Raut, T.E. Patterson, J.C. Wenke, J.O. Hollinger, **The design and use of animal models for translational research in bone tissue engineering and regenerative medicine**, *Tissue Eng., Part B* 16 (2010) 123–145, <https://doi.org/10.1089/ten.teb.2009.0658>.
- 95 J. Aerssens, S. Boonen, G. Lowet, J. Dequeker, **Interspecies differences in bone composition, density, and quality: potential implications for in vivo bone research**, *Endocrinology* 139 (1998) 663–670, <https://doi.org/10.1210/endo.139.2.5751>.
- 96 A. Stavropoulos, A. Sculean, D.D. Bosshardt, D. Buser, B. Klinge, **Pre-clinical in vivo models for the screening of bone biomaterials for oral/craniofacial indications: focus on small-animal models**, *Periodontol* 68 (2015) (2000) 55–65, <https://doi.org/10.1111/prd.12065>.
- 97 G. Pellegrini, Y.J. Seol, R. Gruber, W.V. Giannobile, **Pre-clinical models for oral and periodontal reconstructive therapies**, *J. Dent. Res.* 88 (2009) 1065–1076, <https://doi.org/10.1177/0022034509349748>.
- 98 S. Stübinger, M. Dard, **The rabbit as experimental model for research in implant dentistry and related tissue regeneration**, *J. Invest. Surg.* 26 (2013) 266–282, <https://doi.org/10.3109/08941939.2013.778922>.

- 99 X.-Z. Yan, F. Yang, J.A. Jansen, R.B.M. de Vries, J.J.J.P. van den Beucken, **Cellbased approaches in periodontal regeneration: a systematic review and metaanalysis of periodontal defect models in animal experimental work**, *Tissue Eng., Part B* 21 (2015) 411–426, <https://doi.org/10.1089/ten.teb.2015.0049>.
- 100 S.A. Tassi, N.Z. Sergio, M.Y.O. Misawa, C.C. Villar, **Efficacy of stem cells on periodontal regeneration: systematic review of pre-clinical studies**, *J. Periodontal. Res.* 52 (2017) 793–812, <https://doi.org/10.1111/jre.12455>.
- 101 S. Portron, A. Soueidan, A.-C. Marsden, M. Rakic, C. Verner, P. Weiss, Z. Badran, X. Struillou, **Periodontal regenerative medicine using mesenchymal stem cells and biomaterials: a systematic review of pre-clinical studies**, *Dent. Mater. J.* 38 (2019) 867–883, <https://doi.org/10.4012/dmj.2018-315>.
- 102 M. Böhner, **Resorbable biomaterials as bone graft substitutes**, *Mater. Today Off.* 13 (2010) 24–30, [https://doi.org/10.1016/S1369-7021\(10\)70014-6](https://doi.org/10.1016/S1369-7021(10)70014-6).
- 103 L. Molly, H. Vandromme, M. Quirynen, E. Schepers, J.L. Adams, D. van Steenberghe, **Bone Formation following implantation of bone biomaterials into extraction sites**, *J. Periodontol.* 79 (2008) 1108–1115, <https://doi.org/10.1902/jop.2008.070476>.
- 104 F. Lambert, A. Leonard, G. Lecloux, S. Sourice, P. Pilet, E. Rompen, **A comparison of three calcium phosphate-based space fillers in sinus elevation: a study in rabbits**, *Int. J. Oral Maxillofac. Implants* 28 (2013) 393–402, <https://doi.org/10.11607/jomi.2332>.
- 105 M. Tavoni, M. Dapporto, A. Tampieri, S. Sprio, **Bioactive calcium phosphatebased composites for bone regeneration**, *J. Compos. Sci.* 5 (2021) 227, <https://doi.org/10.3390/jcs5090227>.
- 106 S. Samavedi, A.R. Whittington, A.S. Goldstein, **Calcium phosphate ceramics in bone tissue engineering: a review of properties and their influence on cell behavior**, *Acta Biomater.* 9 (2013) 8037–8045, <https://doi.org/10.1016/j.actbio.2013.06.014>.
- 107 X.D. Zhu, H.J. Zhang, H.S. Fan, W. Li, X.D. Zhang, **Effect of phase composition and microstructure of calcium phosphate ceramic particles on protein adsorption**, *Acta Biomater.* 6 (2010) 1536–1541, <https://doi.org/10.1016/j.actbio.2009.10.032>.

- 108** H. Ma, C. Feng, J. Chang, C. Wu, **3D-printed bioceramic scaffolds: from bone tissue engineering to tumor therapy**, *Acta Biomater.* 79 (2018) 37–59, <https://doi.org/10.1016/j.actbio.2018.08.026>.
- 109** A.A. Vu, D.A. Burke, A. Bandyopadhyay, S. Bose, **Effects of surface area and topography on 3D printed tricalcium phosphate scaffolds for bone grafting applications**, *Addit. Manuf.* 39 (2021) 101870, <https://doi.org/10.1016/j.addma.2021.101870>.
- 110** C. Gao, S. Peng, P. Feng, C. Shuai, **Bone biomaterials and interactions with stem cells**, *Bone Res* 5 (2017) 17059, <https://doi.org/10.1038/boneres.2017.59>.
- 111** M.J. Dewey, B.A.C. Harley, **Biomaterial design strategies to address obstacles in craniomaxillofacial bone repair**, *RSC Adv.* 11 (2021) 17809–17827, <https://doi.org/10.1039/D1RA02557K>.
- 112** Q. Liu, W.F. Lu, W. Zhai, **Toward stronger robocast calcium phosphate scaffolds for bone tissue engineering: a mini-review and meta-analysis**, *Biomater. Adv.* 134 (2022) 112578, <https://doi.org/10.1016/j.msec.2021.112578>.
- 113** T. Koizumi, Y. Komuro, **Influence of mixing blood with calcium phosphate bone paste on hardening**, *J. Craniofac. Surg.* 22 (2011) 329–332, <https://doi.org/10.1097/SCS.0b013e3181f7df69>.
- 114** W. Thein-Han, J. Liu, H.H.K. Xu, **Calcium phosphate cement with biofunctional agents and stem cell seeding for dental and craniofacial bone repair**, *Dent. Mater.* 28 (2012) 1059–1070, <https://doi.org/10.1016/j.dental.2012.06.009>.
- 115** B.T. Smith, M. Santoro, E.C. Grosfeld, S.R. Shah, J.J.J.P. Van Den Beucken, J.A. Jansen, A.G. Mikos, **Incorporation of fast dissolving glucose porogens into an injectable calcium phosphate cement for bone tissue engineering**, *Acta Biomater.* 50 (2017) 68–77, <https://doi.org/10.1016/j.actbio.2016.12.024>.

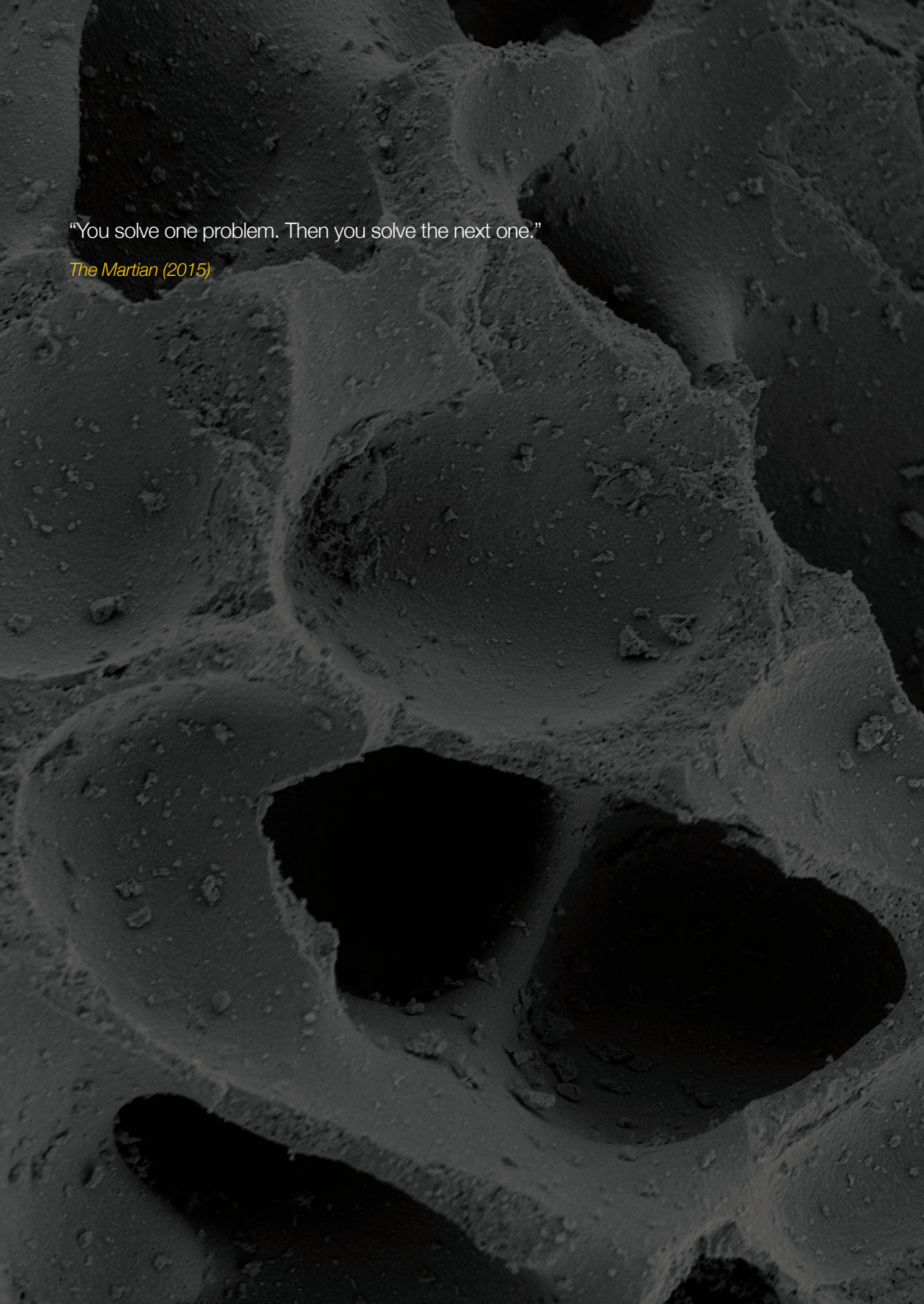
- 116 C.I.A. Van Houdt, R.S. Preethanath, B.A.J.A. Van Oirschot, P.H.W. Zwarts, D.J. O. Ulrich, S. Anil, J.A. Jansen, J.J.J.P. Van Den Beucken, **Toward accelerated bone regeneration by altering poly(d, l -lactic- co -glycolic) acid porogen content in calcium phosphate cement: bone Regeneration by Altering PLGA Porogen in Calcium Phosphate Cement**, J. Biomed. Mater. Res. 104 (2016) 483–492, <https://doi.org/10.1002/jbm.a.35584>.
- 117 A.-M. Yousefi, **A review of calcium phosphate cements and acrylic bone cements as injectable materials for bone repair and implant fixation**, J. Appl. Biomater. Funct. Mater. 17 (2019) 228080001987259, <https://doi.org/10.1177/2280800019872594>.
- 118 E. Sadeghian Dehkord, G. Kerckhofs, P. Compère, F. Lambert, L. Geris, **An empirical model linking physico-chemical biomaterial characteristics to intra-oral bone formation**, J. Funct. Biomater. 14 (2023) 388, <https://doi.org/10.3390/jfb14070388>.
- 119 B. Gaihre, S. Uswatta, A. Jayasuriya, **Reconstruction of craniomaxillofacial bone defects using tissue-engineering strategies with injectable and non-injectable scaffolds**, J. Funct. Biomater. 8 (2017) 49, <https://doi.org/10.3390/jfb8040049>.
- 120 P. Janicki, G. Schmidmaier, **What should be the characteristics of the ideal bone graft substitute? Combining scaffolds with growth factors and/or stem cells**, Injury 42 (2011) S77–S81, <https://doi.org/10.1016/j.injury.2011.06.014>.
- 121 G. Brunello, S. Panda, L. Schiavon, S. Sivoilella, L. Biasetto, M. Del Fabbro, **The impact of bioceramic scaffolds on bone regeneration in preclinical in vivo studies: a systematic review**, Materials 13 (2020) 1500, <https://doi.org/10.3390/ma13071500>.
- 122 C. Hayashi, A. Kinoshita, S. Oda, K. Mizutani, Y. Shirakata, I. Ishikawa, **Injectable calcium phosphate bone cement provides favorable space and a scaffold for periodontal regeneration in dogs**, J. Periodontol. 77 (2006) 940–946, <https://doi.org/10.1902/jop.2006.050283>.
- 123 Y. Shirakata, T. Yoshimoto, H. Goto, Y. Yonamine, H. Kadomatsu, M. Miyamoto, T. Nakamura, C. Hayashi, Y. Izumi, **Favorable periodontal healing of 1-wall infrabony defects after application of calcium phosphate cement wall alone or in combination with enamel matrix derivative: a pilot study with canine mandibles**, J. Periodontol. 78 (2007) 889–898, <https://doi.org/10.1902/jop.2007.060353>.

124

Y. Shirakata, T. Setoguchi, M. Machigashira, T. Matsuyama, Y. Furuichi, K. Hasegawa, T. Yoshimoto, Y. Izumi, **Comparison of injectable calcium phosphate bone cement grafting and open flap debridement in periodontal intrabony defects: a randomized clinical trial**, J. Periodontol. 79 (2008) 25–32, <https://doi.org/10.1902/jop.2008.070141>.

125

J. Lammens, M. Maréchal, H. Delport, L. Geris, H. Oppermann, S. Vukicevic, F. P. Luyten, **A cell-based combination product for the repair of large bone defects**, Bone 138 (2020) 115511, <https://doi.org/10.1016/j.bone.2020.115511>.



"You solve one problem. Then you solve the next one."

The Martian (2015)

2

MANUFACTURING
AND PROOF
OF CONCEPT

Effect of Sintering on In Vivo Biological Performance of Chemically Deproteinized Bovine Hydroxyapatite

Bruno De Carvalho, Eric Rompen, Geoffrey Lecloux, Peter Schupbach, Emilie Dory, Jean-François Art and France Lambert

Keywords:

bone regeneration - hydroxyapatite - osteoconduction - surface microtopography - sintering

ABSTRACT

The influence of the manufacturing process on physicochemical properties and biological performance of xenogenic biomaterials has been extensively studied, but its quantification on bone-to-material contact remains poorly investigated. The aim of this study was to investigate the effect of different heat treatments of an experimental chemically-deproteinized bovine hydroxyapatite in vivo in terms of new bone formation and osteoconductivity. Protein-free hydroxyapatite from bovine origin was produced under sub-critical conditions and then either sintered at 820 °C or 1200 °C. Structural and morphological properties were assessed by scanning electron microscopy (SEM), measurement of surface area and X-ray diffractometry (XRD). The materials were then implanted in standardized alveolar bone defects in minipigs and histomorphometric evaluations were performed using non-decalcified sections. Marked topographical differences were observed by SEM analysis. As the sintering temperature of the experimental material increased, the surface area significantly decreased while crystallite size increased. In vivo samples showed that the highly sintered BHA presented a significantly lower percentage of newly formed bone than the unheated one ($p = 0.009$). In addition, the percentage of bone-to-material contact (BMC) was significantly lowered in the highly sintered group when compared to the unsintered ($p = 0.01$) and 820 °C sintered ($p = 0.02$) groups. Non-sintered or sintered at 820 °C BHA seems to maintain a certain surface roughness allowing better bone regeneration and BMC. On the contrary, sintering of BHA at 1200 °C has an effect on its morphological and structural characteristics and significantly modify its biological performance (osteoconductivity) and crystallinity.

1. INTRODUCTION

Alveolar bone regeneration procedures are often performed to treat bone defects in order to replace missing teeth with dental implants in a multitude of situations in dentistry [1,2]. If autogenous bone grafts are still considered as the “gold standard” due to their osteogenic properties, their use is associated with several limitations, including limited bone supply, high resorption rates [3] and morbidity at the donor site, affecting patient compliance [4,5]. Therefore, biomaterials have become an alternative increasingly used in oral surgery, implantology and periodontology as bone void fillers allowing bone in-growth, acceleration of bone remodeling and osteoconductive structural guidance [6,7].

Calcium-phosphate-based (CaP) materials have received a lot of attention due to their chemical similarity to bone, but also due to their excellent biocompatibility [8,9]. Natural and synthetic CaP materials such as hydroxyapatite (HA) and tricalcium-phosphate (TCP) are the most commonly used biomaterials for alveolar bone regeneration [10]. TCP materials are osteoconductive and support bone growth by delivering calcium and phosphate ions in situ [11,12]; however, their fast biodegradation rate may not be the most appropriate to maintain bone volume overtime, which is indicated in cases of implant site development [12–14]. By contrast, HA which is also highly osteoconductive and similar to the mineral composition of natural bone, has the advantage of being more stable in body fluids [10,14,15]. Natural HA from bovine origin (bovine hydroxyapatites, BHA) remains the most used material in oral surgery, compared to synthetic HA, exhibiting physicochemical properties close to natural bone such as surface structure, morphology and microporosities (1–50 μm) [16,17]. According to some authors, these characteristics may influence the osteoconductivity of the materials and, therefore, their clinical performance [18–21]. Additionally, their slowly resorbable feature is of great interest in the field of dentistry and maxillofacial surgery, as one of the goals of bone regenerative treatment is to maintain the volume over time [22].

For safety reasons, xenogenic materials of bovine origin need to be completely deproteinized through thermal or chemical strategies, to eliminate the risk of transmission of bacteria, virus or prion particles [21,23–25]. One easy and cheap process to deproteinize BHA is sintering at temperatures higher than 1000 °C, ensuring the complete removal of the organic compounds and improving mechanical properties (density, grainsize, compressive, flexural and torsional strength) [26,27]. Commercially available xenogenic HA are manufactured with sintering temperatures up to 1300 °C. Despite the effect of sintering temperature on the physical and chemical characteristics of HA being well documented [16, 26, 28–32], its quantification on bone to material contact is poorly investigated. Thus, it is essential for the clinician to know what is the biological impact of this particular manufacturing process. Sintering hydroxyapatite has a

significant impact on the surface properties; involving surface roughness, on microstructure including the crystallinity and microporosity [33–35] and may, therefore, influence the *in vivo* biological performance of the biomaterial [20,36,37]. Structural characteristics, such as microporosity, surface roughness, and specific surface area were shown to be of great importance in promoting protein adsorption on the material surface and capillary effect, and would consequently influence cell adhesion, proliferation, and bone tissue growth [17,38–42]. The physical characteristics seem to be a key feature, significantly influencing bone-regeneration outcomes [39].

The primary objective of this study was to investigate the effect of different sintering temperatures of chemically-deproteinized bovine hydroxyapatite *in vivo* in terms of biological performance (new bone formation and osteoconductivity). The secondary objective was to correlate the *in vivo* results with the structural and morphological characterization of the studied biomaterials.

2. MATERIALS AND METHODS

2.1. Experimental Biomaterials

Three experimental hydroxyapatite xenografts from bovine origin were tested in the present study for physicochemical characterization and biological evaluations. The three groups were chemically deproteinized, in which the complete elimination of organic residues was performed under sub-critical conditions and a continuous flow of a basic solution following the patented extraction method developed by Wishbone SA (WO 2015/049336 A1) [44]. After elimination of the organic compounds, the two BHA samples were sintered under atmospheric conditions for 30 min at the chosen temperature; after 30 min, the furnace was shut down and the material was retrieved once the furnace had naturally reached room temperature again. All sintering parameters (time and temperature) were controlled by the internal controller of the furnace. The samples, HAN, HA820 and HA1200, were further sterilized in a drying oven at 120 °C. The material was not rinsed between sintering and sterilization. For all samples, small granules (particles size: 250–1000 µm) were used.

2.2. Animals

Yucatan mini-pigs were used for this study because their bone turns over in a similar manner to that of humans. Moreover, these animals are used in dental research due to similarities with humans in tooth development eruption time and sequence of tooth eruption [45]. The animals

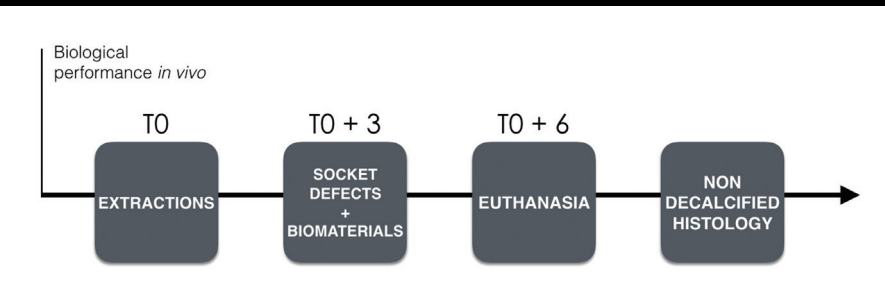
were 18 months old with a weight between 50 to 60 kg and presenting a complete eruption of the permanent teeth. All experimental procedures and protocols used in this investigation were reviewed and approved by the CIRE platform agreement number A37-175-4, Tours, France. The Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines as well as national and European legislation were carefully followed.

2.3. Study Design

Before implantation, the raw experimental biomaterials were characterized by scanning electronic microscopy (SEM) for qualitative analysis, measurement of the surface specific area (BET) and by X-ray diffractometry (XRD) to identify the crystalline phases and measurement of the crystallite size. The biological performance of the biomaterials was then evaluated in vivo in a mini-pig model (Figure 1). Dental extractions (T0) were performed in the maxillae of 5 mini-pigs. After a healing period of 3 months, six standardized bone socket defects were created per animal (3 per hemi-maxilla) and then filled with each of the three experimental biomaterials. A total of 10 samples per condition were considered for analyses. The software GPower was used to calculate the total sample size for 3 groups of biomaterial considering a statistical power of 0.8 and an effect size of 0.6 obtaining a total sample size of 30. Given the number of 6 implantation sites per animal, a total of 5 mini-pigs were chosen for this study. Euthanasia was performed after a healing period of 3 months, and the samples were subjected to non-decalcified histological analysis and histomorphometry.

Figure 1

Study time line on biological performance.



2.4. Scanning Electronic Microscopy (SEM) Analysis

The three biomaterials were mounted on a glass slide double-sided carbon tape with platinum (20 nm) coated in a Balzers SCD030 sputtering unit. The surface characteristics were then captured using a scanning electron microscope ESEM-FEG XL30 (Philips Electron Optics, Hillsboro, OR, USA) at magnification levels of $\times 20,000$, $\times 5000$, and $\times 50$ for descriptive analysis.

2.5. Brunauer–Emmett–Teller (BET) Specific Surface Area Analysis

The specific surface area of each product was measured by N_2 adsorption according to the Brunauer–Emmett–Teller (BET) method. Nitrogen adsorption-desorption isotherms were registered on a TriStar 3000 equipment (Micromeritics, Norcross, GA, USA). Prior to the measurements, the samples were degassed overnight under vacuum at 120 °C on a VacPrep 061 (Micromeritics, Norcross, GA, USA). For each product, the measurement was repeated two times on three independent samples.

2.6. X-ray Diffraction (XRD) Analysis

Powder X-ray diffraction (XRD) was performed in Bragg–Brentano geometry using a Bruker D8 Twin-Twin diffractometer with Cu K α radiation (Billerica, MA, USA). The three products were ground into fine powders using an agate mortar. Three independent diffractograms were collected on each product in the 3°–70° 2θ -range with a 0.02° step size. The TOPAS software [46] was used to estimate the crystallite size; structure parameters for the hydroxyapatite phase were taken from the PDF 01-085-5086 reference (ICCDPDF4+ database) and the fundamental parameters approach was used to model the instrumental contribution to the reflection profiles. The crystallite size contribution to the peak profiles was modeled as lorentzian and convoluted with the instrumental contribution; the crystallite size (CS) and the integral breadth β of the lorentzian (= area under the diffraction peak divided by maximum height of the peak) are related through the equation $CS = \lambda / (\beta \cos \theta)$ where λ is the CuK $_{\alpha 1}$ wavelength and θ is half of the 2θ angle [47].

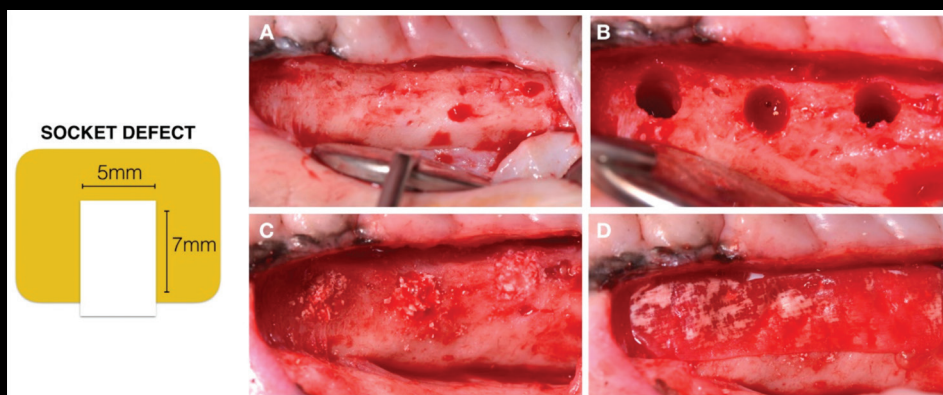
2.7. Surgical Procedure

All the animals were sedated with xylazine (Ronpums, 1 mg/kg, Bayer, Munchen, Germany and imalgene 1000 (Imalgene®, 15 mg/kg, Merial Laboratory, Lyon, France). During the surgery, the

animals inhaled O₂, were surveyed with an electrocardiogram and were maintained with a perfusion of imalgene 1000 (Imalgene®, 15 mg/kg, Merial Laboratory, Lyon, France) in the saline. One hour before surgery, an injection of rimadyl® (carprofen, Kalamazoo, Michigan, USA) 1.5 mg/kg was performed. All the surgical procedures were performed under aseptic conditions. After disinfection of the surgical field with povidone iodine 10 % (Iso-Betadine® titratable iodine, Meda Manufacturing, Merignac, France), a local anesthesia was performed at the maxillae (4 % articaïne hydrochloride with epinephrine 1:100,000 Septanest Sp; Septodont Inc. Créteil, Saint-Maur-des-Fossés, France). An intrasulcular incision and a full thickness flap were made from the canine to the second molar in each hemi arcade. The extractions of the four pre-molars and first molar were made carefully by a technique of root separation. After the extractions, the surgical sites were closed with resorbable sutures (Vicryl 4-0, Ethicon GmbH, Norderstedt, Germany). The following post-surgical treatments and medication were administered: antibiotic coverage of Amoxiciline for 5 days (Streptocillin® Vet, Boehringer Ingelheim, Copenhagen, Denmark), analgesics (buprenorphine, Temgesic Schering-Plough, Brussels, Belgium) and daily mouth spray of chlorhexidine gluconate 0.2 %, (Corsodyl®, GSK, Brentford, UK). After a healing period of 3 months, socket bone defects were created as follows. A supra crestal incision was performed and mucoperiosteal flaps were elevated to expose the right and left edentulous crest in the maxilla (Figure 2A). Three socket defects of 7 mm in depth and 5 mm in diameter were created in each hemi arcade (Figure 2B). The 3 biomaterials were randomly allocated in the standardized socket created at each hemi arcade and a collagen membrane (creos xenoprotect, NobelBiocareAB, Göteborg, Sweden) was sized to cover the 3 bone defects in order to prevent the internal growing of soft tissue (Figure 2C,D).

Figure 2

(A) Maxillae alveolar ridge after 3 months' extraction; (B) Socket defect; (C) Sockets filled with the 3 experimental biomaterials; (D) Collagen membrane covering socket defects.



The mucoperiosteal flaps were sutured with non-resorbable sutures (polypropylene 4-0 FS-2; Ethicon, Edinburgh, UK).

2.8. Euthanasia

After a healing period of 6 months (T0 + 6), euthanasia was performed with an overdose of sodium pentobarbital at 3 %. The maxillary blocks were resected with an oscillating saw autopsy and the specimens containing the biomaterial sites and surrounding hard and soft tissues were placed in a 4 % formaldehyde solution for sample fixation.

3. HISTOLOGY

Histological evaluation was performed on undecalcified polymethylmethacrylate (PMMA) sections. Dehydration was performed using an ascending concentration ethanol bath for 24 h: 1 x 70°, 1 x 80°, 2 x 95° and 3 x 100°, and then by soaking for 24 h in acetone. Subsequently, samples were impregnated with methylmethacrylate for 48 h at 20 °C with a refreshment of the medium. Finally, they were embedded in PMMA at 4 °C for 4 days. The sections were stained using methylene blue (1 %) for 90 s and basic fuchsin (0.3 %) for 25 s for a descriptive analysis. Sections were prepared using the cutting-grinding technique of hard tissues according to Donath and Breuner, 1982 [48].

3.1. Histomorphometry

The resulting sections (Figure 3) were digitalized using a Microscope (Leica 205A stereomicroscope Wetzlar Germany) in order to perform quantitative measurements of mineralized bone and biomaterial using image analysis software (ImageJ, National Institute of Health, Bethesda, MD, USA). The following parameters were calculated:

- Defect area: defined as the total area of the defect (Region Of Interest, ROI1) Figure 4A.
- Regenerated area: defined as the defect area colonized by newly formed bone (ROI2) Figure 4B.
- % of regeneration: proportion of ROI2/ROI1.
- % of newly formed bone, % of biomaterial, % of soft tissue within the overall defect area (ROI1).
- The osteoconductivity characterized by the bone-to-material contact (BMC) defined by percentage of particles perimeter in contact with newly formed bone within the regenerated area (ROI2).

Figure 3

Descriptive histology after 3 months. BP, bone particle; NB, newly formed bone; FT, fibrous connective tissue.

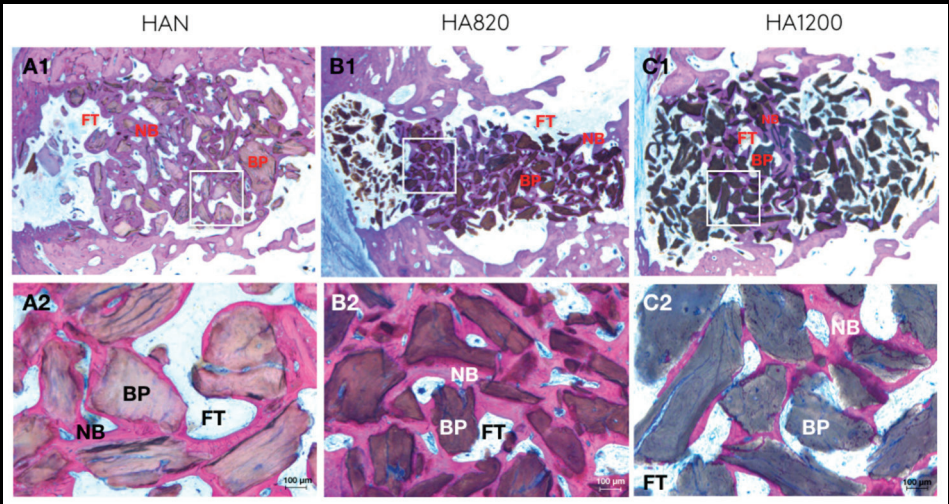


Figure 4

(A) Defect area (ROI1); (B) Regenerated area (ROI2).

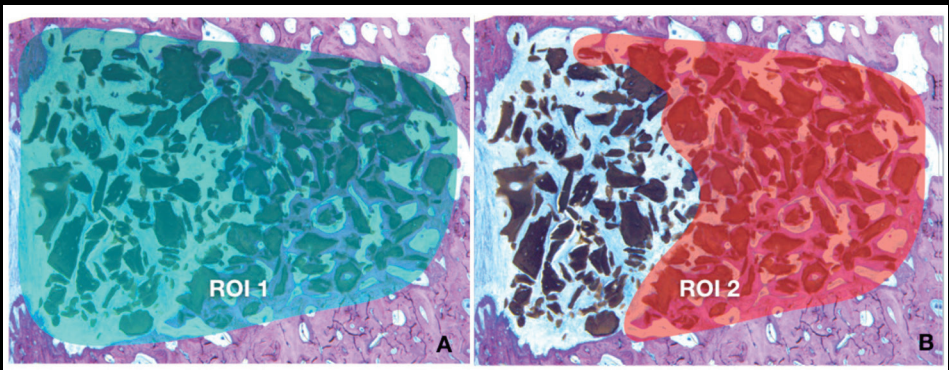
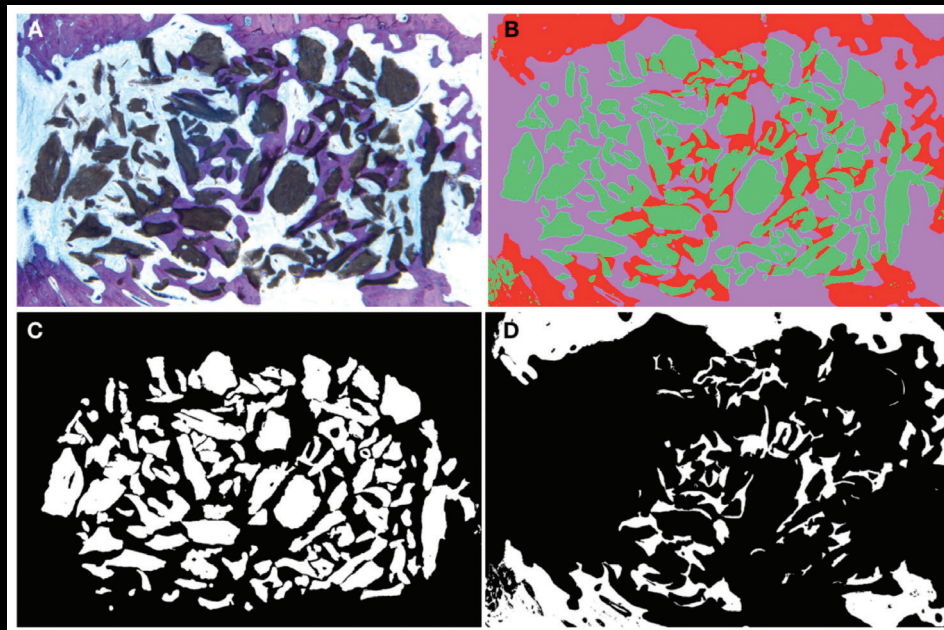


Figure 5

Histomorphometry segmentation steps; A Example of histological cut HA820; B Color segmentation using ImageJ; C Binary image isolating all the particles of bovine hydroxyapatites (BHA); D Binary image isolating the bone content.



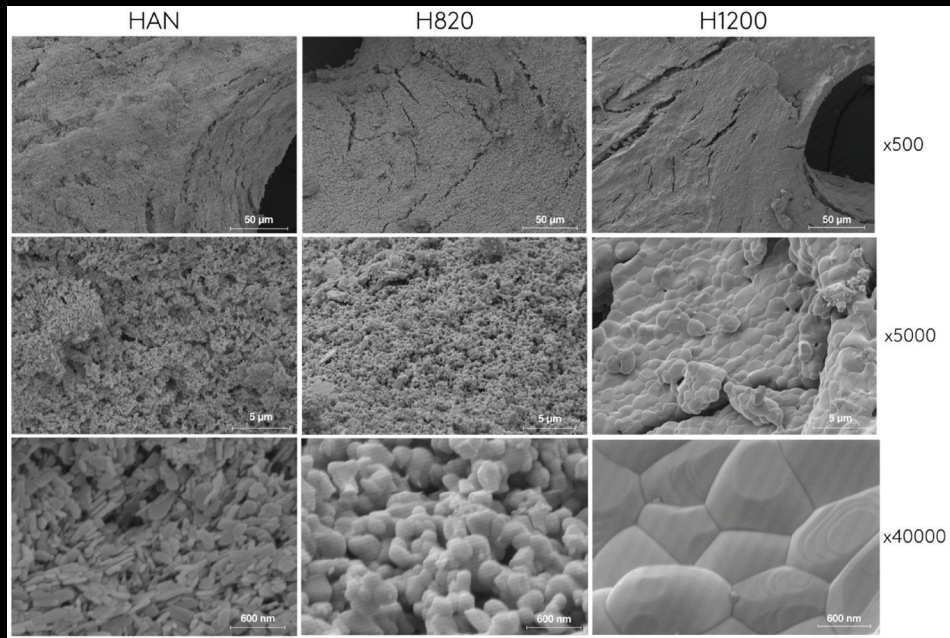
All images were segmented in 3 colors, red, green and purple, identifying respectively bone, biomaterial and soft tissue. The segmented images (Figure 5B) were then transformed into binary images allowing the individualization of each of the segmentations (Figure 5C). The software calculated the % of pixels present in each image. For the BMC, the 2 binary images (bone and biomaterial) were fused using a macrocode, drawing automatically the contact between the 2 segments. The total length of the contact lines was then measured and divided by the total perimeter of the particles contained in the ROI².

3.2. Statistical Analysis

Results were presented as means and standard deviation (SD). A general linear mixed model (GLMM) was used for the comparisons of the parameters between the 3 groups (HAN, HA820 and HA1200) six measurements were done in the same pig. The Scheffé test was used to compare temperatures two by two. The results are considered to be significant at the 5 % level ($p < 0.05$). Statistical analyses were performed using SAS software version 9.4 (SAS Institute, Cary, NC, USA).

Figure 6

Scanning electronic micrographs (SEM) of the studied biomaterials at different magnifications: HAN: non-sintered HA; HA820: 820 °C sintered HA; HA1200: 1200 °C sintered HA.



4. RESULTS

4.1. Study Design

During the healing period, one of the mini-pigs showed signs of paralysis of the hind legs and early euthanasia was performed. Therefore, a total of 24 bone defects and 8 samples per condition were available from the 4 remaining mini-pigs. All samples were assessed quantitatively and descriptively.

4.2. SEM Characterization

The SEM analysis provided a graphic insight of the HA structure with respect to porosity. SEM images of HAN, HA820 and HA1200 are displayed in Figure 6. The bone-like macro-architecture with macropores ($>50\ \mu\text{m}$) was visible in all samples. When looking at higher magnification, micropores could be observed in HAN and HA820 samples. HAN seems to preserve its surface

roughness architecture while H820 changed into grain-like architecture presenting a rougher surface than HA1200 that presents a rather smooth surface and very few micro porosities.

4.3. Surface Area, Pore Size and Pore Volume Analysis

Surface area, pore size and volume are presented in Table 1. BET surface area analysis was carried out on all samples. From the average of two independent BET isotherms, the surface areas of HAN, HA820 and HA1200 were ~ 36 , ~ 4.2 and ~ 0.27 m²/g respectively, which showed that surface area substantially decreased with sintering. Barrett-Joyner-Halenda (BJH) pore size and volume analysis was also carried out. Both pore size and volume decreased with sintering temperature, with HAN, HA820 and HA1200 showing respective average pore sizes of ~ 10 , ~ 5.2 and ~ 4.5 nm, and respective total pore volumes of ~ 0.13 , ~ 0.007 and ~ 0.003 cm³/g.

4.4. XRD Analysis

The powder XRD patterns of representative samples are presented in Figure 7. For HAN, all observed peaks could be indexed to the formation of hydroxyapatite, Ca₁₀(PO₄)₆(OH)₂ (*P6₃m*; $a = 9.424$ Å, $b = 9.424$ Å, $c = 6.879$ Å, $\alpha = 90^\circ$, $\beta = 90^\circ$, $\gamma = 120^\circ$) [49]. For HA820 and HA1200, the majority of peaks could also be indexed to the formation of hydroxyapatite; however two other crystalline phases were detected. In HA820, a small broad peak at $\sim 43^\circ$ 2θ was observed, which may correspond to the formation of NaCaPO₄ (*Pn21a*; $a = 20.397$ Å, $b = 5.412$ Å, $c = 9.161$ Å, $\alpha = 90^\circ$, $\beta = 90^\circ$, $\gamma = 90^\circ$) [50]. In HA1200, several additional peaks corresponding to the presence of NaCaPO₄ were observed, and one additional peak at $\sim 37.5^\circ$ 2θ that may correspond to the formation of CaO (*Fm $\bar{3}m$* ; $a = 4.8152$ Å, $b = 4.8152$ Å, $c = 4.8152$ Å, $\beta = 90^\circ$, $\# = 90^\circ$, $\gamma = 90^\circ$) [51]. With an increase in sintering temperature, peaks in the majority hydroxyapatite phase became sharper and narrower, which was a result of an increase in the average crystallite size. When fitting the reflection with a model taking into account crystallite size and instrumental contributions, the average crystallite size increases from 29 nm in HAN, to ~ 120 nm in HA820 to ~ 230 nm in HA1200.

4.5. Descriptive Histology

After 3 months, newly formed bone was observed in the defect of all samples, mainly in the apical 2/3 and adjacent to the lateral bone walls of the artificially created sockets (Figure 3). The typical structure of a newly formed bone is observed, with the presence of osteocytes entrapped by lamellar bone. Some chains of osteoblasts are also observed close to the newly formed

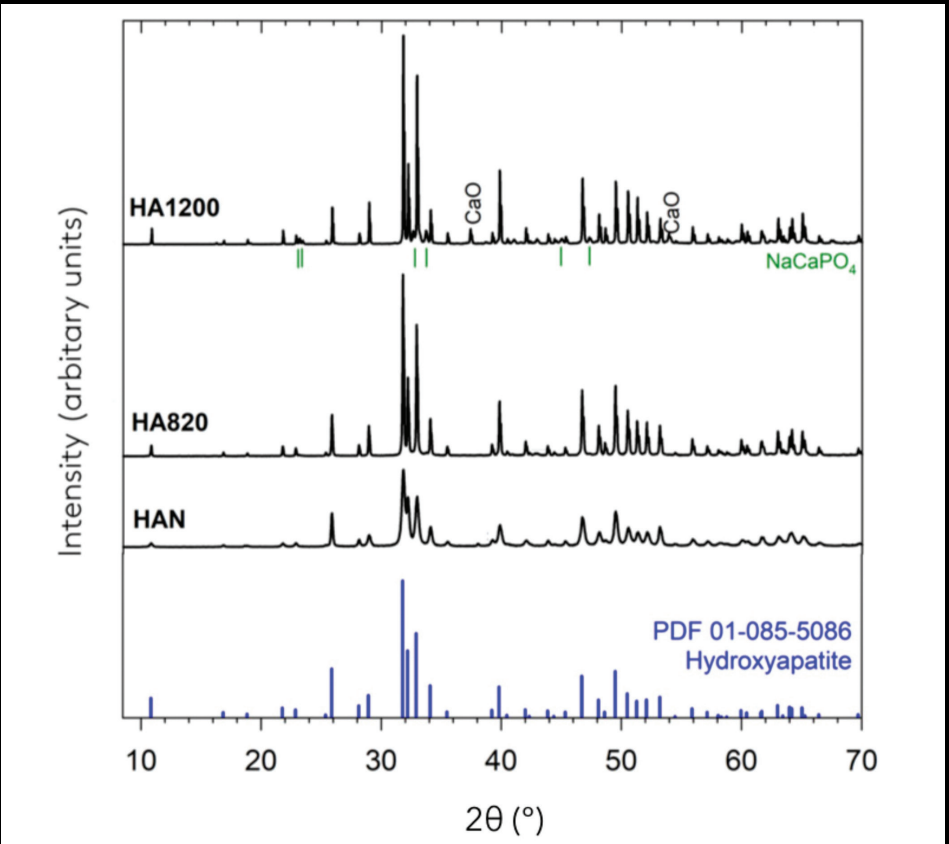
Table 1

Surface area, pore size and pore volume results.

	HAN	HA820	HA1200
Surface area (m ² /g)	~36	~4.27	~0.27
Pore size (nm)	~10	~5.2	~4.5
Total pore volume (cm ³ /g)	~0.13	~0.007	~0.003

Figure 7

Comparison of the X-ray diffraction patterns of HAN, HA820, HA1200 with the reference spectra of hydroxyapatite.



bone as well as some blood vessels, with no visible differences between samples. No signs of inflammation were visible in any of the sections. Generally, samples of HA1200 showed less newly formed bone with more predominance of soft tissue between particles. In all groups it was possible to observe the particles in close contact with newly formed bone; however, this was predominantly observed in HAN and HA820.

4.6. Quantitative Histomorphometric Analysis

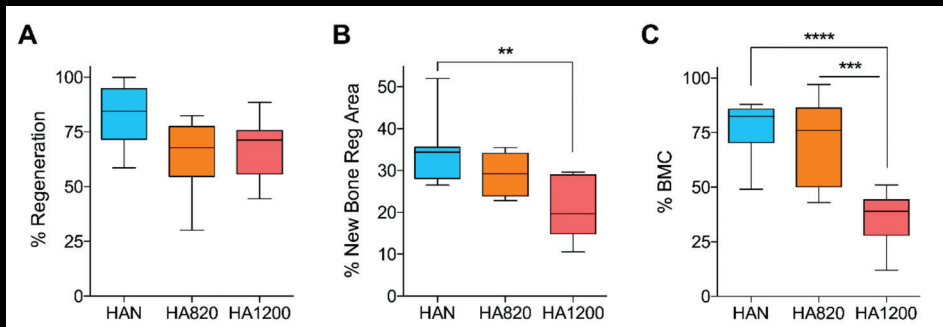
The % of regeneration (Figure 8A) was homogeneous in all groups with no significant differences ($p = 0.10$). In terms of % newly bone formation in the regenerated area (Figure 8B), there were significant differences between HAN and HA1200 ($p = 0.009$), although no significant differences were detected between HA820 and HAN ($p = 0.119$) and between HA820 and HA1200 ($p = 0.115$). The osteoconductivity (bone-to-material contact) (Figure 8C) was also significantly higher in the groups HAN and HA820 compared to HA1200 (HAN vs. HA1200 $p = 0.0192$; HA820 vs HA1200 $p = 0.0234$). There was no significant difference between HAN and HA820 ($p = 0.8876$).

4.7. Correlation Between Structural Characterization and Biological Performance

For all samples, a positive correlation was observed between higher surface area values and % of newly bone ($p = 0.001$) and % of BMC ($p = 0.04$). By contrast, a negative correlation was observed between higher crystallite size values and percentage of newly formed bone ($p = 0.0002$) and percentage of BMC ($p = 0.001$).

Figure 8

(A) % of Regeneration; (B) % of newly formed bone within the ROI2; (C) % of Bone-to-material contact; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.



5. DISCUSSION

The present study emphasized that the sintering process at high temperature modulates not only the structure but also the biological performance of chemically-deproteinized BHA. The sintering process can result in increased crystallinity, appearance of new crystalline phases, decreased porosity and changes in the surface morphology. These characteristics may influence the performance results [26,42]. Nevertheless, the sintering temperature remains an important parameter for the improvement of mechanical properties of BHA [26,27,52]. The analysis of the hydroxyapatite structure by XRD revealed that the main crystalline phase present in all samples is hydroxyapatite. This proves that the chemical deproteinization process used here does not deteriorate the main crystalline phase initially present in bovine bone. The peaks corresponding to hydroxyapatite appear sharper when the sintering temperature increases, which can be interpreted as an increase in crystallite size and is considered a sign of high crystallinity. The crystallinity of a biomaterial is important as it influences its dissolution; therefore, high crystallinity materials display lower biodegradation rates and better volume stability overtime [53–56].

Additional traces of NaCaPO_4 and CaO were also detected in HA1200. The formation of CaO at 1200 °C could result from the decomposition of the carbonate content initially present in natural bone as already observed in other studies [57].

SEM analysis revealed that, from a descriptive point of view, the unsintered particles preserved their surface roughness architecture while the particles sintered at 820 °C displayed a grain-like architecture. By contrast, the particles subjected to 1200 °C suffered from structural changes on the surface morphology, presenting a smoother surface and fewer micro porosities compared to the two other BHA which can be correlated to the fusion of crystals as described by Patel, 2001 [58]. Looking at the porosity using BET analysis, the surface area decreased sharply from 36 to 4.2 m^2/g when the particles were sintered at 820 °C, and even to 0.27 m^2/g when sintered at 1200 °C. The specific surface area has been often mentioned in the literature as a significant parameter for bone regeneration [37,54,58–60]. It is indeed believed that a high specific surface area promotes extracellular matrix protein and cell adsorption, and thus favors bone formation [38]. According to the present results, the in vivo behavior of biomaterials seems to correlate better to their surface morphology than to their specific surface area. When the sample is sintered at low temperature (820 °C) the biological performance was not altered compared to the non-sintered sample. However, a clear change of the biological behavior was observed when the material was sintered at higher temperature (1200 °C) and the surface became smoother. Smooth surfaces are less expected to conduct cell colonization [61]. Indeed, the histomorphometric results of this study showed that the percentage of newly formed

bone and the osteoconductivity (bone-to-material contact, BMC) was equivalent for low and non-sintered hydroxyapatites, despite their significant difference in specific surface area while the BMC was significantly lower for the HA sintered at 1200 °C. Therefore, it seems that the surface morphology of BHA (rather than specific surface area) is a key factor that should be considered when assessing their biological performance. These results seem in accordance with the literature about dental implants as the positive correlation of the roughness of implants and osteoconduction has been well described in several systematic reviews [62,63]. Nevertheless, it should be emphasized that one of the limitations of the present study was the lack of quantitative measurement of the micro-roughness of the experimental materials due to the complexity of this technique on particulate materials such as BHA. Another limitation of this study is that no comparison was made to BHA that did not undergo chemical deproteinization before sintering, which is how the commercially available sintered BHA products are currently made. Such variations in the manufacturing process may affect the physicochemical properties and provide different *in vivo* performance. Also, the specific production process steps might influence the BHA structure and consequently the clinical results. Nevertheless, good osteoconductivity and clinical results of sintered bovine bone has been reported many times [31,32].

The present study is one of the few taking into account the BMC to assess the biological performance of regenerative biomaterials since it is intended that each particle should be able to osteointegrate and have a high contact interface with newly formed bone, forming a tight bone–material network, which plays an important role in the subsequent implant placement and survival rates [42,64,65].

Further studies should correlate the biological performance of biomaterials with different viscoelastic properties, hydrophilicity, physicochemical differences and surface roughness, in order to better explore the impact of these properties in promoting osteoconductivity and long-term volume stability in bone regenerative therapy. Such properties are influenced by the production process and origin of the biomaterial and should be considered as requirements in regenerative procedures [6,42,57].

6. CONCLUSIONS

The sintering temperature affects the surface properties of chemically-deproteinized BHA biomaterials and these parameters influence the in vivo performance in terms of new bone formation and BMC. Non-sintered BHA or BHA sintered at low temperature (820 °C) seem to maintain a certain surface roughness of chemically-deproteinized BHA, allowing better bone regeneration and osteoconductivity (BMC). By contrast, sintering of BHA at 1200 °C has an effect on its morphological and structural characteristics and significantly modifies its biological performance (osteoconductivity) and crystallinity. Despite the presented results being only obtained on chemically deproteinized BHA, they tend to reemphasize the importance of the production process on the in vivo performances of bone regeneration biomaterials. The osteointegration of each particle of BHA plays an important role on the quality of the regenerated bone. Clinicians should take into account that the process of biomaterial manufacturing can significantly influence the degree of bone regeneration, when choosing their biomaterial for regenerative treatments.

Author Contributions:

B.D.C. was involved in the in vivo experiments, histomorphometry and biomaterial characterization. E.R., G.L. and F.L. performed the in vivo experiments and regenerative procedures; P.S. performed the non-decalcified histological analysis; E.D. and J.-F.A. were involved on the fabrication of the experimental biomaterial and characterization of it. All authors contributed to the analyses and discussion of the results as well as preparation of the manuscript.

Funding:

This research received no external funding.

Acknowledgments:

The authors wish to express their gratitude to Laurence SEIDEL, Philippe COMPERE, Miguel QUESADA-GONZALEZ, Bénédicte VERTRUYEN and Andreas KAFIZAS for their input in the present manuscript.

Disclosures:

The co-authors France Lambert, Eric Rompen and Geoffrey Lecloux are integrating members of the Wishbone SA board.

Conflicts of Interest:

The authors declare no conflict of interest.

REFERENCES

- 1 Esposito, M.; Mg, G.; Rees, J.; Karasoulos, D.; Felice, P.; Alissa, R.; Hv, W. **Interventions for replacing missing teeth: Augmentation procedures of the maxillary sinus (Review) SUMMARY OF FINDINGS FOR THE MAIN COMPARISON.** Cochrane Database Syst. Rev. 2010. [CrossRef]
- 2 Donos, N.; Mardas, N.; Chadha, V. **Clinical outcomes of implants following lateral bone augmentation: Systematic assessment of available options (barrier membranes, bone grafts, split osteotomy).** J. Clin. Periodontol. 2008, 35, 173–202. [CrossRef] [PubMed]
- 3 Dasmah, A.; Thor, A.; Ekestubbe, A.; Sennerby, L.; Rasmusson, L. **Particulate vs. block bone grafts: Three-dimensional changes in graft volume after reconstruction of the atrophic maxilla, a 2-year radiographic follow-up.** J. Cranio Maxillofac. Surg. 2012, 40, 654–659. [CrossRef] [PubMed]
- 4 Weibull, L.; Widmark, G.; Ivanoff, C.J.; Borg, E.; Rasmusson, L. **Morbidity after chin bone harvesting—A retrospective long-term follow-up study.** Clin. Implant Dent. Relat. Res. 2009, 11, 149–157. [CrossRef]
- 5 Burchardt, H. **The Biology of Bone Graft Repair.** Clin. Orthop. Related Res. 1983, 174, 28–42. [CrossRef]
- 6 Trajkovski, B.; Jaunich, M.; Müller, W.D.; Beuer, F.; Zafiropoulos, G.G.; Houshmand, A. **Hydrophilicity, viscoelastic, and physicochemical properties variations in dental bone grafting substitutes.** Materials (Basel) 2018, 11, 215. [CrossRef]
- 7 Kolk, A.; Handschel, J.; Drescher, W.; Rothamel, D.; Kloss, F.; Blessmann, M.; Heiland, M.; Wolff, K.D.; Smeets, R. **Current trends and future perspectives of bone substitute materials - From space holders to innovative biomaterials.** J. Cranio Maxillofac. Surg. 2012, 40, 706–718. [CrossRef]
- 8 Hench, L.L. **Bioceramics: From Concept to Clinic.** J. Am. Ceram. Soc. 1991, 74, 1487–1510. [CrossRef]

- 9** Epple, M.; Ganesan, K.; Heumann, R.; Klesing, J.; Kovtun, A.; Neumann, S.; Sokolova, V. **Application of calcium phosphate nanoparticles in biomedicine.** *J. Mater. Chem.* 2010, 20, 18–23. [CrossRef]
- 10** Al-Sanabani, J.S.; Madfa, A.A.; Al-Sanabani, F.A. **Application of calcium phosphate materials in dentistry.** *Int. J. Biomater.* 2013, 2013. [CrossRef]
- 11** Metsger, D.S.; Dephillip, R.; Hayes, T.G. **An Autoradiographic Study of Calcium Phosphate Ceramic Bone Implants in Turkeys.** *Clin. Orthop. Relat. Res.* 1993, 283–294. [CrossRef]
- 12** Koepp, H.E.; Schorlemmer, S.; Kessler, S.; Brenner, R.E.; Claes, L.; Günther, K.-P.; Ignatius, A.A. **Biocompatibility and osseointegration of β -TCP: Histomorphological and biomechanical studies in a weight-bearing sheep model.** *J. Biomed. Mater. Res. Part B Appl. Biomater.* 2004, 70B, 209–217. [CrossRef] [PubMed]
- 13** Kwon, S.-H.; Jun, Y.-K.; Hong, S.-H.; Lee, I.-S.; Kim, H.-E.; Won, Y.Y. **Calcium Phosphate Bioceramics with Various Porosities and Dissolution Rates.** *J. Am. Ceram. Soc.* 2004, 85, 3129–3131. [CrossRef]
- 14** Chiba, S.; Anada, T.; Suzuki, K.; Saito, K.; Shiwaku, Y.; Miyatake, N.; Baba, K.; Imaizumi, H.; Hosaka, M.; Itoi, E.; et al. **Effect of resorption rate and osteoconductivity of biodegradable calcium phosphate materials on the acquisition of natural bone strength in the repaired bone.** *J. Biomed. Mater. Res. Part A* 2016, 104, 2833–2842. [CrossRef]
- 15** Ribeiro, N.; Sousa, S.R.; Monteiro, F.J. **Influence of crystallite size of nanophased hydroxyapatite on fibronectin and osteonectin adsorption and on MC3T3-E1 osteoblast adhesion and morphology.** *J. Colloid Interface Sci.* 2010, 351, 398–406. [CrossRef]
- 16** Gunduz, O.; Erkan, E.M.; Daglilar, S.; Salman, S.; Agathopoulos, S.; Oktar, F.N. **Composites of bovine hydroxyapatite (BHA) and ZnO.** *J. Mater. Sci.* 2008, 43, 2536–2540. [CrossRef]

- 17 Figueiredo, M.; Henriques, J.; Martins, G.; Guerra, F.; Judas, F.; Figueiredo, H. **Physicochemical characterization of biomaterials commonly used in dentistry as bone substitutes—Comparison with human bone.** *J. Biomed. Mater. Res. Part B Appl. Biomater.* 2010, 92, 409–419. [CrossRef]
- 18 Wu, J.; Li, B.; Lin, X. **Histological outcomes of sinus augmentation for dental implants with calcium phosphate or deproteinized bovine bone: A systematic review and meta-analysis.** *Int. J. Oral Maxillofac. Surg.* 2016, 45, 1471–1477. [CrossRef]
- 19 Jensen, S.S.; Broggini, N.; Hjørtting-Hansen, E.; Schenk, R.; Buser, D. **Bone healing and graft resorption of autograft, anorganic bovine bone and β -tricalcium phosphate. A histologic and histomorphometric study in the mandibles of minipigs.** *Clin. Oral Implants Res.* 2006, 17, 237–243. [CrossRef]
- 20 Accorsi-Mendonça, T.; Conz, M.B.; Barros, T.C.; de Sena, L.Á.; Soares, G.d.; Granjeiro, J.M. **Physicochemical characterization of two deproteinized bovine xenografts.** *Braz. Oral Res.* 2008, 22, 5–10. [CrossRef]
- 21 Lei, P.; Sun, R.; Wang, L.; Zhou, J.; Wan, L.; Zhou, T.; Hu, Y. **A new method for xenogeneic bone graft deproteinization: Comparative study of radius defects in a rabbit model.** *PLoS ONE* 2015, 10, 1–17. [CrossRef] [PubMed]
- 22 Lambert, F.; Lecloux, G.; Rompen, E. **One-Step Approach for Implant Placement and Subantral Bone Regeneration Using Bovine Hydroxyapatite: A 2- to 6-Year Follow-up Study.** *Int. J. Oral Maxillofac. Implants* 2010, 25, 598–606. [PubMed]
- 23 Wenz, B.; Oesch, B.; Horst, M. **Analysis of the risk of transmitting bovine spongiform encephalopathy through bone grafts derived from bovine bone.** *Biomaterials* 2001, 22, 1599–1606. [CrossRef]
- 24 Baldini, N.; de Sanctis, M.; Ferrari, M. **Deproteinized bovine bone in periodontal and implant surgery.** *Dent. Mater.* 2011, 27, 61–70. [CrossRef]
- 25 Uklejewski, R.; Winiecki, M.; Musielak, G.; Tokłowicz, R. **Effectiveness of various deproteinization processes of bovine cancellous bone evaluated via mechano-biostructural properties of produced osteoconductive biomaterials.** *Biotechnol. Bioprocess Eng.* 2015, 20, 259–266. [CrossRef]

- 26 Goller, G.; Oktar, F.N.; Agathopoulos, S.; Tulyaganov, D.U.; Ferreira, J.M.F.; Kayali, E.S.; Peker, I. **Effect of sintering temperature on mechanical and microstructural properties of bovine hydroxyapatite (BHA)**. *J. Sol-Gel Sci. Technol.* 2006, 37, 111–115. [CrossRef]
- 27 Witek, L.; Smay, J.; Silva, N.R.F.A.; Guda, T.; Ong, J.L.; Coelho, P.G. **Sintering effects on chemical and physical properties of bioactive ceramics**. *J. Adv. Ceram.* 2013, 2, 274–284. [CrossRef]
- 28 Knowles, J.C.; Horton, J.A.; Bonfield, W. **Rietveld Analysis on the Effect of Sintering Conditions on the Structure of Hydroxyapatite**. *Bioceramics* 1994, 23–28. [CrossRef]
- 29 Barone, A.; Todisco, M.; Ludovichetti, M.; Gualini, F.; Aggstaller, H.; Torres-Lagares, D.; Rohrer, M.; Prasad, H.; Kenealy, J. **A Prospective, Randomized, Controlled, Multicenter Evaluation of Extraction Socket Preservation Comparing Two Bovine Xenografts: Clinical and Histologic Outcomes**. *Int. J. Periodontics Restor. Dent.* 2013, 33, 795–802. [CrossRef]
- 30 Spies, C.K.G.; Schnürer, S.; Gotterbarm, T.; Breusch, S.J. **Efficacy of Bone Source™ and Cementek™ in comparison with Endobon™ in critical size metaphyseal defects, using a minipig model**. *J. Appl. Biomater. Biomech.* 2010, 8, 175–185. [CrossRef]
- 31 Tawil, G.; Barbeck, M.; Unger, R.; Tawil, P.; Witte, F. **Sinus Floor Elevation Using the Lateral Approach and Window Repositioning and a Xenogeneic Bone Substitute as a Grafting Material: A Histologic, Histomorphometric, and Radiographic Analysis**. *Int. J. Oral Maxillofac. Implants* 2018, 33, 1089–1096. [CrossRef]
- 32 Takauti, C.A.Y.; Futema, F.; Junior, R.B.d.; Abrahao, A.C.; Costa, C.; Queiroz, C.S. **Assessment of Bone Healing in Rabbit Calvaria grafted with three different Biomaterials**. *Braz. Dent. J.* 2014, 25, 379–384. [CrossRef]
- 33 Joschek, S.; Nies, B.; Krotz, R.; Göpferich, A. **Chemical and physicochemical characterization of porous hydroxyapatite ceramics made of natural bone**. *Biomaterials* 2000, 21, 1645–1658. [CrossRef]

- 34** Figueiredo, M.; Fernando, A.; Martins, G.; Freitas, J.; Judas, F.; Figueiredo, H. **Effect of the calcination temperature on the composition and microstructure of hydroxyapatite derived from human and animal bone.** *Ceram. Int.* 2010, 36, 2383–2393. [CrossRef]
- 35** Etok, S.E.; Valsami-Jones, E.; Wess, T.J.; Hiller, J.C.; Maxwell, C.A.; Rogers, K.D.; Manning, D.A.C.; White, M.L.; Lopez-Capel, E.; Collins, M.J.; et al. **Structural and chemical changes of thermally treated bone apatite.** *J. Mater. Sci.* 2007, 42, 9807–9816. [CrossRef]
- 36** Li, X.; van Blitterswijk, C.A.; Feng, Q.; Cui, F.; Watari, F. **The effect of calcium phosphate microstructure on bone-related cells in vitro.** *Biomaterials* 2008, 29, 3306–3316. [CrossRef] [PubMed]
- 37** Habibovic, P.; Kruyt, M.C.; Juhl, M.V.; Clyens, S.; Martinetti, R.; Dolcini, L.; Theilgaard, N.; van Blitterswijk, C.A. **Comparative in vivo study of six hydroxyapatite-based bone graft substitutes.** *J. Orthop. Res.* 2008, 26, 1363–1370. [CrossRef] [PubMed]
- 38** Rouahi, M.; Champion, E.; Gallet, O.; Jada, A.; Anselme, K. **Physico-chemical characteristics and protein adsorption potential of hydroxyapatite particles: Influence on in vitro biocompatibility of ceramics after sintering.** *Colloids Surfaces B Biointerfaces* 2006, 47, 10–19. [CrossRef]
- 39** Espanol, M.; Mestres, G.; Luxbacher, T.; Dory, J.B.; Ginebra, M.P. **Impact of Porosity and Electrolyte Composition on the Surface Charge of Hydroxyapatite Biomaterials.** *ACS Appl. Mater. Interfaces* 2016, 8, 908–917. [CrossRef]
- 40** LeGeros, R.Z. **Properties of osteoconductive biomaterials: Calcium phosphates.** *Clin. Orthop. Relat. Res.* 2002, 395, 81–98. [CrossRef]
- 41** Klenke, F.M.; Liu, Y.; Yuan, H.; Hunziker, E.B.; Siebenrock, K.A.; Hofstetter, W. **Impact of pore size on the vascularization and osseointegration of ceramic bone substitutes in vivo.** *J. Biomed. Mater. Res. Part A* 2008, 85, 777–786. [CrossRef]
- 42** Lambert, F.; Bacevic, M.; Layrolle, P.; Schüpbach, P.; Drion, P.; Rompen, E. **Impact of biomaterial microtopography on bone regeneration: Comparison of three hydroxyapatites.** *Clin. Oral Implants Res.* 2017, 28, e201–e207. [CrossRef]

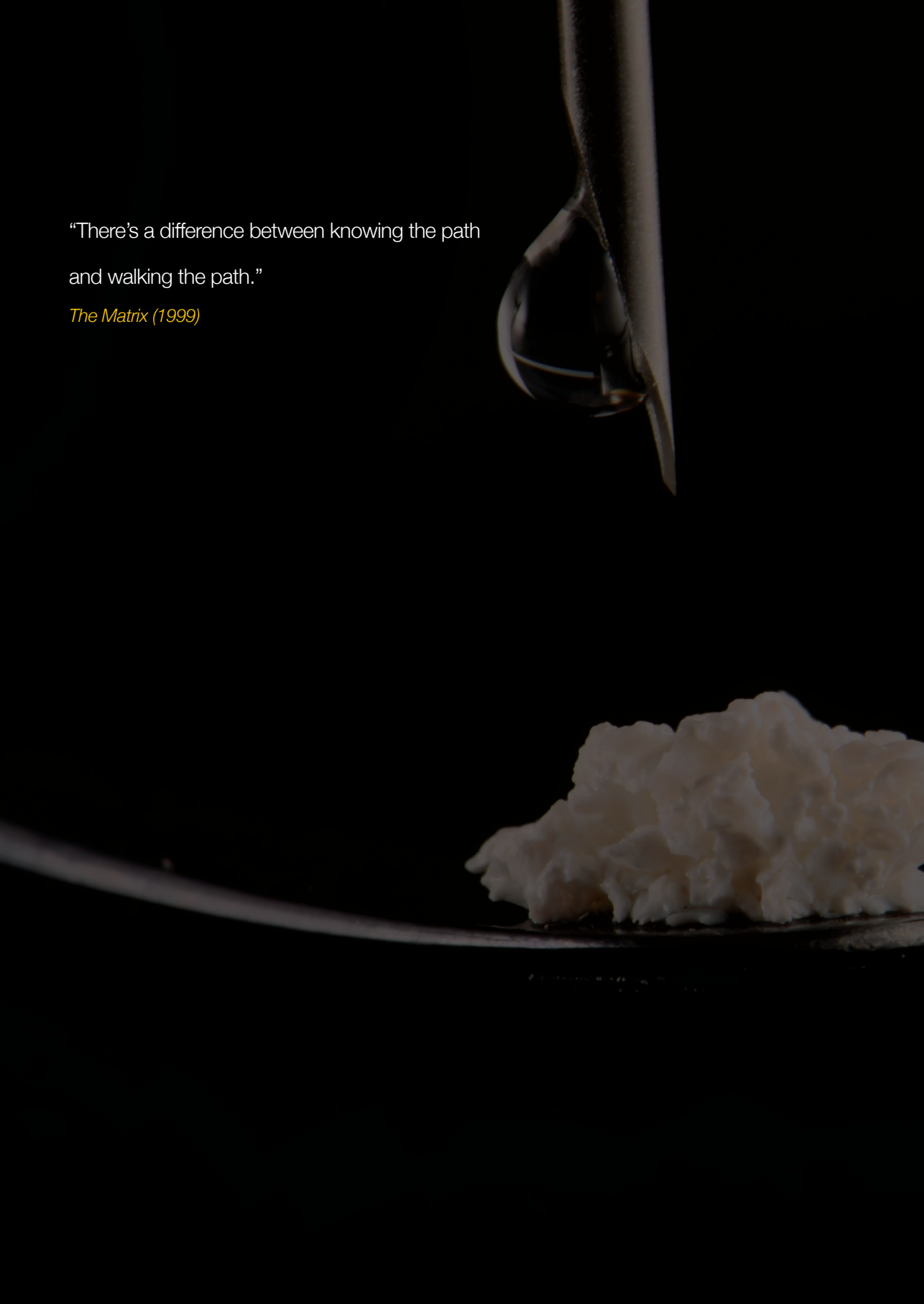
- 43 Kurkcu, M.; Benlidayi, M.E.; Cam, B.; Sertdemir, Y. **Anorganic Bovine-Derived Hydroxyapatite vs β -Tricalcium Phosphate in Sinus Augmentation: A Comparative Histomorphometric Study.** J. Oral Implantol. 2012, 38, 519–526. [CrossRef]
- 44 Rompen, E.; Lambert, F.; Lecloux, G.; Moniotte, P. **Materiau De Regeneration Osseuse Et Son Procede De Fabrication.** Pat. WO 2015/049336 A1, 2015.
- 45 Wang, S.; Liu, Y.; Fang, D.; Shi, S. **The miniature pig: A useful large animal model for dental and orofacial research.** Oral Dis. 2007, 13, 530–537. [CrossRef]
- 46 Cheary, B.Y.R.W.; Coelho, A. **A Fundamental Parameters Approach to X-ray Line-Profile Fitting.** J. Appl. Crystallogr. 1992, 109–121. [CrossRef]
- 47 Krill, C.E.; Haberkorn, R.; Birringer, R. **Specification of microstructure and characterization by scattering techniques, Handb. Nanostruct. Mater. Nanotechnol.** 2000, 155–211. [CrossRef]
- 48 Donath, K.; Breuner, G. **A method for the study of undecalcified bones and teeth with attached soft tissues*.** J. Oral Pathol. Med. 1982, 11, 318–326. [CrossRef] [PubMed]
- 49 Sudarsanan, K.; Young, R.A. **Significant precision in crystal structural details. Holly Springs hydroxyapatite.** Acta Crystallogr. Sect. B 1969, 25, 1534–1543. [CrossRef]
- 50 Amara, M.B.; Vlasse, M.; le Flem, G.; Hagenmuller, P. **Structure of the low-temperature variety of calcium sodium orthophosphate, NaCaPO₄.** Acta Crystallogr. Sect. C 1983, 39, 1483–1485. [CrossRef]
- 51 Shen, C.H.; Liu, R.S.; Lin, J.G.; Huang, C.Y. **Phase stability study of La_{1.2}Ca_{1.8}Mn₂O₇.** Mater. Res. Bull. 2001, 36, 1139–1148. [CrossRef]
- 52 Yetmez, M.; Erkmen, Z.E.; Kalkandelen, C.; Fical, A.; Oktar, F.N. **Sintering effects of mullite-doping on mechanical properties of bovine hydroxyapatite.** Mater. Sci. Eng. C 2017, 77, 470–475. [CrossRef]

- 53 Kusrini, E.; Sontang, M. **Characterization of x-ray diffraction and electron spin resonance: Effects of sintering time and temperature on bovine hydroxyapatite.** *Radiat. Phys. Chem.* 2012, 81, 118–125. [CrossRef]
- 54 Bohner, M. **Calcium orthophosphates in medicine: From ceramics to calcium phosphate cements.** *Injury* 2000, 31. [CrossRef]
- 55 Riachi, F.; Naaman, N.; Tabarani, C.; Aboelsaad, N.; Aboushelib, M.N.; Berberi, A.; Salameh, Z. **Influence of material properties on rate of resorption of two bone graft materials after sinus lift using radiographic assessment.** *Int. J. Dent.* 2012, 2012. [CrossRef]
- 56 Fernández, M.P.R.; Gehrke, S.A.; Martinez, C.P.A.; Guirado, J.L.C.; de Aza, P.N. **SEM-EDX study of the degradation process of two xenograft materials used in sinus lift procedures.** *Materials (Basel)* 2017, 10, 542. [CrossRef] [PubMed]
- 57 Tadic, D.; Epple, M. **A thorough physicochemical characterisation of 14 calcium phosphate-based bone substitution materials in comparison to natural bone.** *Biomaterials* 2004, 25, 987–994. [CrossRef]
- 58 Patel, N.; Gibson, I.R.; Ke, S.; Best, S.M.; Bonfield, W.; Materials, B.; Mary, Q.; College, W.; Road, M.E. **Calcining influence on the powder properties of hydroxyapatite.** *J. Mater. Sci. Mater. Med.* 2001, 2, 181–188. [CrossRef] [PubMed]
- 59 Tan, C.Y.; Ramesh, S.; Aw, K.L.; Yeo, W.H.; Hamdi, M.; Sopyan, I. **Effect of powder calcination on the sintering of hydroxyapatite.** *Med. J. Malaysia* 2008, 63 (Suppl A), 87–88. [CrossRef]
- 60 Wang, A.J.; Lu, Y.P.; Zhu, R.F.; Li, S.T.; Xiao, G.Y.; Zhao, G.F.; Xu, W.H. **Effect of sintering on porosity, phase, and surface morphology of spray dried hydroxyapatite microspheres.** *J. Biomed. Mater. Res. Part A* 2008, 87, 557–562. [CrossRef]
- 61 Deligianni, D.D.; Katsala, N.D.; Koutsoukos, P.G.; Missirlis, Y.F. **Effect of surface roughness of hydroxyapatite on human bone marrow cell adhesion, proliferation, differentiation and detachment strength.** *Biomaterials* 2000, 22, 87–96. [CrossRef]

- 62 Wennerberg, A.; Albrektsson, T. **Effects of titanium surface topography on bone integration: A systematic review.** Clin. Oral Implants Res. 2009, 20, 172–184. [CrossRef]
- 63 Shalabi, M.M.; Gortemaker, A.; van Hof, M.A. **Critical reviews in oral biology & medicine Implant Surface Roughness and Bone Healing.** J. Dent. Res. 2006, 496–501. [CrossRef]
- 64 Molly, L.; Vandromme, H.; Quirynen, M.; Schepers, E.; Adams, J.L.; van Steenberghe, D. **Bone Formation Following Implantation of Bone Biomaterials Into Extraction Sites.** J. Periodontol. 2008, 79, 1108–1115. [CrossRef] [PubMed]
- 65 Lambert, F.; Leonard, A.; Lecloux, G.; Sourice, S.; Pilet, P.; Rompen, E. **A Comparison of Three Calcium Phosphate-Based Space Fillers in Sinus Elevation: A Study in Rabbits.** Int. J. Oral Maxillofac. Implants 2013, 28, 393–402. [CrossRef] [PubMed]

“There’s a difference between knowing the path
and walking the path.”

The Matrix (1999)



3

PRECLINICAL
BIOLOGICAL
VALIDATION

Biological performance of a novel bovine hydroxyapatite in a guided bone regeneration model: A preclinical study in a mandibular defect in dogs

Bruno De Carvalho, Emilie Dory DDS, MS, Caroline Trus DDS, Justine Pirson DDS, Loïc Germain DDS, Geoffrey Lecloux DDS, MS, France Lambert DDS, MS, PhD, Eric Rompen DDS, MS, PhD

Keywords:

biomaterials - bone regeneration - hydroxyapatite - osteoconduction

ABSTRACT

Objectives: This preclinical model study aims to evaluate the performance and safety of a novel hydroxyapatite biomaterial (Wishbone Hydroxyapatite, WHA) on guided bone regeneration compared to a commercially available deproteinized bovine bone mineral (Bio-Oss, BO).

Material and Methods: Twenty-four beagle dogs were allocated to three time point cohorts (4, 12, and 26 weeks) of eight animals each. In all animals, four critical-sized, independent wall mandibular defects were created (32 defects/cohort). Each animal received all four treatments, allocated randomly to separated defects: WHA + collagen membrane (M), BO + M, no treatment (Sham, Sh), and Sh + M. At each time point, the specimens were harvested for histologic and histomorphometric analyses to determine the newly formed bone and osteoconductivity.

Results: At 4 weeks, bone regeneration was significantly higher for WHA + M (46.8 %) when compared to BO + M (21.4 %), Sh (15.1 %), and Sh + M (23.1 %) ($p < 0.05$); at 12 and 26 weeks, regeneration was similar for WHA and BO. Bone-to-material contact increased over time similarly for WHA + M and BO + M. From a safety point of view, inflammation attributed to WHA + M or BO + M was minimal; necrosis or fatty infiltrate was absent.

Conclusions: WHA + M resulted in higher bone regeneration rate than BO + M at 4 weeks. Both BO + M and WHA + M were more efficient than both Sh groups at all time points. Safety and biocompatibility of WHA was favorable and comparable to that of BO.

1. INTRODUCTION

The use of dental implants to replace missing teeth has increased over the last decades [1]. A common problem encountered in the dental implant rehabilitation is the lack of sufficient bone volume and alveolar bone augmentation procedures are often necessary prior to or simultaneously to implant placement [2–7].

Autogenous bone has long been regarded as the gold standard for bone augmentation procedures [8–11] as the material is histocompatible and possesses osteoinductive, osteoconductive, and osteogenic properties. However, its use is limited by availability, increased morbidity and scarring at the donor site, and high resorption rates [8,12–15]. Guided bone regeneration using biomaterials as bone fillers represent an alternative used in oral surgery, implantology and periodontology as they allow bone ingrowth and osteoconductive structural guidance [3,16,17]. Currently, several types of bone graft substitute biomaterials are available, each with their own advantages and disadvantages [7,17,18]. Natural and synthetic calcium phosphate bone graft substitutes such as hydroxyapatite (HA) and tricalcium-phosphate are the most widely used biomaterials for alveolar bone regeneration [15,19,20]. Natural HA of bovine origin has some advantages over synthetic scaffolds because of its physicochemical properties such as surface structure, morphology and microporosities that closely resemble those of natural bone, if not manufactured at high sintering temperature [21–23]. Indeed, high sintering temperature of bovine HA have an influence of the surface topography and therefore on the osteoconductive potential [24]. Additionally, the slowly resorbable feature of natural bovine HA materials is of great interest in the field of dentistry, as one of the goals of bone regenerative treatments is to maintain the volume over time and to allow a denser composite regenerated bone tissue [16,25]. Several natural HA biomaterials are available; among these, DBBM has been distinguished itself as the most documented natural HA bone substitute of bovine origin in regenerative dentistry for over 30 years [26].

Recently, a novel HA bone substitute has been granted marketing approval by the United States Food and Drug Administration [27] in which the complete elimination of the organic residues is performed under subcritical conditions and a continuous flow of a basic solution following the patented extraction method (WO 2015/049336 A1) [28]. Both biomaterials are xenograft biomaterials composed of HA of bovine origin, intended for the filling, augmentation, or reconstruction of alveolar bone defects [27,29]. The aim of this study was to evaluate the biocompatibility and biological performance (new bone formation and osteoconductivity) of novel HA bone substitute on guided bone regeneration in comparison with a control biomaterial as it is considered as the gold standard of xenogeneic materials in regenerative dentistry.

2. MATERIALS AND METHODS

2.1. Biomaterials characterization

Two xenografts composed of HA of bovine origin were used in the present study: Wishbone Hydroxyapatite (WHA, Wishbone SA, Liège, Belgium) and Bio-Oss (BO, Geistlich Pharma AG, Wolhusen, Switzerland). Physicochemical characterization was performed in both xenografts in terms of Scanning Electron Microscopy (SEM) x-ray diffraction (XRD), specific surface area (SSA), remnant organic compounds, elemental concentration, and volumetric porosity.

SEM provided the observation of the micro-organization of the surface morphology at magnification 500x and 1000x (ESEM-FEG XL30 (Philips Electron Optics, Hillsboro, OR, USA)). XRD identified the phase composition, crystallite size and crystallinity ratio present in the samples and was acquired by means of Rietveld refinement, using the crystal structure model for HA of PDF card 01-076-0694. The SSA of each product was measured by N₂ adsorption according to the Brunauer–Emmett–Teller (BET) method analyzed on the TriStar 3000 equipment. For the analysis of the remnant of organic compounds, three samples of each material were heated successively to three different temperature stages in a furnace, resulting in thermal oxidation and conversion of carbon into gaseous CO₂ which is measured by means of an infrared detector. A temperature measurement of total carbon was applied to quantify both the total organic carbon released up to 400 °C. Elemental composition of Ca, P, Mg, and Na were calculated in which three samples of each material were digested in concentrated HNO₃ and diluted 1:100 in a dilute acidic solution for trace/impurity element determination, and 1:1000 for Ca and P determination. Calibration solutions are prepared by diluting certified multi- and single-element standard solutions. Element concentrations in the solutions are quantified on an Agilent 7700x ICP-MS and evaluated using Agilent “Masshunter” software. To determine volumetric porosity 1.5 g granules were filled into a container and tapped 1000 times under specified conditions on a pneumatic device. Subsequently, the granules protruding a defined upper edge of the container are scraped off and the mass of the granules inside the container is determined on an analytical scale. Finally, the tapped density is obtained by division of the mass by the volume of the container (1.4 cm³).

2.2. Experimental animals

Twenty-four 14–16-month-old female Beagle dogs (Marshall Bioresources, New York, USA) weighing ~6 to 8 kg were allocated to three time point cohorts (4, 12, and 26 weeks) of eight

animals each. The canine model was chosen due to its bone density, mechanical properties, morphological structure and healing capacity similar to the human bone [30].

2.3. Ethics statement

The study protocol was reviewed and approved by the Committee Review of the testing facility, Charles River Laboratories Montreal ULC, Boisbriand, Canada under the ethical approval number 2889-416G. The review ensured compliance with appropriate regulations. The testing facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and the Canadian Council on Animal Care. The reporting of this preclinical study follows the recommendations contained in the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines.

2.4. Study design

For each animal, four independent wall defects were created (two per hemimandible), resulting in a total of 32 defects for each time point (4, 12, and 26 weeks) (Figure 1). Each animal received all four treatments. One material-filled defect (test: WHA + membrane [M] or control: BO + M) and one sham control defect with or without a collagen membrane (sham [Sh] or Sh + M) were randomly allocated to each side of the mandible. Randomization was carried out based on a predetermined allocation plan.

2.5. Surgical procedure

Tooth extraction, defect creation and regenerative procedures were performed in a single step under general anesthesia. Four premolars were extracted on each side of the mandible, and two independent critical-sized wall defects of 10.0 mm (mesio-distally), 8.0 mm (vertically), 6.0 mm (bucco-lingually) were created on each side by totally removing the buccal alveolar plate and partially the lingual one by means of a bone saw disc (Figure 2). No template was used to perform the critical-sized defects. The surgeons calculated the available space of the crest after extraction by means of a periodontal probe, assuring the protocolized measurements of the critical-sized defects as they were making the defect cut. For anatomical reasons, back and frontal defects in each hemimandible slightly differed in size, but this had no impact on the results generated in the study since the attribution of the defects to a control or test group was randomized.

An intraoral scan (IOS) imaging of the edentulous area and of the empty defect was performed to have the baseline dimensions, and then the defect was randomly allocated to one of the four groups. In the groups allocated for a regenerative procedure (WHA and BO), the defect was filled with the biomaterials and a resorbable collagen membrane (Creos Xenoprotect, Nobel Biocare, Switzerland) stabilized with anchor pins (Bone Tac Screw 5.0 mm GDT implants, Israel). The sham defects in control groups were left empty and one was covered with the collagen membrane also fixed with anchor pins.

The muco-periosteal flaps were sutured with nonabsorbable sutures (Seraflex 4/0, Serag Wiessner). To prevent postoperative infection, animals received a prophylactic antibiotic (Cefazolin 25 mg/kg, IV) during the preparation procedures. In the postoperative period, the animals received Carprofen (Rimadyl) 3–5 mg/kg PO (per os) to control postoperative pain and inflammation. Following surgical procedures, the animals were housed in individual cages. Housing was modified to prevent the dogs from chewing on housing material/ devices and possibly damage the surgical site.

2.6. IOS and defect wall homogeneity evaluation

For each defect created, an optical impression from a buccal, coronal, and lingual view (from the first remaining canine tooth to the first remaining molar tooth, both included) was taken using an intraoral scanner (Trios 3[®], 3Shape, Copenhagen, Denmark). Each hemimandible was scanned before defect creation and after defect creation (Figure 3A,B).

IOS scans were used to consistently select bone areas for histological processing and analysis. The scans taken before and after defect creation were superimposed based on the position of the remaining canine and first molar teeth (Figure 3C). The exact size and volume of the defects were calculated using surface-based analysis software (GOM Inspect Professional). The cross-sectional area of the mandibular bone that was present in the scan taken before defect creation but absent in the scan taken after defect creation was used to define the original defect area, named as region of interest (ROI) 1.

2.7. Histology processing

Nondecalcified histology of the retrieved specimens was performed. To match the intraoral scanning data with the specimen, the distance from the center of the bone ridge in between the defects to the region of interest was used to match the slide position. Samples were fixed in 10 % neutral-buffered formalin solution, followed by dehydration in a series of graded ethanol solutions, and finally embedded in methyl-metacrylate resin. For each defect, six sections

Figure 1

Study design: N, number of dogs/specimens; D, day; R, randomization; W, week; n, number of defects. The red arrows indicate extracted teeth (premolar [PM]1–PM4); red rectangles denote the bone defects created.

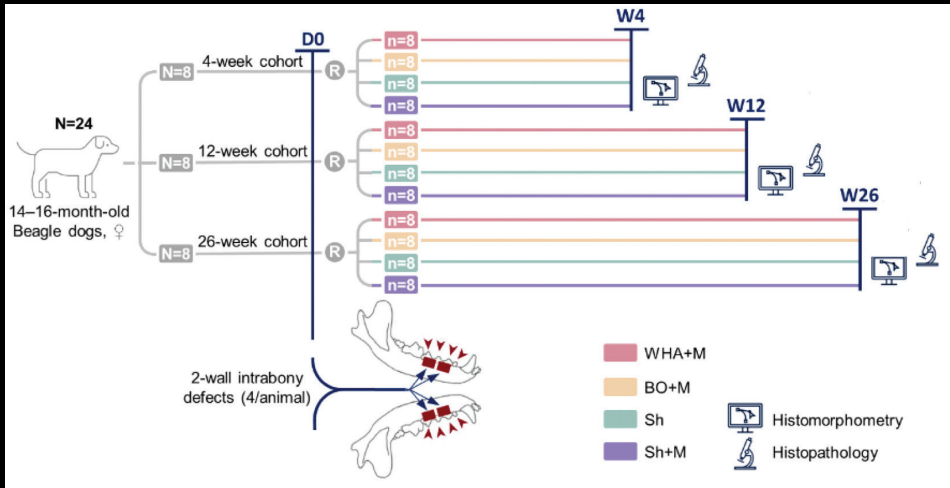
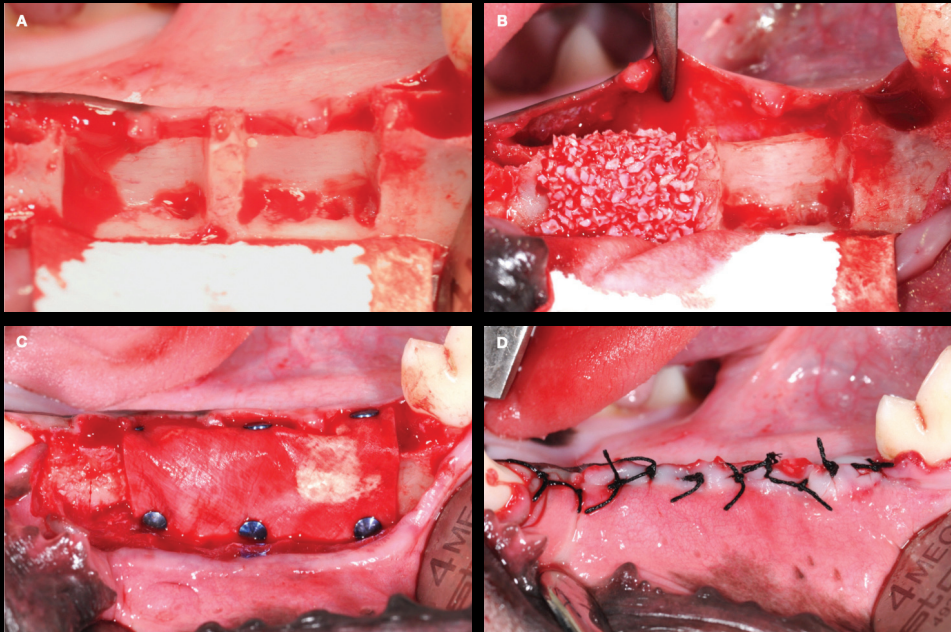


Figure 2

Critical bone defects of each hemimandible. (A) Critical bone defects perform in a hemimandible. (B) BO and Sh + M allocation. (C) Collagen membrane placement anchored with pins. (D) Flap closure.



(20–40 μm thick) were performed in each specimen (two sections located at the center of the defect, two other sections located in the mesial end and two others in the distal end of each defect) (Figure 3D). From these sections, three sections were stained with hematoxylin and eosin for histopathology analysis, and three others were stained with Stevenel's blue for both histomorphometry and histopathology analysis.

2.8. Histomorphometry evaluation

All sections from all bone defects were digitally captured (Clemex Vision Lite Software) to obtain whole-section images by microscope with a 10x objective (Nikon Eclipse LV100ND, Tokyo, Japan). To evaluate the extent of bone regeneration, histomorphometric measurements were performed on selected scans using Image-Pro Premier 9.2 software (© Media Cybernetics, Inc., USA). Depending on the structure to analyze, both manual tracing or automatically measured based on the color and color intensity differences were used.

The defect area (ROI1) was defined precisely for each defect based on IOS measurements. The regenerated area (ROI2) was defined by the surface of the original defect colonized by newly formed bone (Figure 4). For the histomorphometric, analyses were made based on the mean of three sections per defect and the following variables were collected:

- Defect area: defined as the total area of the defect (Region of Interest, ROI1) (Figure 4B).
- The regenerated area (ROI2), defined as the portion of the defect area (ROI1) colonized with newly formed bone (Figure 4C).
- The percentage of newly formed bone (B), biomaterial (M), and soft tissue (S) in the defect area (ROI1).
- The percentage of bone-to-material contact (BMC) characterizing the osteoconductivity and defined as the percentage of the biomaterial perimeter in contact with newly formed bone within the regenerated area (ROI2).

2.9. Descriptive and histopathological evaluation

Descriptive analyses as well as safety and biocompatibility semiquantitative analyses of the implanted materials were evaluated on both Stevenel's blue and Hematoxylin–Eosin stained histology sections of the bone defects by a single pathologist. Sections were analyzed and graded according to the presence of different cell types and tissue responses, including necrosis, fibrosis, and neovascularization (Table A1). An irritancy scoring system adapted from the ISO 10993-6 Annex E [31] was used to compare the biocompatibility of the different study materials. Average irritancy scores for each group were calculated at each time point. Based on

Figure 3

(A) Baseline IOS from the edentulous crest after dental extraction; (B) IOS of the critical bone defects; (C) superimposition of the baseline and critical bone defects IOS's; (D) definition of the area for the histological sections for histomorphometry and histopathology.

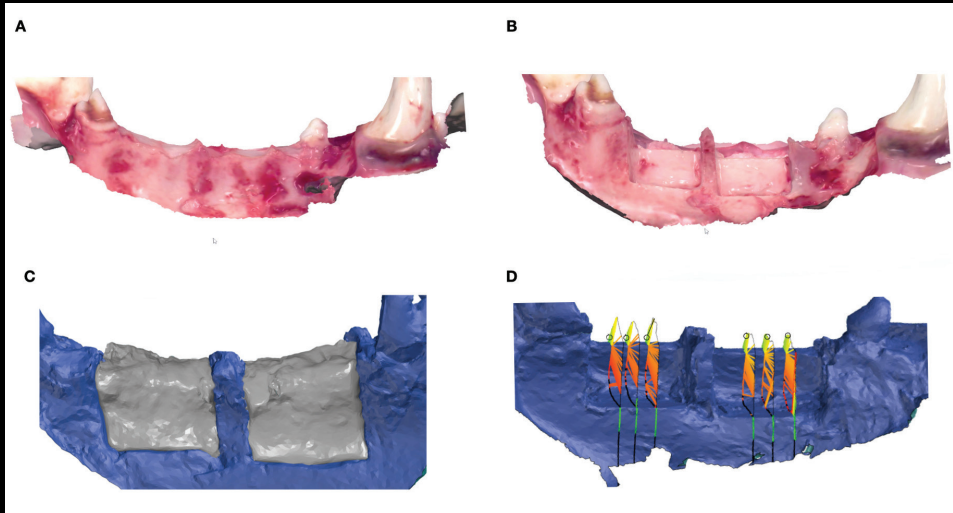


Figure 4

Tracing of ROI1 and ROI2 for histomorphometric analyses. (A) WHA + M at 12 weeks; (B) defect area highlighted in blue (ROI1); (C) regenerated area highlighted in green (ROI2).

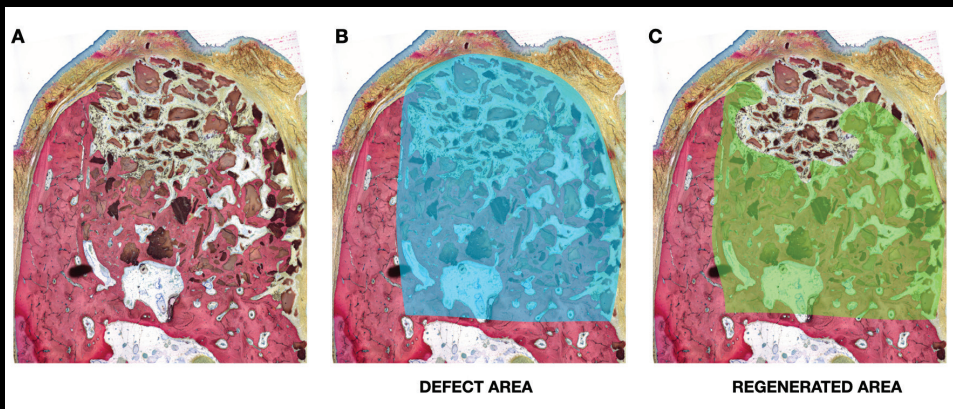


Table 1

Biomaterials characterization: Organic content, phase composition, crystal size, porosity, and specific surface area.

Biomaterial	Organic content	Phase composition (% of HA)	Crystal size (nm)	Porosity (%)	Specific surface area (m ² /g)
WHA	<0.1	100	54.4	82.3	16.0
BO	0.1	100	15.5	83.6	81.7

the difference between the irritancy score of WHA + M and that of the comparator treatments, WHA + M was to be considered as nonirritant at ≤ 2.9 points difference, a slight irritant at 3.0–8.9 points difference, a moderate irritant at 9.0–15.0 points difference, and a severe irritant at >15 points difference.

2.10. Statistics analyses

2.10.1. Sample size calculation

The sample size was calculated using the software Gpower 3.1 [32]. Assuming an effect size of 0.6, an alpha error of 0.004 (corrected for multiple comparisons), and a power of 95 %, the obtained total sample size was 95. Thus, 24 animals received one of each four conditions and were randomly divided into three cohorts for the three observation periods (4, 12, and 26 weeks), giving a total sample size of 96.

2.10.2. STATISTICAL TESTS

Means and standard derivations were reported for quantitative outcomes. A mixed effect model with individual animal as a random effect and treatment, location, time and the interaction between week and treatment was used to compare WHA against BO and sham controls. Analysis of variance was performed using SAS 9.4 (SAS Institute Inc., NC, USA) to identify the differences of least square means between the treatments. The Tukey–Kramer method of multiple testing correction was used.

3. RESULTS

3.1. Study design

All study animals survived until the scheduled sacrifice. Their body weight remained stable over the course of the study and was consistent with the experimental model. IOS measurements demonstrated that the size of the defects, as well as the defect walls, were homogenous. For anatomical reasons, back and front defects slightly differed in size, but this had no impact on the results generated in the study, since attribution of the defects to a group was randomized. At 12-weeks, one animal had notably higher inflammation associated with both WHA and BO defects, as well as inflammation associated with the sham defects. In addition, there was significant migration of particles out of one of the WHA defects in another animal. To present the effect of implanted biomaterials on bone regeneration more accurately, results from these five outlier defects were excluded from the analysis; therefore, week 12 data are based on six defects for the WHA group and on seven defects for the BO, Sh and Sh + M groups.

3.2. Physicochemical characterization

The XRD analysis demonstrate that the crystallographic phase composition of all WHA and BO samples consists in 100 % of hydroxyapatite with no secondary phase (limit of detection ≤ 0.88 wt %). Hence, regarding phase composition, the WHA and BO products are equivalent. The crystal size is lower in BO compared to WHA samples which is a parameter influenced by the heat treatment at high temperatures increasing the crystal size and decreasing solubility of hydroxyapatite bone substitutes. Specific surface area analysis showed that the mean for WHA and BO samples are $16 \text{ m}^2/\text{g}$ and $81.7 \text{ m}^2/\text{g}$, respectively.

These results demonstrate that WHA presents a moderate specific surface area while BO presents a high specific surface. The results of organic carbon released up to 400°C showed that a low amount, if any, of organic residues are found in both WHA and BO products given that the values for the 6 samples are below or equal to 0.1 wt % where <0.1 designates the limit of determination. Volumetric porosity analysis reveals that WHA and BO products have a similar porosity.

The results of the above-mentioned characterizations are presented in Table 1. Regarding the elemental composition (Table 2), the results obtained for the six samples show that Ca, P, Mg, and Na concentrations in WHA and Bio-Oss products are similar.

3.3. SEM characterization

The micro-organization of the surface was observed at magnification 500x and 1000x. SEM images of WHA and BO are displayed in Figure 5. Fibril-like structures are observed in both materials with a periodical organization of the collagen fibrils and a close contact between collagen and hydroxyapatite within the bone matrix. The observation of this micro-organization demonstrates that WHA like Bio-Oss, retrieves the main characteristics of bone hydroxyapatite with its microporous and rough surface.

3.4. Quantitative histomorphometric analysis

3.4.1. % REGENERATED AREA (ROI2/ROI1) (4, 12, AND 26 WEEKS)

At 4 weeks, the percentage of regeneration in the defect area ROI2 was significantly higher in the WHA group (46.8 %) compared to the BO group (21.4 %) and the sham control groups (Sh (15.1 %), Sh + M (23.1 %)) ($p < 0.05$) (Figure 6). At 12 weeks, bone regeneration was significantly higher in both the WHA (70.7 %) and BO (73.9 %) compared to the sham control groups (respectively, Sh (17.6 %), Sh + M (33.2 %)) with no significant differences between the two biomaterial groups. At 26 weeks, a higher percentage of regeneration was observed in the WHA + M (59.9 %) compared to both sham control groups as well as in the BO + M (54.3 %) group compared to the Sh group.

3.4.2. NEWLY FORMED BONE + BIOMATERIAL WITHIN DEFECT AREA (ROI1) (4, 12, AND 26 WEEKS)

At 4 weeks, the percentage of newly formed bone was significantly higher in the WHA group (9.5 %) compared to the BO group (5.8 %) (Figure 7). The BO group (5.8 %) displayed less newly formed bone than the two shams with a significant difference with the Sh + M (9.3 %). At 12 weeks, the percentage of newly formed bone was no significantly different in the two biomaterial groups (WHA: 23.4 %, BO: 30.9 %). Moreover, the Sh + M (21.9 %) presented significantly more newly formed bone than the Sh without membrane. At 26 weeks, the percentage of newly formed bone was stable compared to the 12 weeks cohort and no significant differences are observed in between the biomaterial groups (WHA: 23.8 %, BO: 25.8 %).

The percentage of biomaterial presented no significant differences in between implanted groups in the three time points (Figure 7). However, a decrease was observed at 26 weeks in the BO groups.

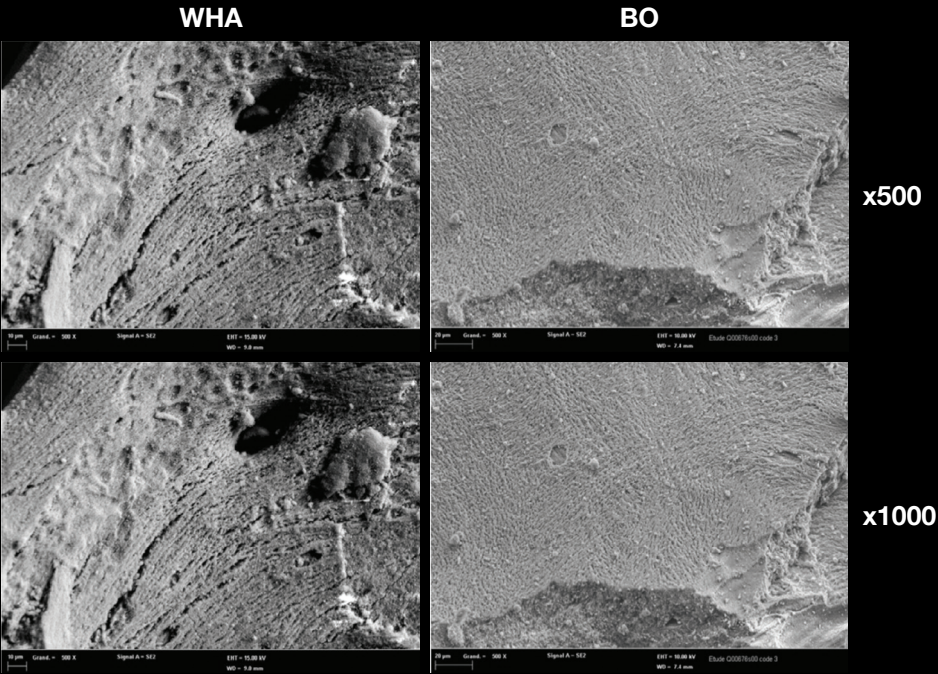
Table 2

Elemental composition analysis for Ca, P, Mg, and Na.

Biomaterial	Ca (%w/w)	P (%w/w)	Mg (%w/w)	Na (%w/w)
WHA	38.9	18.1	0.64	0.38
BO	37.3	17.0	0.59	0.46

Figure 5

Scanning electronic micrographs (SEM) of the studied biomaterials at different magnifications: WHA and BO with 500x and 1000x magnification. WHA and Bio-Oss, presenting both microporous and rough surface.



3.5. BMC (ROI2) (4, 12, and 26 weeks)

The bone-to-material contact within regenerated area ROI2 increased over the three time points at similar rates in the WHA and the BO groups with no significant differences between the two groups (Figure 8).

3.6. Descriptive, safety, and biocompatibility analyses

After 4, 12, and 26 weeks, newly formed bone was found in the defect of all samples. Macroscopically (Figure 9), in both sham groups, woven bone was observed at 4 weeks while at 12 and 26 weeks a more cortical lamellar bone was observed with a noticeable lack of buccal bone volume. In the biomaterial groups, woven bone was predominately present in the WHA group at 4 weeks when compared to BO, nevertheless, at 12 and 26 weeks both groups present more mature bone in between the particles in the regenerated area.

Microscopically (Figure 10), in the implanted groups (WHA and BO) at 4 weeks the newly formed bone was immature and with thick trabeculae. At 12 and 26 weeks, the newly formed bone was in close contact with the biomaterial particles in both groups and bridged the particles together. At 12 weeks, the newly formed bone was still immature compared to the bone at 26 weeks displaying a lamellar organization. Some chains of osteoblasts are also observed close to the newly formed bone as well as some blood vessels, with no visible differences between the groups.

Histopathological examination revealed minimal inflammation within the defect area across all three time points and in all four groups. At all three time points, there was minimal inflammation attributed to either WHA + M or BO + M, and no necrosis or fatty infiltrate was observed. WHA + M was considered a nonirritant compared to BO + M at all three time points, and a slight irritant compared to Sh and Sh + M at 12 and 26 weeks (Table 3). At the 12-week time point, one animal (W12-02) exhibited an unexplained, generalized inflammatory reaction across all four of its defects, and one additional WHA + M defect (W12-06 BL) showed marked particle migration. Following the testing facility's approach, the histomorphometric outcomes were summarized both including and excluding these outliers. The exclusion affected only the 12-week cohort; the 4-week and 26-week results were unchanged. A sensitivity comparison confirmed that the exclusion did not alter any conclusion: the percentage of regeneration remained statistically comparable between WHA + M and BO + M whether the outliers were included ($63.8 \pm 24.8\%$ vs $68.3 \pm 26.1\%$) or excluded ($70.7 \pm 23.0\%$ vs $73.9 \pm 22.4\%$), and bone-to-material contact within ROI2 likewise remained equivalent between the two grafts in both analyses (66.8% vs 65.1% included; 70.6% vs 67.5% excluded). Including the outliers increased outcome variability,

Figure 6

Evolution of defect area regeneration over time. Week 4 is marked by significant differences in between WHA + M and all other groups ($p < 0.05$). At week 12 both WHA + M and BO + M present significant differences in between both Sh and Sh + M groups. Week 26 WHA + M maintains its significant differences in between Sh and Sh + M. BO + M show significant differences only to Sh group.

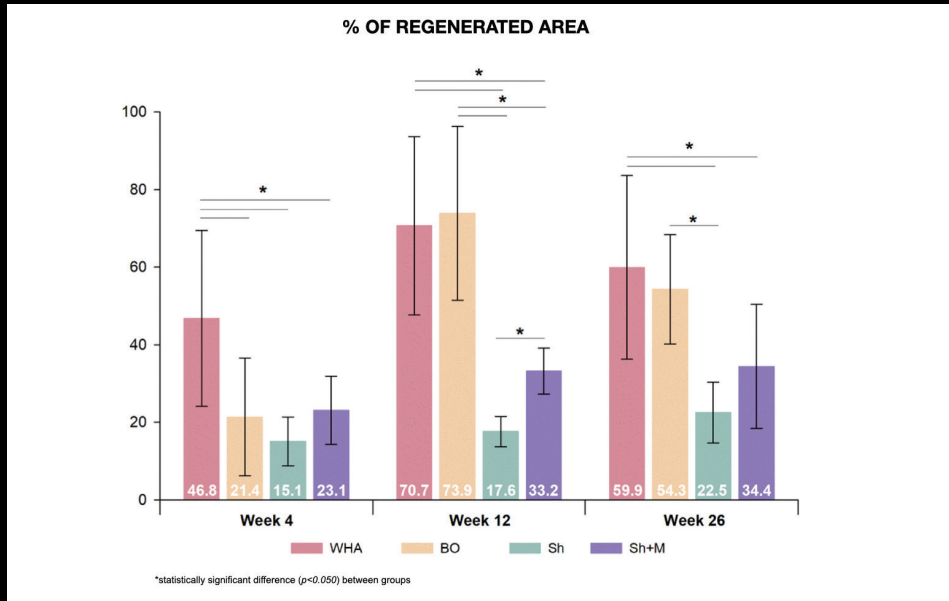


Figure 7

(A) Evolution of % newly formed bone overtime in all groups. At week 4 statistically significant differences in between WHA + B and BO + M ($p < 0.05$) and between Sh + M and BO + M ($p < 0.05$). At week 12 statistically significant differences in both WHA + M and BO + M when compared to Sh ($p < 0.05$) and significant differences in between Sh + M and Sh. At week 26 significant differences and maintained in both WHA + M and BO + M when compared to Sh ($p < 0.05$). (B) % of biomaterial over time within ROI1 in WHA + M and BO + M with no statistically significant differences in between groups during the 3 time points.

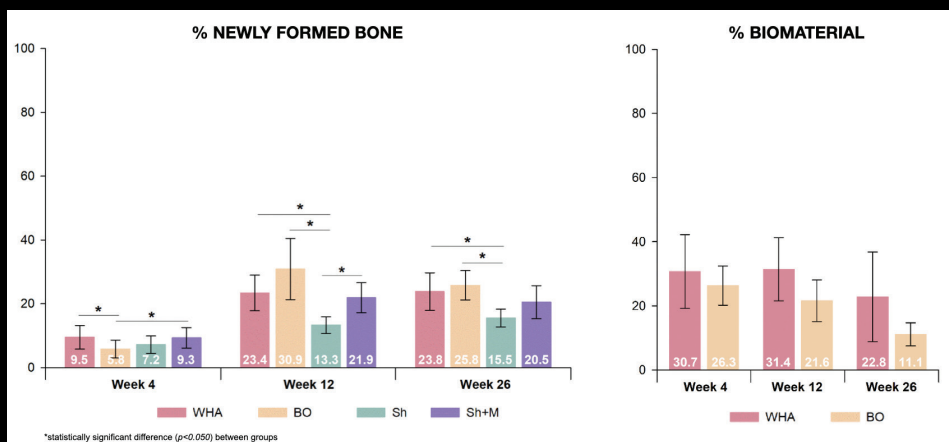


Figure 8

Evolution of bone-to-material contact within ROI2 in the groups WHA + M and BO + M during the 3 time points. No statistically significant differences in between groups.

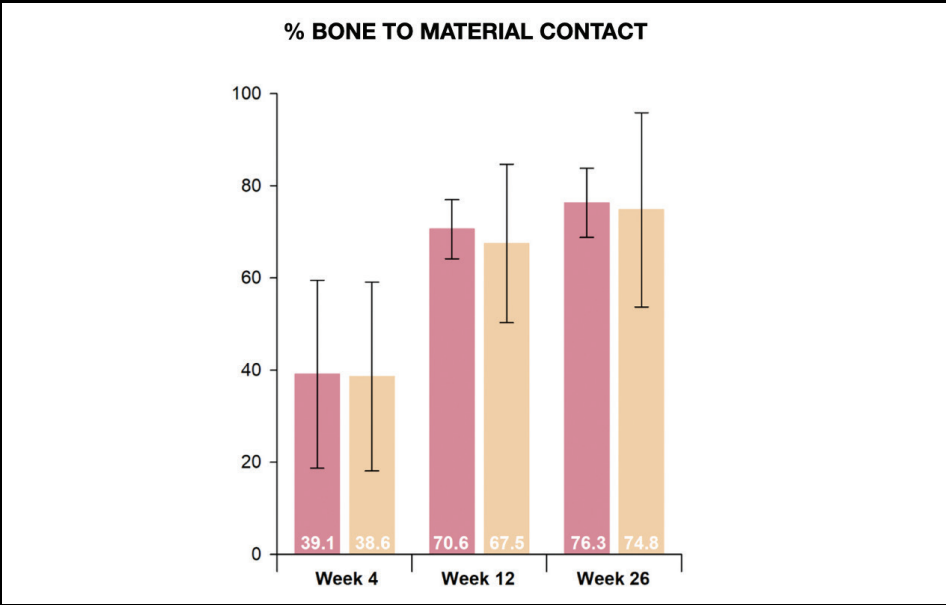


Figure 9

Descriptive histology at 4, 12, and 26 weeks. BP, bone particle; FT, fibrous connective tissue; NB, newly formed bone. The red squares correspond to the magnification areas presented in Figure 10.

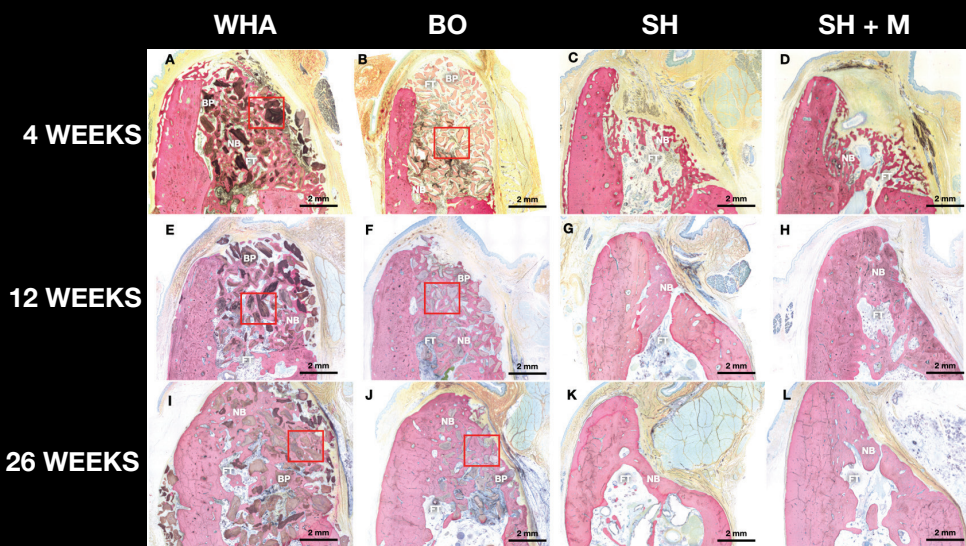


Figure 10

High magnification histological samples of both WHA and BO groups in the different cohorts, corresponding to the red squares areas illustrated in Figure 9. At 4 weeks it is possible to observe that WHA presents a more osteoconductive behavior in between particles when compared to BO. At 12 and 26 weeks both biomaterials presents a similar osteoconductive behavior in between particles.

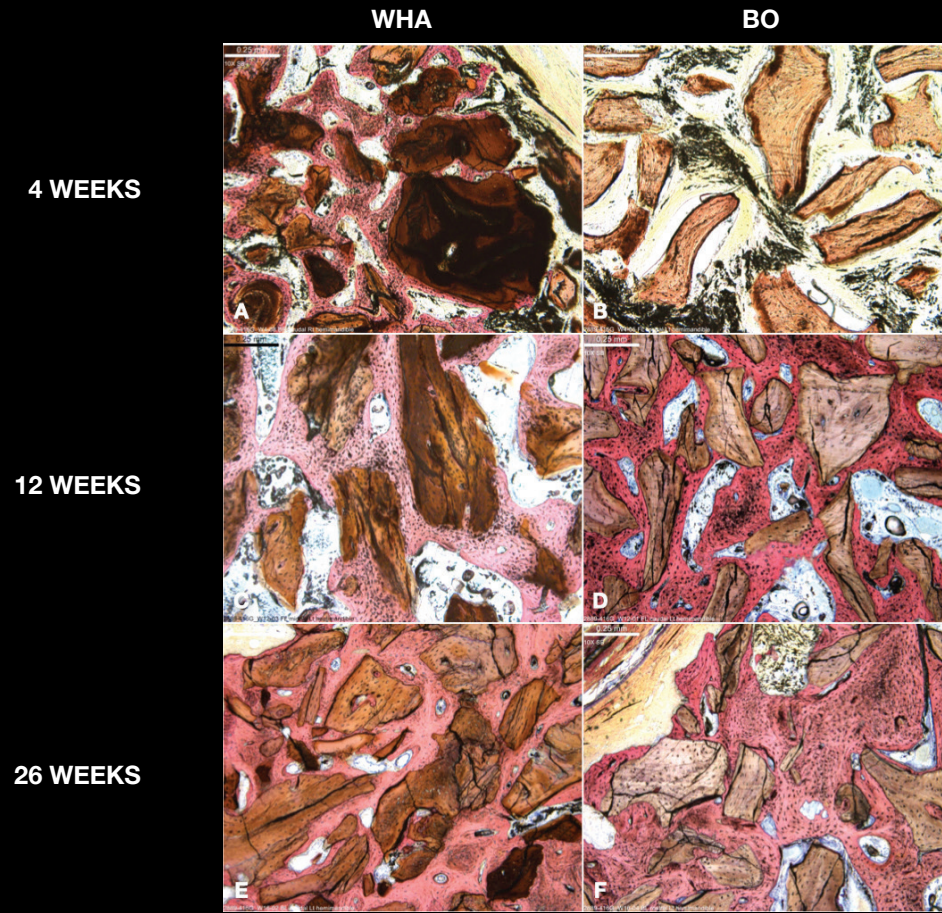


Table 3

Average histopathological irritancy scoring
Abbreviations: BO, control hydroxyapatite; M, membrane; Sh, sham; WHA, test hydroxyapatite.

Time point	Treatments			
	WHA + M	BO + M	Sh	Sh + M
4 weeks	11.0	17.1	14.6	14.3
12 weeks	9.9	8.4	2.7	5.0
26 weeks	9.6	7.1	1.6	4.3

as expected, but preserved the direction and significance of all comparisons, confirming the robustness of the reported findings.

The higher irritancy scores in the WHA + M group at weeks 12 and 26 were partly due to higher scores for fibrosis and neovascularization (data not shown). In the WHA + M group, fibrosis was observed under the membrane, within the defect area, and surrounded the WHA granules outside of the area of new bone formation; conversely, for the Sh and Sh + M groups, the periosteum/ membrane generally directly (or almost directly) overlaid the area of new bone formation that was associated with little fibrosis.

In all groups, there was low amount of neovascularization and fibrosis over time; in the WHA + M and BO + M groups, it was due to the growing area of new bone and marrow that replaced the fibrous tissue surrounding the granules, while in the sham control groups, it was largely due to the healing process and further collapse of the membrane/periosteum over the growing area of new bone.

4. DISCUSSION

The present study was able to assess the biocompatibility and biological performance of WHA for guided bone regeneration in comparison to a widely used DBBM (BO), to the absence of filling biomaterial with and without a collagen membrane in a canine mandibular critical-sized bone defect model. Both biomaterials promoted bone regeneration in a similar extent and were not suspected to cause any predisposing factors to the pathogenesis of any of the observed clinical signs, revealing an excellent safety profile. Nevertheless, at 4 weeks, almost 50 % of the defects were regenerated in the WHA group while only 21 % of regeneration was found in the BO group, suggesting a different regenerative kinetic and higher osteoconductive properties for WHA. One hypothesis for this regenerative behavior at 4 week time point could be the different specific surface area (SSA) between the materials (BO 79.7 m²/g [33] vs WHA 16.0 m²/g according to the manufacturer). Specific surface area has been often mentioned in the literature as a significant parameter for bone regeneration [34–36]. However, these different SSA values, translates in different micro and nanoporosities between the materials. The higher micro and nanoporosities of BO might increase Ca²⁺ uptake from the extracellular fluids, decreasing its availability in the extracellular environment. WHA having less micro and nanoporosities, may allow more extracellular Ca²⁺ availability enhancing the effect of BMP-2 on Osteocalcin, Runx2 and Osterix expression, promoting faster bone regeneration in vivo [37,38].

At 4 weeks, BO showed significantly less % of newly formed bone of when compared to Sh + M, This results are in accordance to the literature [39,40] where in early stages of healing BO can delay the healing when compared to nongrafted sites.

The crystal size is lower for BO samples compared to WHA samples. It is indeed known that heating treatments at high temperatures increase the crystal size and decrease the solubility of hydroxyapatite bone substitutes, which is a parameter that might only impact the performances [41]. However, as demonstrated by our group similar performances for the unsintered hydroxyapatite and the hydroxyapatite sintered at mid-range temperature (820 °C) proving that a crystal size up to 120 nm has no impact on the performances of hydroxyapatite from bovine origin. Based on that, the higher crystal size of WHA compared to BO has no impact on its performances.

Moreover, bone-to-material-contact (BMC) was similar in the WHA and BO groups and appeared to increase over time at a similar rate. This parameter allows the evaluation of the osteointegration and osteoconductive behavior of the bone substitutes and can be correlated to surface topography and/or to sintering temperatures used in the manufacturing process, as suggested in our previous data. The manufacturing process of both bone fillers used in the present study allow to preserve a certain surface roughness, favoring cell colonization, osteoconductivity and better bone regeneration [15,24]. High osteoconductive properties are relevant from a regenerative point of view since a tight network between bone and biomaterial play an important role in implant primary stability and implant survival rates [15,42,43].

From week 12 onwards, the use of either bone filler materials resulted in increased bone regeneration when compared to Sh and Sh + M group. This shows that the use of a bone filler material under collagen membrane enhances bone regeneration supporting mineralization process and remodeling, when compared to the use of a membrane alone or no membrane as described in several studies [44–46]. The key role of barrier membranes for GBR procedures in preventing the ingress of cells from the surrounding soft tissues, favoring osteoprogenitor cells for new bone formation is well described on the literature [47,48]. The results of the Sh + M group in the present study clearly show the role of the barrier membrane in the % of new bone formation, since this group performs significantly better than BO + M at 4 weeks, significantly better than Sh group at 12 weeks and presented at 26 weeks equivalent results than WHA + M and BO + M.

At 26 weeks, the % of regenerated area in the biomaterial groups decreases from the previous time point at 12 weeks. This can be explained by a residual remodeling of the crest, resorption of the biomaterial at 26 weeks [49] and the fact that the region of interest that was used to calculate this variable was the ROI1, corresponding to the baseline ROI before defect creation.

Volume stability over time also represents a crucial factor for the success of bone regeneration procedures and it is affected by the biodegradation rate of biomaterials [37,50] which depends on various factors like microporosities, chemical structure, crystallinity and local biological environment [51,52]. In the present study, it was not possible to assess the biodegradation rate of the studied biomaterials. Nevertheless, at 26 weeks WHA group presented a % of biomaterial with no significant differences when compared to BO group described in the literature as a biomaterial with high dimensional maintenance, [25,39,53] suggesting that WHA might be suitable for longterm volume stability at the regenerated sites.

The main strength of the study lies in the extent of data collected, with regards to time, space, and treatment groups. Bone regeneration was evaluated over the course of 6 months, which is a relatively long period for an *in vivo* study. Another strength was the IOS methodology used to record the original defect area (ROI1) consistently and precisely between defects in different treatment groups. This registration was particularly important to overcome limitations imposed by the collapsing of membranes in Sh + M group and the collapse of the soft tissue in Sh group. The IOS methodology was able to precisely define the actual physical surface of the mandible prior to and after creation of the defects and based upon the measurements done during histologic processing to determine the precise location of the slide area within the defect [54]. To our knowledge this is the first study using this volumetric methodology contributing for the precision of the histomorphometric results. In addition, the study was designed to include three control groups: one positive control treated with BO + M, and two negative controls (with or without a resorbable membrane) and for each defect, three histological slides were analyzed for histomorphometry. These characteristics allowed a more comprehensive view of the bone regeneration induced by the study materials.

The present study presents some limitations. It should be emphasized that the collection of the central and two lateral histological slides, due to technical limitations, was not always possible. However, the likelihood of any bias arising from this limitation is low, since it occurred in all treatment groups. Another limitation is related to the absence of immunohistochemical analysis. This could provide information about the bone regeneration cellular mechanism in both tested materials, providing broader information about the variations observed at 4 weeks with WHA + M and BO + M.

5. CONCLUSIONS

WHA and BO resulted in similar at 12 and 26 weeks, with higher regenerative performance when compared to the sham controls. However, at 4 weeks, the defect regeneration was significantly enhanced by WHA compared to all other treatments. WHA showed excellent safety and biocompatibility at all three time points, which was comparable to that of BO.

Author Contributions:

Emilie Dory, Caroline Trus, Geoffrey Lecloux, France Lambert, and Eric Rompen designed the study; Geoffrey Lecloux, Bruno De Carvalho, and Eric Rompen carried out the study interventions and partially collected the data; Loïc Germain and Caroline Trus analyzed the data results outsourced; Bruno De Carvalho led the writing. All authors critically revised the manuscript and have given their approval for the final version to be published.

Acknowledgments:

The authors thank AccellAB for conducting the experimental part of the study, Pharmalex, Timea Kiss, and Souheil Salem.

Funding information:

This work was funded by Wishbone SA. Wishbone SA was involved in all stages of the conduct and analysis of the studies and covered the costs associated with the development and publishing of the present manuscript.

Conflicts of Interest statement:

This study was funded by Wishbone SA. E.D., J.P. and C.T. are currently employees of Wishbone SA. L.G. was an employee of Wishbone SA during the study. F.L., G.L. and E.R. are founders and shareholders of Wishbone SA, and E.R. is a founder and shareholder of Wishbone Cy; their contribution to the study was limited and neither of them were involved in the analysis and interpretation of the results. For the histological processing and performance analyses, this study was outsourced in GLP conditions. B.D.C. declares no competing interests.

Data availability statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Bruno De Carvalho  <https://orcid.org/0000-0001-9527-7616>

REFERENCES

- 1 Elani HW, Starr JR, Da Silva JD, Gallucci GO. **Trends in dental implant use in the U.S., 1999–2016, and projections to 2026.** J Dent Res. 2018;97(13):1424-1430. doi:10.1177/0022034518792567
- 2 Aghaloo TL, Moy PK. **Which hard tissue augmentation techniques are the most successful in furnishing bony support for implant placement?** Int J Oral Maxillofac Implants. 2007;22(suppl):49-70.
- 3 Esposito M, Grusovin MG, Rees J, et al. **Interventions for replacing missing teeth: augmentation procedures of the maxillary sinus.** Cochrane Database Syst Rev. 2010;3:CD008397. doi:10.1002/14651858.Cd008397
- 4 Jensen SS, Terheyden H. **Bone augmentation procedures in localized defects in the alveolar ridge: clinical results with different bone grafts and bone-substitute materials.** Int J Oral Maxillofac Implants. 2009;24-(suppl):218-236.
- 5 Jepsen S, Schwarz F, Cordaro L, et al. **Regeneration of alveolar ridge defects. Consensus report of group 4 of the 15th European workshop on periodontology on bone regeneration.** J Clin Periodontol. 2019;46-(suppl 21):277-286. doi:10.1111/jcpe.13121
- 6 Naenni N, Lim HC, Papageorgiou SN, Hämmerle CHF. **Efficacy of lateral bone augmentation prior to implant placement: a systematic review and meta-analysis.** J Clin Periodontol. 2019;46(suppl 21):287-306. doi:10.1111/jcpe.13052
- 7 Sanz-Sánchez I, Ortiz-Vigón A, Sanz-Martín I, Figuero E, Sanz M. **Effectiveness of lateral bone augmentation on the alveolar crest dimension: a systematic review and meta-analysis.** J Dent Res. 2015; 94(9 suppl):128s-142s. doi:10.1177/0022034515594780
- 8 Chappuis V, Rahman L, Buser R, Janner SFM, Belser UC, Buser D. **Effectiveness of contour augmentation with guided bone regeneration: 10-year results.** J Dent Res. 2018;97(3):266-274. doi:10.1177/0022034517737755

- 9** Chiapasco M, Abati S, Romeo E, Vogel G. **Clinical outcome of autogenous bone blocks or guided bone regeneration with the PTFE membranes for the reconstruction of narrow edentulous ridges.** Clin Oral Implants Res. 1999;10(4):278-288. doi:10.1034/j.1600-0501.1999.100404.x
- 10** Reynolds MA, Aichelmann-Reidy ME, Branch-Mays GL. **Regeneration of periodontal tissue: bone replacement grafts.** Dent Clin N Am. 2010;54(1):55-71. doi:10.1016/j.cden.2009.09.003
- 11** Shamsoddin E, Houshmand B, Golabgiran M. **Biomaterial selection for bone augmentation in implant dentistry: a systematic review.** J Adv Pharm Technol Res. 2019;10(2):46-50. doi:10.4103/japtr.JAPTR_327_18
- 12** Dasmah A, Thor A, Ekestubbe A, Sennerby L, Rasmusson L. **Particulate vs. block bone grafts: three-dimensional changes in graft volume after reconstruction of the atrophic maxilla, a 2-year radiographic follow-up.** J Craniomaxillofac Surg. 2012;40(8):654-659. doi:10.1016/j.jcms.2011.10.032
- 13** Esposito M, Felice P, Worthington HV. **Interventions for replacing missing teeth: augmentation procedures of the maxillary sinus.** Cochrane Database Syst Rev. 2014;5:CD008397. doi:10.1002/14651858.CD008397.pub2
- 14** Hallman M, Thor A. **Bone substitutes and growth factors as an alternative/complement to autogenous bone for grafting in implant dentistry.** Periodontol 2000. 2008;2000(47):172-192. doi:10.1111/j.1600-0757.2008.00251.x
- 15** Lambert F, Bacevic M, Layrolle P, Schüpbach P, Drion P, Rompen E. **Impact of biomaterial microtopography on bone regeneration: comparison of three hydroxyapatites.** Clin Oral Implants Res. 2017;28(10): e201-e207. doi:10.1111/clr.12986
- 16** Kolk A, Handschel J, Drescher W, et al. **Current trends and future perspectives of bone substitute materials – from space holders to innovative biomaterials.** J Craniomaxillofac Surg. 2012;40(8):706-718. doi:10.1016/j.jcms.2012.01.002

- 17 Trajkovski B, Jaunich M, Müller WD, Beuer F, Zafiropoulos GG, Houshmand A. **Hydrophilicity, viscoelastic, and physicochemical properties variations in dental bone grafting substitutes.** *Materials (Basel)*. 2018;11(2):215. doi:10.3390/ma11020215
- 18 Berberi A, Samarani A, Nader N, et al. **Physicochemical characteristics of bone substitutes used in oral surgery in comparison to autogenous bone.** *Biomed Res Int*. 2014;2014:320790. doi:10.1155/2014/320790
- 19 Al-Sanabani JS, Madfa AA, Al-Sanabani FA. **Application of calcium phosphate materials in dentistry.** *Int J Biomater*. 2013;2013:876132. doi:10.1155/2013/876132
- 20 Epple M, Ganesan K, Heumann R, et al. **Application of calcium phosphate nanoparticles in biomedicine.** *J Mater Chem*. 2010;20(1):18-23. doi:10.1039/B910885H
- 21 Filippi M, Born G, Chaaban M, Scherberich A. **Natural polymeric scaffolds in bone regeneration.** *Front Bioeng Biotechnol*. 2020;8:474. doi:10.3389/fbioe.2020.00474
- 22 Gunduz O, Erkan EM, Daglilar S, Salman S, Agathopoulos S, Oktar FN. **Composites of bovine hydroxyapatite (BHA) and ZnO.** *J Mater Sci*. 2008;43(8):2536-2540. doi:10.1007/s10853-008-2497-1
- 23 Sanz M, Dahlin C, Apatzidou D, et al. **Biomaterials and regenerative technologies used in bone regeneration in the craniomaxillofacial region: consensus report of group 2 of the 15th European workshop on periodontology on bone regeneration.** *J Clin Periodontol*. 2019;46-(suppl 21):82-91. doi:10.1111/jcpe.13123
- 24 De Carvalho B, Rompen E, Lecloux G, et al. **Effect of sintering on in vivo biological performance of chemically deproteinized bovine hydroxyapatite.** *Materials (Basel)*. 2019;12(23):3946. doi:10.3390/ma12233946
- 25 Lambert F, Léonard A, Drion P, Sourice S, Layrolle P, Rompen E. **Influence of space-filling materials in subantral bone augmentation: blood clot vs. autogenous bone chips vs. bovine hydroxyapatite.** *Clin Oral Implants Res*. 2011;22(5):538-545. doi:10.1111/j.1600-0501.2010.02069.x

- 26 Baldini N, De Sanctis M, Ferrari M. **Deproteinized bovine bone in periodontal and implant surgery.** Dent Mater. 2011;27(1):61-70. doi:10.1016/j.dental.2010.10.017
- 27 US FDA. **Wishbone HA marketing authorization approval.** 2021. https://www.accessdata.fda.gov/cdrh_docs/pdf21/K211551.pdf
- 28 Rompen E, Lambert F, Lecloux G, Moniotte P. **Materiau de régénération osseuse et son procédé de fabrication.** Patent WO 2015/049336 A1(12). 2015.
- 29 US FDA. GEISTLICH BIO-OSS®. 510(k) **Summary.** 2013. https://www.accessdata.fda.gov/cdrh_docs/pdf12/k122894.pdf
- 30 Aerssens J, Boonen S, Lowet G, Dequeker J. **Interspecies differences in bone composition, density, and quality: potential implications for in vivo bone research.** Endocrinology. 1998;139(2):663-670. doi:10.1210/endo.139.2.5751
- 31 International Organization for Standardization. ISO 10993-6:2016 (en). **Biological evaluation of medical devices—Part 6: Tests for local effects after implantation.** 2016. <https://www.iso.org/obp/ui#iso:std:iso:10993:-6:ed-3:v1:en>
- 32 Faul F, Erdfelder E, Lang A-G, Buchner A. **G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences.** Behav Res Methods. 2007;39(2):175-191. doi:10.3758/bf03193146
- 33 Weibrich G, Trettin R, Gnoth SH, Götz H, Duschner H, Wagner W. **Determining the size of the specific surface of bone substitutes with gas adsorption.** Mund Kiefer Gesichtschir. 2000;4(3):148-152. doi:10.1007/s100060050187
- 34 Böhner M. **Calcium orthophosphates in medicine: from ceramics to calcium phosphate cements.** Injury. 2000;31(suppl 4):37-47. doi:10.1016/s0020-1383(00)80022-4
- 35 Habibovic P, Kruijt MC, Juhl MV, et al. **Comparative in vivo study of six hydroxyapatite-based bone graft substitutes.** J Orthop Res. 2008;26(10):1363-1370. doi:10.1002/jor.20648

- 36** Patel N, Gibson IR, Ke S, Best SM, Bonfield W. **Calcining influence on the powder properties of hydroxyapatite.** J Mater Sci Mater Med. 2001;12(2):181-188. doi:10.1023/a:1008986430940
- 37** Aquino-Martínez R, Artigas N, Gámez B, Rosa JL, Ventura F. **Extracellular calcium promotes bone formation from bone marrow mesenchymal stem cells by amplifying the effects of BMP-2 on SMAD signalling.** PLoS One.2017;12(5):e0178158. doi:10.1371/journal.pone.0178158
- 38** Kwon S-H, Jun Y-K, Hong S-H, Lee I-S, Kim H-E, Won YY. **Calcium phosphate bioceramics with various porosities and dissolution rates.** J Am Ceram Soc. 2002;85(12):3129-3131. doi:10.1111/j.1151-2916.2002.tb00599.x
- 39** Araújo M, Linder E, Lindhe J. **Effect of a xenograft on early bone formation in extraction sockets: an experimental study in dog.** Clin Oral Implants Res. 2009;20(1):1-6. doi:10.1111/j.1600-0501.2008.01606.x
- 40** Cardaropoli G, Araújo M, Hayacibara R, Sukekava F, Lindhe J. **Healing of extraction sockets and surgically produced – augmented and nonaugmented – defects in the alveolar ridge. An experimental study in the dog.** J Clin Periodontol. 2005;32(5): 435-440. doi:10.1111/j.1600-051X.2005.00692.x
- 41** Liu Q, Huang S, Matinlinna JP, Chen Z, Pan H. **Insight into biological apatite: physiochemical properties and preparation approaches.** Biomed Res Int. 2013; 2013:929748. doi:10.1155/2013/929748
- 42** Lambert F, Leonard A, Lecloux G, Sourice S, Pilet P, Rompen E. **A comparison of three calcium phosphate-based space fillers in sinus elevation: a study in rabbits.** Int J Oral Maxillofac Implants. 2013;28(2):393-402. doi:10.11607/jomi.2332
- 43** Molly L, Vandromme H, Quirynen M, Schepers E, Adams JL, van Steenberghe D. **Bone formation following implantation of bone biomaterials into extraction sites.** J Periodontol. 2008;79(6):1108-1115. doi:10.1902/jop.2008.070476

- 44 Dimitriou R, Mataliotakis GI, Calori GM, Giannoudis PV. **The role of barrier membranes for guided bone regeneration and restoration of large bone defects: current experimental and clinical evidence.** BMC Med. 2012;10:81. doi:10.1186/1741-7015-10-81
- 45 Friedmann A, Gissel K, Soudan M, Kleber BM, Pitaru S, Dietrich T. **Randomized controlled trial on lateral augmentation using two collagen membranes: morphometric results on mineralized tissue compound.** J Clin Periodontol. 2011;38(7):677-685. doi:10.1111/j.1600-051X.2011.01738.x
- 46 Merli M, Moscatelli M, Mariotti G, Pagliaro U, Raffaelli E, Nieri M. **Comparing membranes and bone substitutes in a one-stage procedure for horizontal bone augmentation. Three-year post-loading results of a double-blind randomised controlled trial.** Eur J Oral Implantol. 2018;11(4):441-452.
- 47 Aprile P, Letourneur D, Simon-Yarza T. **Membranes for guided bone regeneration: a road from bench to bedside.** Adv Healthc Mater. 2020;9(19):e2000707.doi:10.1002/adhm.202000707
- 48 Retzepi M, Donos N. **Guided bone regeneration: biological principle and therapeutic applications.** Clin Oral Implants Res. 2010;21(6):567-576. doi:10.1111/j.1600-0501.2010.01922.x
- 49 Elnayef B, Porta C, Suárez-López Del Amo F, Mordini L, Gargallo-Albiol J, Hernández-Alfaro F. **The fate of lateral ridge augmentation: a systematic review and meta-analysis.** Int J Oral Maxillofac Implants. 2018;33(3):622-635. doi:10.11607/jomi.6290
- 50 Chiba S, Anada T, Suzuki K, et al. **Effect of resorption rate and osteoconductivity of biodegradable calcium phosphate materials on the acquisition of natural bone strength in the repaired bone.** J Biomed Mater Res A. 2016;104(11):2833-2842. doi:10.1002/jbm.a.35828
- 51 Hannink G, Arts JJ. **Bioresorbability, porosity and mechanical strength of bone substitutes: what is optimal for bone regeneration?** Injury. 2011;42(suppl 2):S22-S25. doi:10.1016/j.injury.2011.06.008

52

Wach T, Kozakiewicz M. **Fast-versus slow-resorbable calcium phosphate bone substitute materials-texture analysis after 12 months of observation.** Materials (Basel). 2020;13(17):3854. doi:10.3390/ma13173854

53

Kim YJ, Saiki CET, Silva K, et al. **Bone formation in grafts with Bio-Oss and autogenous bone at different proportions in rabbit Calvaria.** Int J Dent. 2020;2020:2494128. doi:10.1155/2020/2494128

54

Lo Russo L, Caradonna G, Troiano G, Salamini A, Guida L, Ciavarella D. **Three-dimensional differences between intraoral scans and conventional impressions of edentulous jaws: a clinical study.** J Prosthet Dent. 2020;123(2):264-268.doi:10.1016/j.prosdent.2019.04.004

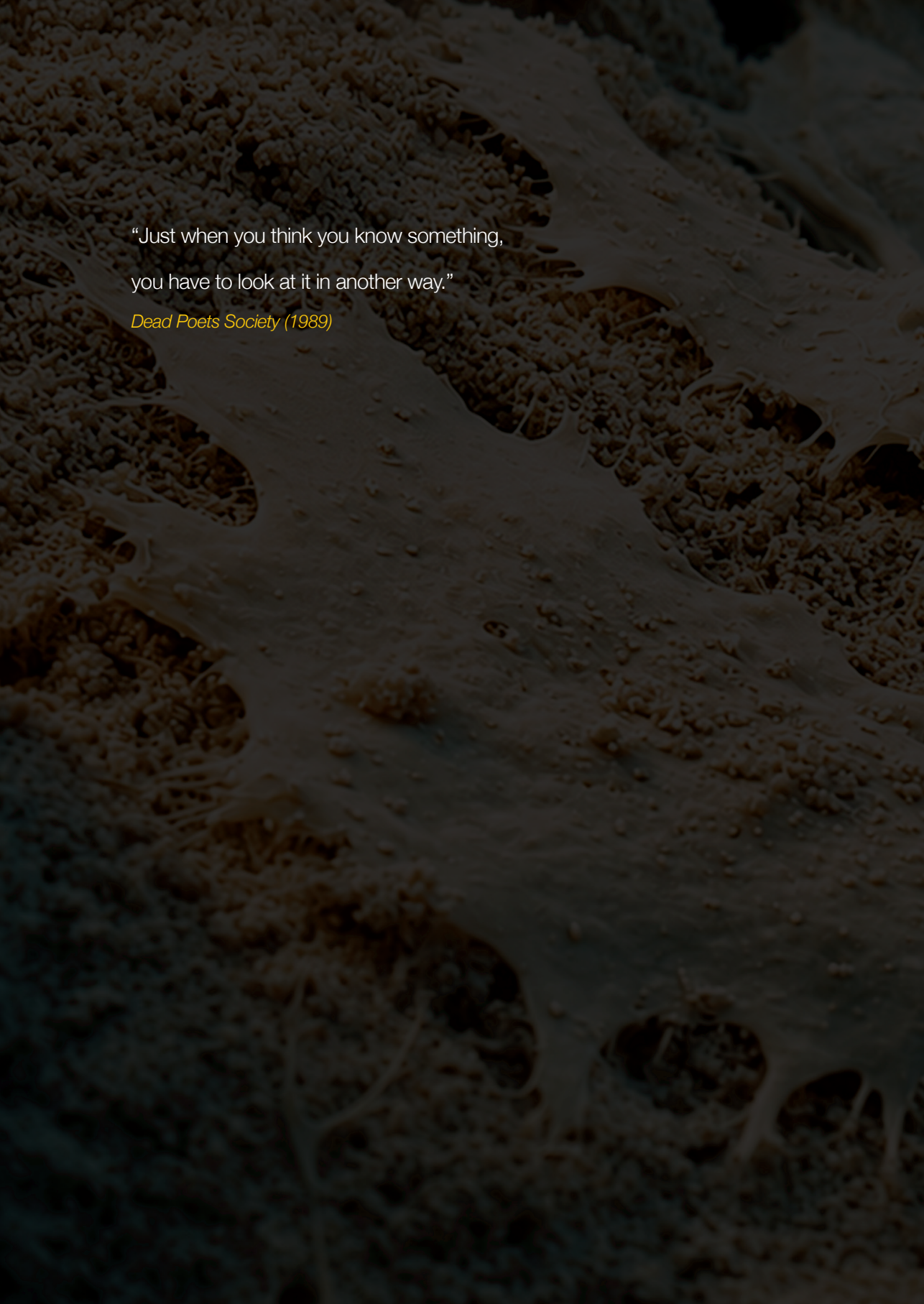
How to cite this article: De Carvalho B, Dory E, Trus C, et al. Biological performance of a novel bovine hydroxyapatite in a guided bone regeneration model: A preclinical study in a mandibular defect in dogs. Clin Implant Dent Relat Res. 2023; 1-14. doi:10.1111/cid.13260

Table A1

Histologic evaluation system for cell type/tissue response.

Note: Minimal: observation was barely perceptible microscopically and was not believed to have clinical significance; Mild: observation was visible but involved a minor proportion of the tissue, and the clinical consequences of the observation were most likely subclinical; Moderate: observation was clearly visible and involved a significant proportion of the tissue; it was likely to have some clinical manifestations, but which were generally expected to be minor; Marked: observation was clearly visible and involved a major proportion of the tissue; clinical manifestations were probable and may have been associated with significant tissue.
Abbreviation: hpf, high powered (400) field.

Response	Score				
	0	1	2	3	4
Polymorphonuclear cells	Absent	Rare, 1–5/hpf	6–10/hpf	Heavy infiltrate	Packed
Lymphocytes	Absent	Rare, 1–5/hpf	6–10/hpf	Heavy infiltrate	Packed
Plasma cells	Absent	Rare, 1–5/hpf	6–10/hpf	Heavy infiltrate	Packed
Macrophages	Absent	Rare, 1–5/hpf	6–10/hpf	Heavy infiltrate	Packed
Giant cells	Absent	Rare, 1–2/hpf	3–5/hpf	Heavy infiltrate	Packed
Necrosis	Absent	Minimal	Mild	Moderate	Marked
Fibrinous exudates	Absent	Minimal	Mild	Moderate	Marked
Tissue degeneration	Absent	Minimal	Mild	Moderate	Marked
Neovascularization	Absent	Minimal capillary proliferation, focal, 1–3 buds	Groups of 4–7 capillaries with supporting fibroblastic structures	Broad band of capillaries with supporting structures	Extensive band of capillaries with fibroblastic structures
Fibrocytes/ fibroconnective tissue, fibrosis	Absent	Narrow band	Moderately thick band	Thick band	Extensive band
Fatty infiltrate	Absent	Minimal amount of fat associated with fibrosis	Several layers of fat and fibrosis	Elongated and broad accumulation of fat cells around the implant site	Extensive fat completely surrounding the implant



“Just when you think you know something,
you have to look at it in another way.”

Dead Poets Society (1989)

A dark, high-contrast microscopic image of biological tissue, possibly a cross-section of a bone or cartilage, showing a porous, fibrous structure. The image is dimly lit, with some areas appearing slightly brighter than others, creating a textured, almost abstract appearance.

4

FURTHER
UNDERSTANDING
THE BIOLOGICAL
OUTCOMES

Influence of in vitro physicochemical and biological characteristics on hydroxyapatite xenografts performance.

Bruno De Carvalho, DDS, MSc, PhD Fellow, Head of Clinic, Department of Periodontology, Oro-Dental and Implant surgery, Dental biomaterial research Unit, University of Liège, Liège, Belgium

Sandy Anania, PhD, Department of Periodontology, Oro-Dental and Implant surgery, Dental biomaterial research Unit, University of Liège, Liège, Belgium

Dorien Van Hede, PhD, Department of Periodontology, Oro-Dental and Implant surgery, Dental biomaterial research Unit, University of Liège, Liège, Belgium

Emilie Dory, Ir, Chief Executive Officer, Wishbone SA, Liège, Belgium

Eric Rompen, DDS, MSc, PhD, Research consultant, Wishbone SA, Liège, Belgium

France Lambert, DDS, MSc, PhD, Professor and Head, Department of Periodontology, Oro-Dental and Implant surgery, Dental biomaterial research Unit, University of Liège, Liège, Belgium

Keywords:

Bovine hydroxyapatite - Physicochemical characterization - Cellular response - Bone regeneration

ABSTRACT

Aims: Bovine hydroxyapatite (BHA) is widely used in bone regeneration due to its biocompatibility and osteoconductivity. Variations in physicochemical properties from different manufacturing processes can impact their biological performance. This study aims to compare two commercialized BHA materials, by evaluating their physicochemical and in vitro biological characteristics and to link these findings with a previously published in vivo performance study.

Methods: Two BHA biomaterials (BO and WHA) were analyzed. Physicochemical characterization involved BET analysis for specific surface area, ion release via ICP-MS, FTIR spectroscopy for surface composition, SEM analysis for morphology, and capillarity tests for hydrophilicity. The in vitro biological characterization assays included chemotaxis using RAW 264.7 macrophages, protein adsorption, cell attachment and viability using MC3T3 cells, and ALP activity to assess osteogenic differentiation.

Results: WHA exhibited significantly higher magnesium and calcium ion release and protein adsorption (both total serum proteins and albumin) than BO. Chemotaxis assays revealed that BO attracted more macrophages than WHA. However, WHA demonstrated higher cell attachment and proliferation rates. ALP activity was comparable between the two materials, with a notable increase for WHA on day 5. Capillarity tests showed faster blood capillary rise in WHA, indicating better hydrophilicity.

Conclusion: The in vitro results highlight differences in the biological responses of the two BHA materials. The findings underscore that refining manufacturing processes can optimize the physicochemical properties and in vitro biological characteristics, improving the regenerative potential of BHA biomaterials.

1. INTRODUCTION

In modern dentistry, the development of advanced solutions for oral bone regeneration is crucial to improve the success of surgical interventions, promoting tissue regeneration, and ultimately restoring dental function. Alveolar bone augmentation procedures are often necessary prior to, or concurrently with, dental implant placement. The selection of appropriate bone substitutes for these procedures is a critical factor that significantly impacts clinical outcomes. These substitutes must possess osteoconductive properties that trigger a cascade of molecular events, including the migration of bone cells into the defect site, which leads to osteogenic differentiation and accelerates bone formation (Albrektsson and Johansson, 2001, Gani et al., 2023).

Xenogeneic biomaterials, particularly hydroxyapatite (HA), are widely utilized in guided bone regeneration due to their structural similarity to human bone. These materials provide volumetric stability, exhibit slow resorption rates, and offer an osteoconductive matrix that promotes bone regeneration by supporting cell adhesion, migration, and proliferation (Abou Fadel et al., 2018, Carmagnola et al., 2003, Serrano Méndez et al., 2017). The performance of HA-based biomaterials is primarily influenced by their composition, crystallinity, morphology, wettability, and particle size, among other factors (Sun et al., 2017). These physicochemical properties are critical in determining the biological responses and, consequently, the material's effectiveness in promoting successful bone regeneration and integration within host tissues (Mitragotri and Lahann, 2009, Sadeghian Dehkord et al., 2024).

The manufacturing process of biomaterials can also profoundly affect their properties, which in turn influences the regenerative outcomes. Numerous studies have demonstrated how different production techniques impact the material's surface microtopography, mechanical properties,

crystallinity, and in vivo osteoconductivity (Abdelmoneim et al., 2022, De Carvalho et al., 2019, Lambert et al., 2017, Tadic and Epple, 2004). Changes in surface microtopography can significantly alter protein adsorption, capillarity, cell adhesion, and tissue growth (Klenke et al., 2008, Lambert et al., 2017, Walther et al., 2014), all of which are essential for initiate the healing and regeneration processes.

Recently, a novel bovine HA bone substitute designed for filling, augmenting, or reconstructing alveolar bone defects received marketing approval from the United States Food and Drug Administration (FDA, 2021). This material is produced using a patented extraction method (WO 2015/049336 A1) that involves the complete removal of organic residues under subcritical conditions with a continuous flow of a basic solution (Rompen et al., 2015). A recent preclinical study by our group (De Carvalho et al., 2023) identified significant differences in early-stage bone regeneration (4 weeks post-implantation) between this novel material and a widely used xenograft (Bio-Oss, Geistlich, Switzerland). Beyond chemical composition, the physicochemical and in vitro biological properties of these commercially available bovine hydroxyapatites may play a pivotal role in their in vivo biological performance, thereby influencing their clinical effectiveness.

This study aims to investigate how the physicochemical and in vitro biological characteristics of two commercially available HA materials correlate with their previously observed in vivo behaviors. By conducting a comprehensive physicochemical and in vitro evaluation, and correlating these findings with earlier in vivo results, this study seeks to enhance the understanding of the impact of specific material properties on in vivo efficacy and regenerative outcomes, thus improving the clinical applicability of BHA-based biomaterials in oral bone regeneration.

2. METHODS

2.1. Study design

Two commercially available hydroxyapatite xenografts from bovine origin manufactured with different processes were used in this study: Bio-Oss (BO, Geistlich Pharma AG, Wolhusen, Switzerland), a xenograft used as a control reference and Wishbone Hydroxyapatite (WHA, Wishbone SA, Liège, Belgium). The two xenografts were assessed to identify their physicochemical characteristics, in vitro biological behaviors and in vivo biological performance.

2.2. Physicochemical characterization BET (Brunauer–Emmett–Teller)

The specific surface area of each bovine hydroxyapatite sample was analyzed using the TriStar 3000 equipment, following the standard operating procedure at the COMOC, University of Ghent. Approximately 1.0 g of each sample was placed in a clean 3/8-inch BET tube, sealed with a rubber O ring. The exact mass of the sample was recorded before connecting the tube to a VacPrep 061 degas system (Micrometrics), where it was degassed overnight (minimum 8 hours) at 120 °C under vacuum with a temperature ramp of 10 °C/min. An isothermal jacket was placed on the BET tube, which was then connected to the TriStar 3000. The Dewar flask was filled with liquid nitrogen, and the BET tubes were immersed in it. Full adsorption and desorption data were recorded to determine the specific surface area, pore diameter, and pore volume.

2.3. Ions captation/release

Cumulative calcium and magnesium concentrations obtained from biomaterial dissolution (WHA, BO and medium) under static conditions were investigated after 1, 2, 4, 8, and 12h and after 1, 5, 10, 15, and 21 days of incubation. The bone grafts substitutes (0.05 g) were soaked in 1 ml of α -MEM culture medium (with 10 % FBS and 1 % penicillin/streptomycin) and were maintained in an incubator at 37 °C, under static conditions. At each time point, 1 ml of the culture medium was collected for Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) and replaced with fresh medium. Afterward, 200 μ l of collected medium were mixed with 200 μ l of 70 % distilled HNO_3 and incubated overnight at 65 °C. Then, the mixed collected medium was centrifuged for 10 minutes at 15 000 g and 5 μ l of the supernatant was added to 4.995 ml of 2 % distilled HNO_3 at room temperature. The samples were vigorously mixed three times (15 s each time) with 40 μ l of an internal standard (provided by the Department of Geology of the University of Pittsburgh) in a Vortex-mixer, the mixture was ready for ICP-MS isotope analysis (NexION 300).

2.4. FTIR (Fourier-transformed Infrared)

The FTIR analysis was conducted to investigate the biomaterial composition, protein adsorption, and to determine if the decrease in calcium ions (Ca^{2+}) in the medium during dissolution contributed to re-mineralization on the material surface. First, these materials underwent a dissolution analysis to simulate physiological conditions and understand their behavior in a biological environment. The materials were initially pre-treated and soaked in cell culture

medium (α -MEM containing 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin). The soaking process was conducted over incubation periods of 30 and 90 days. Following the incubation, the materials were dried under vacuum at room temperature. The FTIR spectra were then collected using a Bruker Vertex 70 spectrometer, where each sample was prepared by mixing 100 mg of potassium bromide (KBr) with 600 ng of the bone graft material. The spectra were recorded at room temperature, covering a range from 400 to 4000 cm^{-1} , to identify characteristic absorption bands and assess the presence of phosphate (PO_4^{3-}) and carbonate (CO_3^{2-}) groups, as well as to evaluate the protein adsorption profiles by detecting amide group peaks.

2.5. Capillarity test

Qualitative observations were used to assess the capillarity of the two biomaterials and performed in triplicates. To avoid electrostatic potential, the particles were precisely weighted using a sterile 15 ml falcon tube with a special beak lid. The beak lid was used to pour the particles in a 1.1 ml pipette (LP Italiana, 161111, Milano) until reaching the 0.5 ml tag. Along with the pipette, a 3.5 cm petri dish with 6 ml pig blood diluted 1:2 in PBS solution was prepared. The pipettes were immersed at the same exact time in the blood. The blood capillary rise speed and rise height were recorded.

2.6. Scanning electron microscope (SEM) analysis

Assays were performed by soaking 0.05 g of particles of each product in 1 ml of cell culture medium (5 %) for a static dissolution in a 1.5 ml Eppendorf tube at 37 °C. After incubation periods of 1, 3, and 7 days, the samples were fixed in 2.5 % (v/v) glutaraldehyde to preserve cellular structures. The cells were then dehydrated in a graded series of ethanol solutions (25 %, 30 %, 50 %, 60 %, 70 %, 80 %, and 90 % ethanol) for 20 minutes each. This was followed by three dehydrations in 100 % ethanol to ensure the complete removal of water. The samples were treated with hexamethyldisilazane (HMDS) and left to dry overnight in a fume hood. Once dried, the samples were mounted on aluminum stubs and sputter-coated with gold to create a conductive layer. Finally, the samples were imaged using a JEOL JSM6330F scanning electron microscope (SEM) (Tokyo, Japan) to obtain high-resolution images of the cell surfaces.

2.7. In vitro biological characterization

2.7.1. CELLS MC3T3 cells (ATCC, Manassas, Virginia, USA) were cultured in α -minimum essential medium (α -MEM, Lonza, Basel, Switzerland) supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. hBMSC cells, purchased from the A&M Institute for Regenerative Medicine (Texas, USA), were grown in α -MEM supplemented with 16.5 % FBS, 2mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

RAW 264.7 (ATCC, Manassas, Virginia, USA) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Lonza, Basel, Switzerland) supplemented with 10 % FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

TABLE 1

Cell lines used for in vitro biological characterization. Three established cell lines of different species and phenotype were selected to probe complementary aspects of the early biological response to the biomaterials.

CELL LINE	SPECIES	PHENOTYPE	USED FOR
MC3T3-E1	Mouse	Pre-osteoblast	Cell attachment; viability / proliferation; ALP activity (osteogenic differentiation)
hBMSC	Human	Bone-marrow mesenchymal stromal cells	Cell viability / proliferation; adhesion (SEM)
RAW 264.7	Mouse	Macrophage	Chemotaxis (immune / inflammatory recruitment)

2.7.2. CHEMOTAXIS Before the experiment, bone graft substitutes were pre-wetted in a DMEM culture medium and incubated for 24 hours at 37 °C. Then, RAW 264.7 cells (5×10^5 cells/well) were seeded, in DMEM, in the insert of Boyden's chambers (CLS3422, Sigma, Bornem, Belgium). 150 mg of pre-wetted bone graft were placed in the bottom compartment and immersed in 1.2 ml of DMEM. Cells were allowed to migrate for 24 hours or 48 hours in a humidified 5 % CO₂ incubator at 37 °C. Then, the cells at the upper side of the membrane (8 μ m pore size, 15 μ m thickness) were wiped off with Q-tips, while the cells at the bottom side of the membrane were stained with DAPI (Invitrogen Catalog number: D1306, 0.1 μ g/ml, Calbiochem, San Diego, CA). Pictures (100x magnification, 5 random fields) were acquired using a Nikon

TE2000-E microscope. As a negative control, no biomaterials were placed in the bottom compartment of the Boyden chambers.

2.7.3. ALKALINE PHOSPHATASE ACTIVITY (ALP) Bone graft particles (15 mg) were placed in a 96-well plate with an ultra-low attachment surface (Corning), UV-sterilized, and soaked in α -MEM for 24 h before use. MC3T3 cells (4×10^4 per well) were seeded onto the particles. Particles without seeded cells served as an additional (material-only) control to account for background signal from the biomaterial. Cells were initially cultured in α -MEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10 % FBS. Twenty-four hours after seeding, the growth medium was replaced with osteogenic differentiation medium (α -MEM further supplemented with 50 μ g/ml ascorbic acid, 10 mM β -glycerophosphate, and 10^{-7} M dexamethasone), and refreshed every other day. After 1, 5, 10, 14, and 21 days ($n = 6$), samples were washed twice with PBS and lysed with 150 μ l of lysis buffer for 15 min. ALP activity was measured with the SensoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec): 50 μ l of cell lysate was mixed with 50 μ l of pNPP substrate solution, incubated at room temperature for 30 min, stopped with 50 μ l of stop solution, and read at 405 nm on a BioTek microplate reader. A separate aliquot of lysate (25 μ l) was used for total protein quantification with the BCA protein assay kit (Pierce), read at 562 nm against a standard curve. ALP activity was normalized to total protein content and expressed as μ g/g protein.

2.7.4. TOTAL PROTEIN ADSORPTION 50 mg of non-pre-wetted bone graft substitutes were incubated with 1 ml of α -MEM containing 10 % FBS and 1 % penicillin/streptomycin, under continuous rotation in a humidified 5 % CO_2 incubator at 37 °C. After 1, 2, and 4 days of incubation, bone graft substitutes were washed three times with PBS and 500 μ l of 1 % Triton X-100 were added for 15 minutes to each sample for protein elution. After centrifugation (5 minutes, 10000 g), a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, USA) was used on cell lysate (supernatant) for protein quantification, following the manufacturer's instructions.

2.7.5. ALBUMIN ADSORPTION 50 mg of non-pre-wetted bone graft substitutes were incubated with albumin (1 mg/ml of PBS, pH 7.4) under continuous rotation in a humidified 5 % CO_2 incubator at 37 °C. After 2, 12, and 24 hours of incubation, bone graft substitutes were washed three times with PBS and 500 μ l of 1 % Triton X-100 were added for 15 minutes to each sample for protein elution. After centrifugation (5 minutes, 10000 g), the supernatant was used for protein quantification using a Bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, USA), following the manufacturer's instructions.

2.7.6. CELL ATTACHMENT Before the experiment, bone graft substitutes were pre-wetted in an α -MEM culture medium and incubated for 24 hours at 37°C. Then, 15 mg per well of pre-wetted bone graft substitutes were added to a 96-well plate with an ultralow attachment surface. Following this step, 3.3×10^4 of MC3T3 cells were seeded per well on top of bone graft substitutes. After 4 hours of incubation in a humidified 5 % CO₂ incubator at 37 °C, the wells were washed twice with phosphate-buffered saline (PBS) to remove non-adhering cells. An Alamar blue assay was performed to assess cell attachment on bone graft substitutes. Tissue culture polystyrene surface (TCPS) was used as a positive control for cell attachment and its mean was set at 100 % for cell attachment. Alamar blue was added in each well (10 % v/v) and cells were further incubated for 2 hours. After incubation, the fluorescence intensity was measured using alamarBlue™ Cell Viability Reagent, Promega Catalog number: DAL1025, with excitation and emission wavelength of 560 and 590 nm, respectively. Correction for background fluorescence was realized by including control wells containing only cell culture medium.

2.7.7. CELL VIABILITY/PROLIFERATION Bone graft particles were placed in a 24-well plate with ultralow attachment surface. They were soaked in culture medium and were incubated for 48 hours at 37 °C. The plate was placed under UV light for sterilization. 1×10^5 hBMSC or 1×10^4 MC3T3 cells were seeded per well and incubated for 1, 3, and 7 days. An Alamar blue assay was performed to assess cell viability and proliferation regarding bone graft substitutes as described above. TCPS was used as a positive control for cell attachment. Proliferation of hBMSCs on biomaterials was also assessed by SEM.

2.8. Statistical analysis

A one-way analysis of variance (ANOVA) was performed when assessing the impact of one independent variable on a response variable. When two independent variables impacted a response variable, a two-way ANOVA was performed. For multiple comparisons, Sidak (for small number of comparisons) or Tukey (for a large number of comparisons) tests were applied. A p-value < 0.05 was considered statistically significant.

3. RESULTS

3.1. Physicochemical characterization

3.1.1. SPECIFIC SURFACE AREA (BET) Specific surface area analysis showed that the mean for WHA and BO samples were $16.0 \pm 0.0 \text{ m}^2/\text{g}$ and $81.7 \pm 0.6 \text{ m}^2/\text{g}$ respectively.

3.1.2. BIOMATERIALS IONS RELEASE (CA, MG) In the static dissolution experiment, distinct calcium and magnesium release profiles were observed. Cumulative calcium concentration was significantly higher in the control medium compared to WHA and BO (Fig. 1A), with a rapid release in the first 24 hours (Fig. 1B). WHA exhibited a consistent release with significant increases compared to BO at later time points ($p < 0.05$). Magnesium release from WHA remained significantly higher than BO and the control medium under static conditions ($p < 0.05$) (Fig. 1C), highlighting WHA's capacity for sustained magnesium enrichment throughout the 21 days ($p < 0.05$).

3.1.3. CHEMICAL COMPOSITION AND RE-MINERALIZATION (FTIR) The FTIR test showed that WHA and BO were composed of similar PO_4^{3-} or CO_3^{2-} groups (Fig. 2). Both materials displayed crystalline surfaces and re-mineralization process was not observed on their surfaces at 30 or 90 days.

3.1.4. SURFACE TOPOGRAPHY AND CELL ADHESION (SEM) SEM analysis was performed to observe the morphology and microstructure of the biomaterials after static dissolution (Fig. 3). The surface topography of the WHA did not appear to change after soaking in the medium. In contrast, more nano-sized pores appeared in some areas of BO. Fibril-like structures with periodic collagen organization and close collagen-hydroxyapatite contact are observed in both materials. This micro-organization shows that WHA, like Bio-Oss, retains key bone hydroxyapatite characteristics, including a microporous and rough surface.

3.1.5. HYDROPHILICITY (CAPILLARITY TEST) The capillary rise speed and height were assessed on both WHA and BO using pig's blood, demonstrating significant differences in their performance (Fig. 4). The capillary rise speed for BO was found to be $0.2 \pm 0.05 \text{ mm/sec}$ whereas WHA showed a much faster rise speed of $0.4 \pm 0.1 \text{ mm/sec}$ ($p < 0.05$). The blood capillary rise height for BO was measured at $20.0 \pm 5.0 \text{ mm}$ while WHA exhibited a substantially higher rise height of $45.0 \pm 5.0 \text{ mm}$ ($p < 0.001$) (Fig. 4B).

Figure 1

Cumulative calcium and magnesium concentrations obtained from biomaterial dissolution (WHA, BO and control) under static conditions. Magnesium and calcium concentrations were assessed after 1, 2, 4, 8 and 12h and after 1, 5, 10, 15 and 21 days of incubation. (A) Graph showing the cumulative calcium concentration (mg/L) for WHA (green), BO (grey) and the control (blue), from 1h to 21 days. The statistical significance between 1 and 24h is shown in (B), which constitutes an enlarged version of panel A in the first 24h. (C) Graph showing the cumulative magnesium concentration (mg/L) for the WHA, BO and control conditions from 1h to 21 days. The statistical significance between 1 to 24h is represented in (D), which constitutes an enlarged version of panel C in the first 24h. A two-way ANOVA was used for statistical analyses. The test of Tukey was used for multiple comparisons. ****: p -value < 0.0001. ***: p -value < 0.001. **: p -value < 0.01. *: p -value < 0.05. Each point corresponds to the mean and, the error bars represent the standard deviation (SD) computed for 4 replicates.

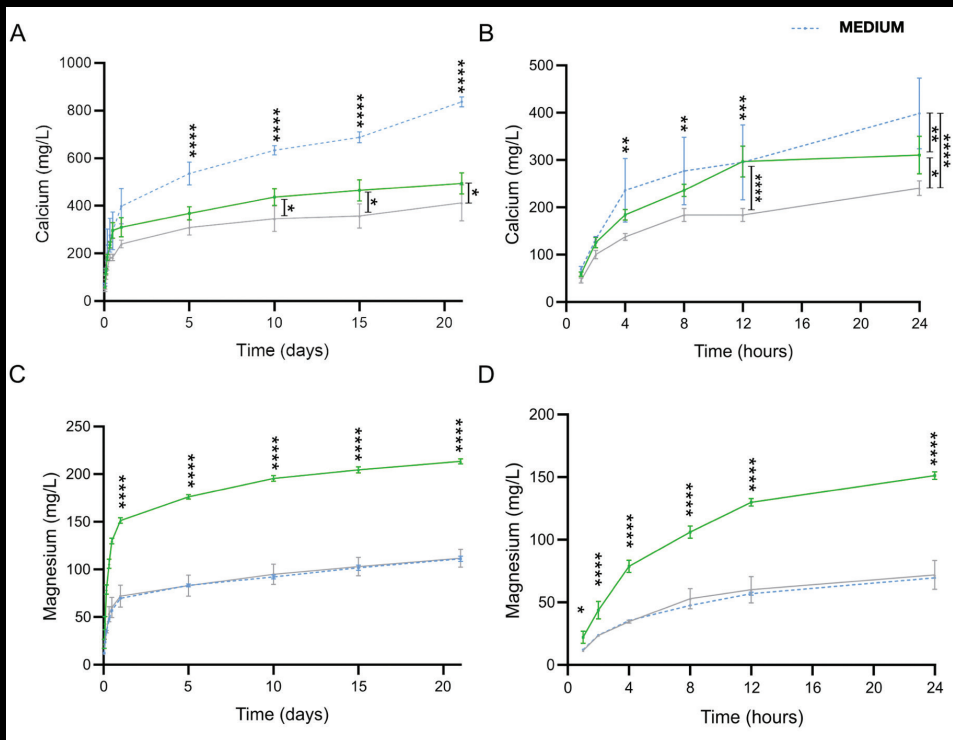


Figure 2

A: FTIR spectra KBR of WHA at day 30 and 90 of dissolution.

B: FTIR spectra KBR of BO at day 30 and 90 of dissolution.

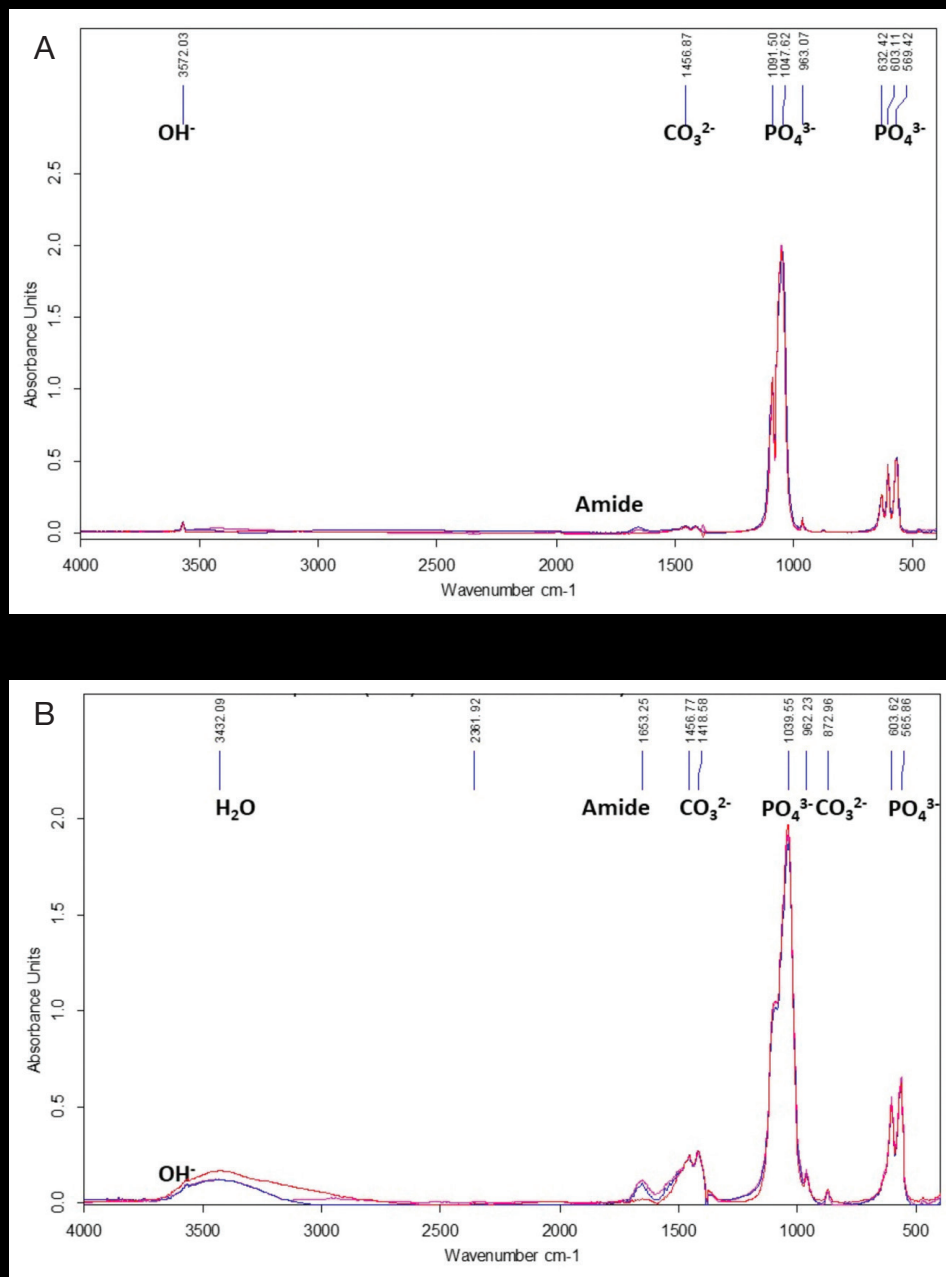
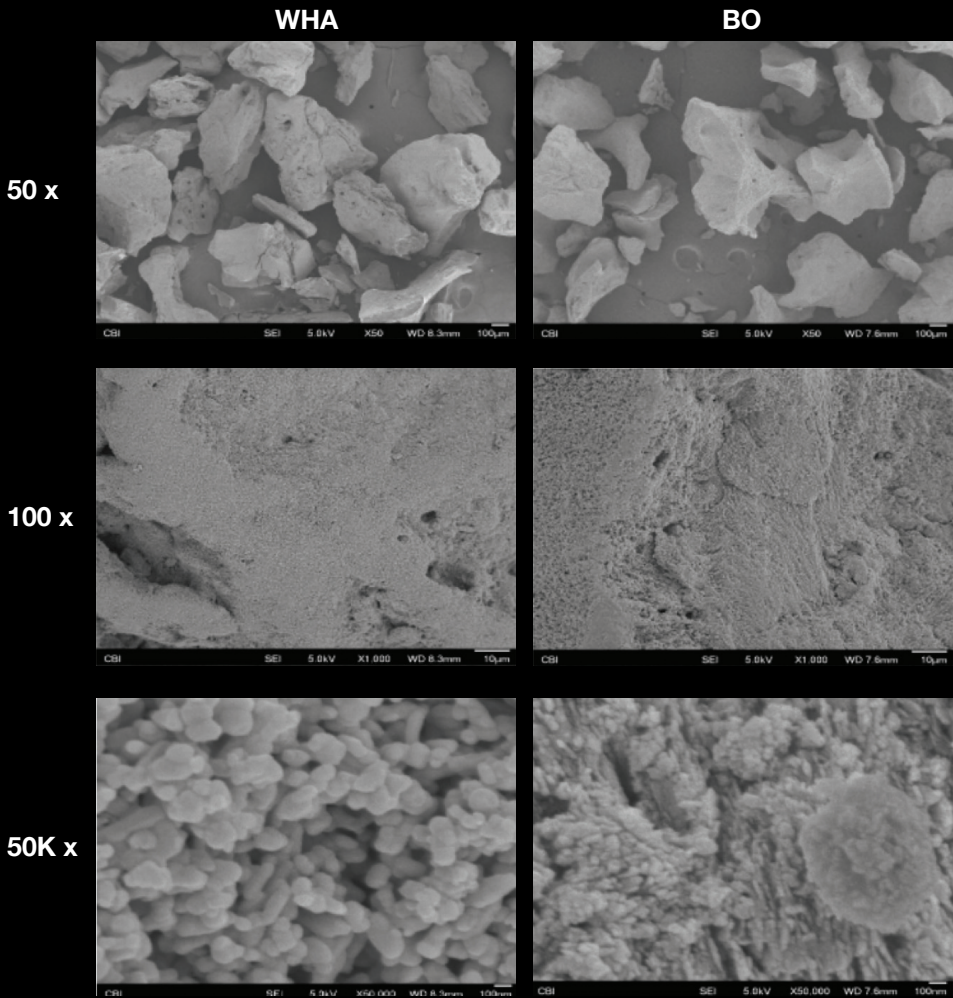


Figure 3

Scanning electron microscopy (SEM) of the studied biomaterials at different magnifications: WHA and BO with 50, 1000 and 50K magnification. WHA and Bio-Oss, presenting both microporous and rough surface.



3.2. Biological characterization

3.2.1. CHEMOTAXIS The results showed that WHA bone graft particles did not impact RAW 264.7 cells migration, since there was no difference with the negative control at both 24 and 48h (Fig. 5). In contrast, the BO biomaterial attracted significantly more macrophages than the negative control, at both time points (p values).

3.2.2. ALP ACTIVITY The results of the ALP assay on MC3T3 cells when using WHA and BO biomaterials show no differences for the ALP activity between HA and BO biomaterials, except after 5 days of incubation, where the activity was significantly higher for WHA compared to BO. In addition, the ALP activity was the highest for both WHA and BO at 5 and 10 days of culture (Fig. 6).

Figure 4

*A: Comparative Blood Capillary Rise in BO and WHA. WHA exhibits a significantly faster and higher blood capillary rise compared to BO, indicating better hydrophilicity. B: Blood capillary rise height and speed results. WHA demonstrates a significantly higher blood capillary rise height (** $p < 0.001$) and faster blood capillary rise speed (* $p < 0.05$) compared to BO.*

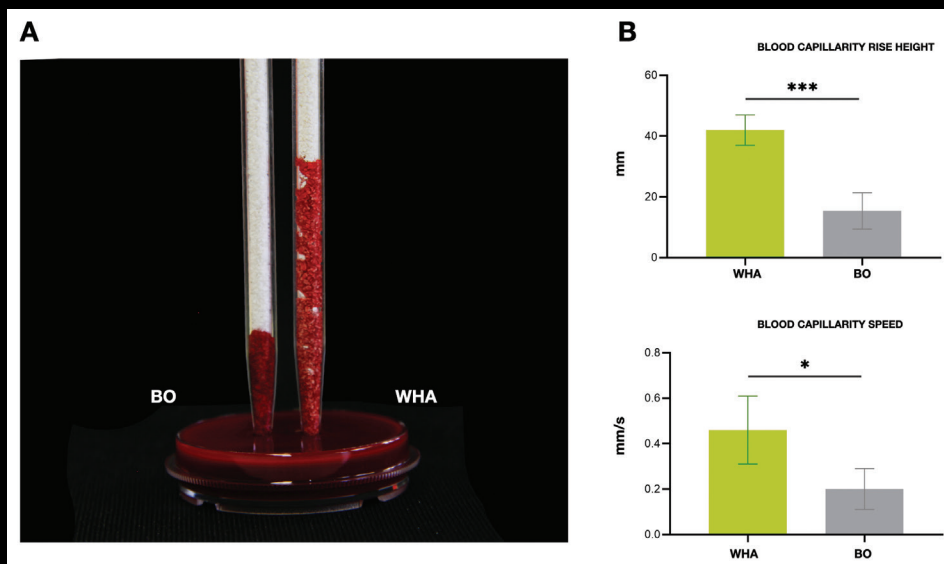


Figure 5

Chemotaxis of RAW 264.7 toward biomaterials (WHA and BO) using Boyden's chambers. As a negative control, no biomaterials were placed in the bottom compartment of the Boyden's chambers. Cell migration was assessed after 24 and 48h. A two-way ANOVA was performed for statistical analysis. The test of Tukey was used for multiple comparisons. ****: p -value < 0.0001 , ***: p -value < 0.001 , ns: non-significant. Mean $\pm$ SD is represented. $n=5$ for each biomaterial and at each time point.

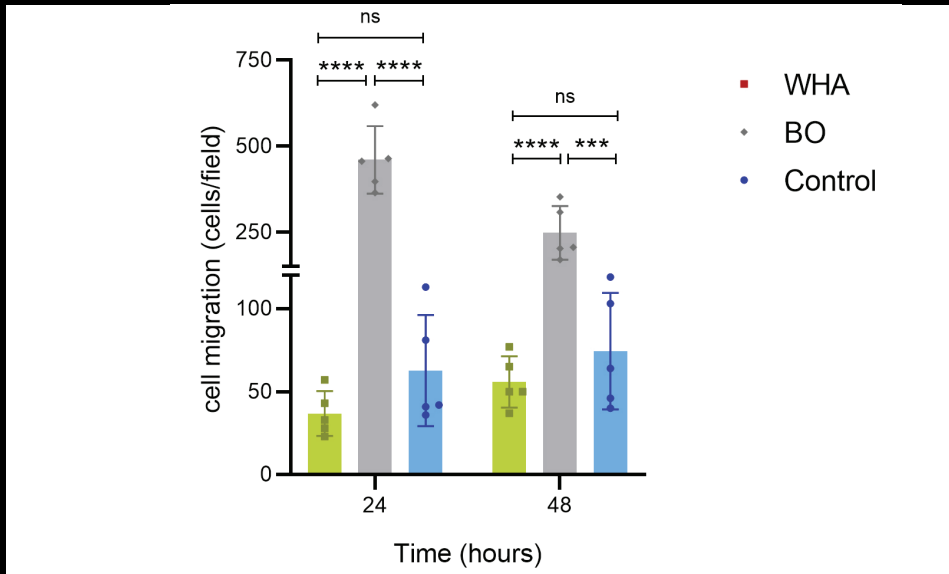
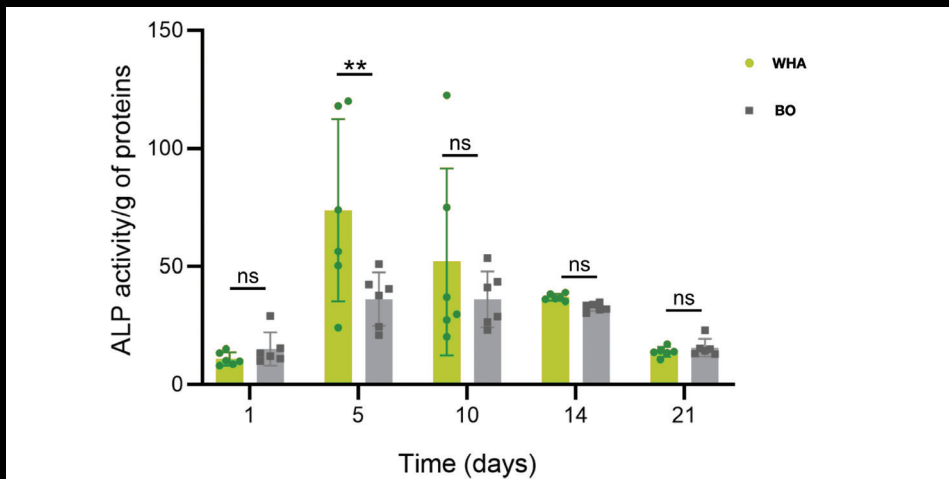


Figure 6

The ALP activity of MC3T3 cells after 1, 5, 10, 14 and 21 days of culture on WHA or BO biomaterials. A two-way ANOVA was performed for statistical analysis. Sidak's test was used for multiple comparisons. **: p -value < 0.01 , ns: non-significant. Mean $\pm$ SD is represented. $n=6$ for each biomaterial and at each time point.



3.2.3. TOTAL PROTEIN AND ALBUMIN ADSORPTION Total protein adsorption was assessed on both WHA and BO groups. Results show that the WHA biomaterial adsorbed significantly more serum proteins on its surface than BO over time (from 1 to 4 days) (Fig. 7A). Interestingly, this observation was also true when assessing albumin adsorption. Albumin was significantly more adsorbed on WHA than BO from 2h to 24h (Fig. 7B).

3.2.4. CELL ATTACHMENT MC3T3 cell attachment on WHA and BO was investigated when cultured in a medium with serum (FBS) (Fig. 8). For this purpose, MC3T3 cells were incubated for 4 h with biomaterials. TCPS was used as a positive control and the mean of its replicates was set at 100 % for cell attachment (Fig. 6). The percentage of attached cells on WHA was of 76 ± 6 %, while it was only of 55 ± 3 % for BO. Both WHA and BO had a significantly lower percentage of cell attachment than the control (p values). However, the percentage of attached cells was significantly higher on WHA than BO ($p < 0.0001$).

3.2.5. CELL VIABILITY/PROLIFERATION Cell viability/proliferation regarding bone graft substitutes was assessed after 1, 3, and 7 days of incubation. For both MC3T3 and hBMSC cells, cell viability was significantly higher on WHA than BO at each time point (Fig. 9). While the cell viability decreased over time when using hBMSC cells on WHA and BO (decreased

Figure 7

Quantity (μg) of proteins adsorbed on 50 mg of WHA (green) and BO (grey) biomaterials. (A) Total protein adsorption on HA and BO after 1, 2 and 4 days of incubation. (B) Albumin adsorption on the HA and BO surfaces after 2, 12 and 24 hours of incubation. A two-way ANOVA was used for statistical analyses. The test of Sidak was used for multiple comparisons. ****: p -value < 0.0001 . ***: p -value < 0.001 . **: p -value < 0.01 . *: p -value < 0.05 . Mean $\pm$ SD is represented. $n=3$ for each condition and at each time point.

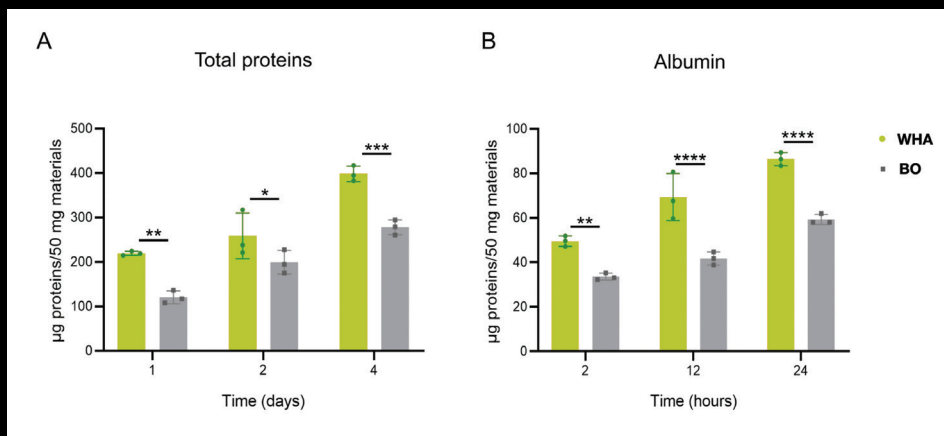


Figure 8

Percentage of MC3T3 cell attachment on WHA (green) and BO (grey) biomaterials after 4 h of incubation. TCPS (pink) was used as a positive control and the mean of its replicates was set at 100 %. A one-way ANOVA was used for statistical analysis. Tukey's test used for multiple comparisons. ****: p -value < 0.0001. Mean $\pm$ SD is represented. $n=6$ for each condition and at each time point.

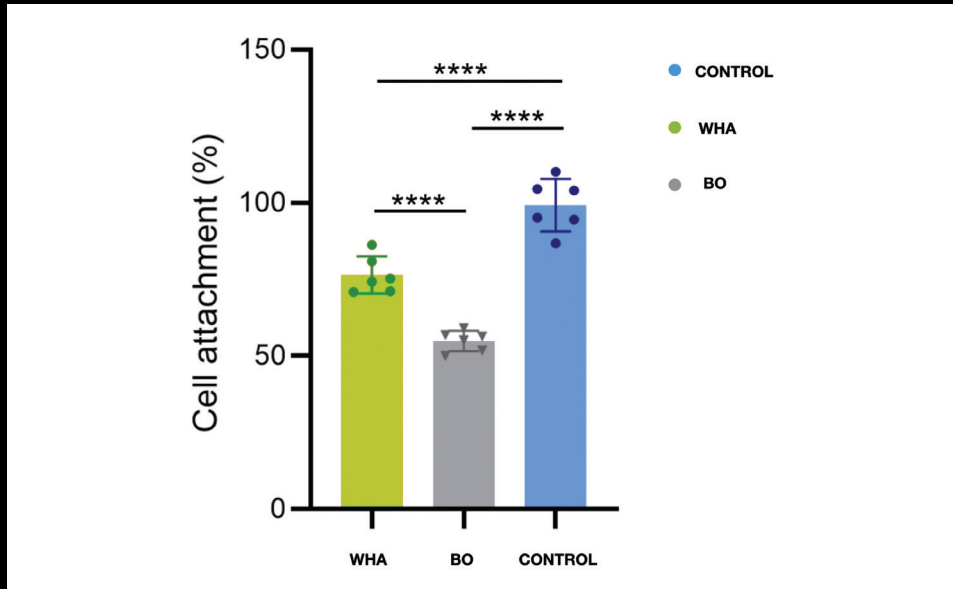
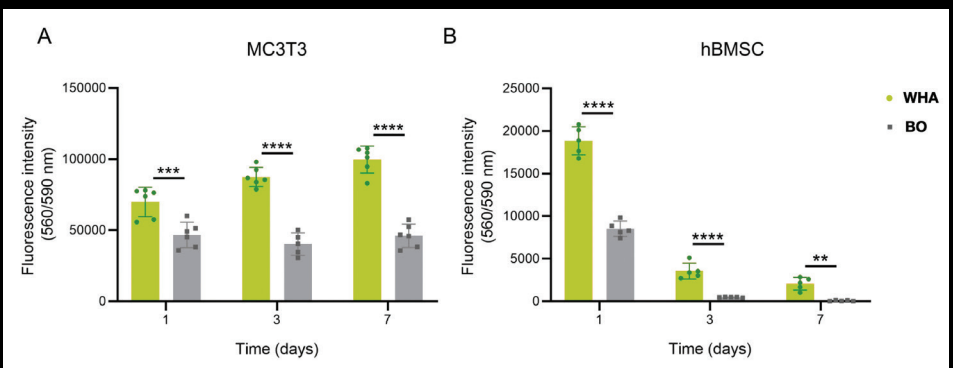


Figure 9

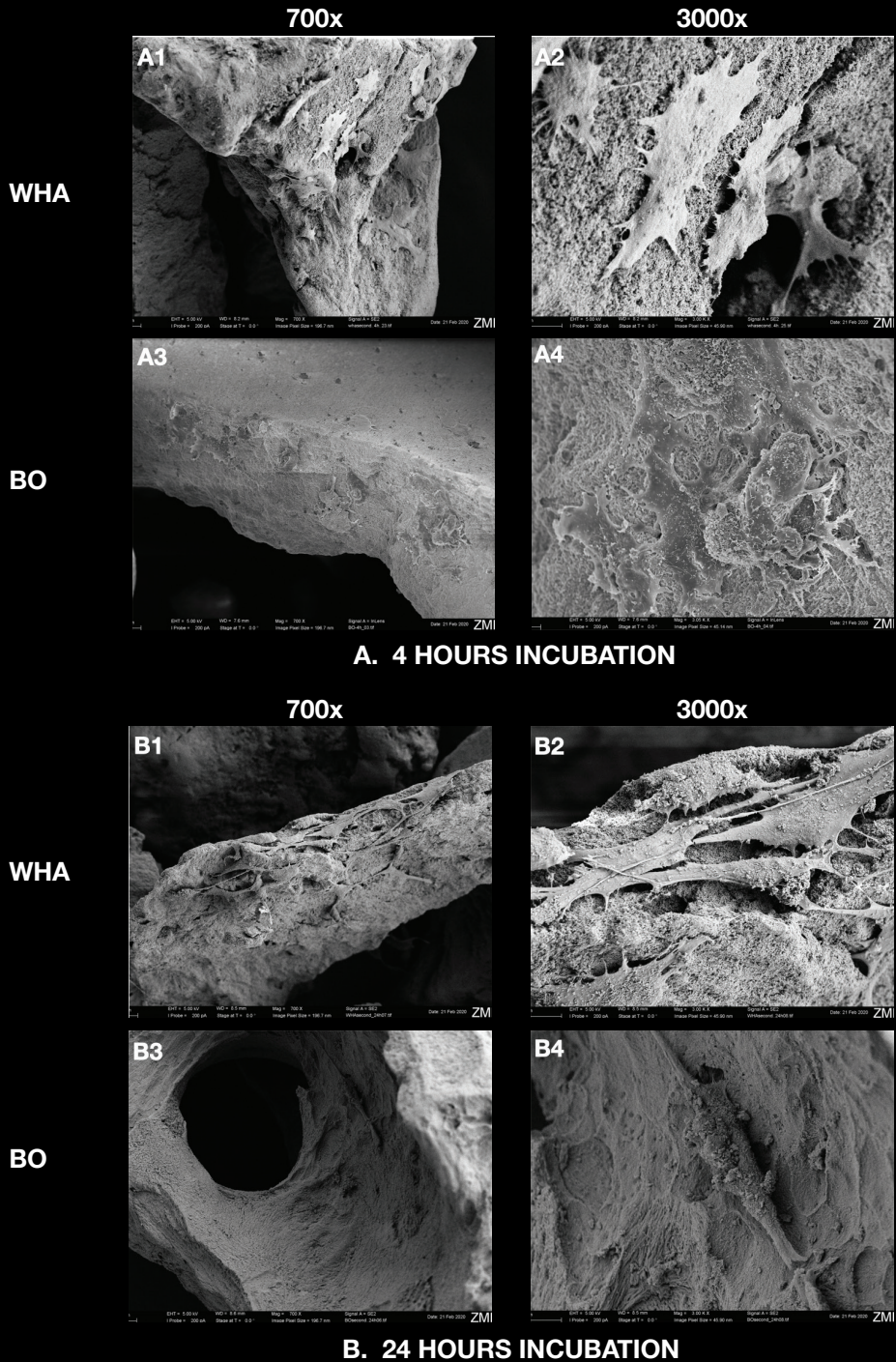
Cell viability on WHA (green) and BO (grey) biomaterials after 1, 3 and 7 days of incubation using Alamar blue assay. (A) MC3T3 viability on HA and BO biomaterials. (B) hBMSC viability regarding WHA and BO biomaterials. A two-way ANOVA was performed for statistical analyses. The test of Sidak was used for multiple comparisons. ****: p -value < 0.0001, ***: p -value < 0.001, **: p -value < 0.01. Mean $\pm$ SD is represented. $n=6$ for each biomaterial and at each time point when using MC3T3 (except for BO, where $n=5$ after 3 days of incubation). $n=5$ for both WHA and BO and at each time point when using hBMSC.



fluorescence intensity) (Fig. 9B), it remained stable for BO or even increased for WHA when using MC3T3 cells (stable or increased fluorescence intensity) (Fig. 9A). Proliferation of hBMSCs was confirmed by SEM, where both materials were covered with cells after 4 hours culture. (Fig. 10)

Figure 10

SEM images of hBMSC cell adhesion on surfaces of WHA and BO after 4 hours (A) and 24 hours (B) of incubation.



4. DISCUSSION

The present study highlights that biomaterials originating from the same source but subjected to different manufacturing processes exhibit distinct physical and biological behaviors (Table 1). WHA showed better capillarity, less Ca^{2+} captation and higher magnesium ion release, which could enhance bone healing and regeneration. WHA also demonstrated superior protein and albumin adsorption, cell attachment, viability, and proliferation compared to BO. In contrast, BO attracted more macrophages, suggesting a stronger initial inflammatory response. These *in vitro* characteristics and properties allow for a better understanding of some of the *in vivo* results presented in our earlier research (De Carvalho et al., 2023), more specifically, regarding the higher bone regeneration rate at 4 weeks in WHA when compared to BO.

In the present study, the FTIR analysis confirmed that both WHA and BO shared similar phosphate (PO_4^{3-}) and carbonate (CO_3^{2-}) groups indicating that both materials could potentially perform similarly in biological environments. Nevertheless, when evaluating their dissolution, our results show that the cumulative calcium concentration over time was significantly higher in the medium without biomaterials, compared to the WHA and BO conditions. This suggests that both WHA and BO biomaterials adsorb calcium on their surfaces, thus decreasing the amount of calcium in the medium. Interestingly, the cumulative calcium concentration was significantly higher in the WHA group than the BO group, suggesting that WHA adsorbs less calcium than BO. This difference may be attributed to variations in surface properties, such as specific surface area or porosity, between the two biomaterials. As mentioned in our previous study (De Carvalho et al., 2023), the different specific surface areas (SSA) of the two biomaterials translate into different micro and nanoporosity. Thus, the higher SSA of BO can enhance the material's capacity to adsorb calcium ions from the surrounding fluids. If the biomaterial adsorbs a less significant amount of calcium from the extracellular environment, it can increase the stimulation required for osteoblast proliferation and differentiation, potentially accelerating the bone regeneration process. The higher calcium availability in the WHA group could have enhanced bone morphogenetic protein (BMP) signaling and upregulated osteogenic transcription factors such as Runx2 and Osterix (Ielo et al., 2022, Aquino-Martínez et al., 2017). This mechanism might explain the superior early-stage bone regeneration observed with WHA at 4 weeks when compared to BO in our earlier study. These facts could be correlated with those reported by Hassan, M. (2024) (Hassan et al., 2024), exploring the association between Ca^{2+} release over time by 3D-printed HA-based templates and osteoconductivity. The HA templates, which released Ca^{2+} at a mild rate, demonstrated higher osteoconductivity evidenced by higher mineralized ECM secretion and greater new bone formation *in vitro* and *in vivo* compared to the templates with high Ca^{2+} releasing.

TABLE 2

Comparative Analysis of Physicochemical Properties and In Vitro Biological Characteristics of Bio-Oss (BO) and Wishbone Hydroxyapatite (WHA)

ANALYSIS	BO results	WHA results	INTERPRETATION
BET	81.7m ² /g	16m ² /g	BO has a higher specific surface area, indicating a higher level of microporosity compared to WHA.
Capillarity	Slower blood capillarity rise	Faster blood capillarity rise	Better hydrophilicity of WHA suggests improved protein adhesion and cellular attachment, which can enhance bone healing and regeneration.
Dissolution Ca+ / Mg+	Release less Ca and Mg to the medium	Release more Ca and Mg to the medium	Higher Ca+ and Mg+ release in WHA could contribute to faster bone regeneration and better process.
FTIR	Similar PO ₄ ³⁻ and CO ₃ ²⁻ groups no re-mineralization, higher protein adsorption	Similar PO ₄ ³⁻ and CO ₃ ²⁻ groups no re-mineralization,	Both materials share similar functional groups. Higher protein adsorption for BO might influence tissue integration.
Chemotaxis	Significantly attracted more macrophages	No significant macrophages migration remodeling.	BO's higher macrophage attraction indicates a stronger initial inflammatory response which could influence bone
ALP	Lower than WHA at 5 days, similar after	Higher than BO at 5 days, similar after	Higher ALP activity in WHA suggests better osteogenic differentiation and bone formation at early stages.
Total proteins adsorption	Lower than WHA	Significantly higher than BO	WHA's higher protein adsorption suggests a more hydrophilic surface, leading to better initial cell adhesion and growth.
Albumin Adsorption	Lower than WHA	Significantly higher than BO	Higher albumin adsorption in WHA suggests better protein binding properties.
Cell Attachment	55 % attachment rate	76 % attachment rate	Higher cell attachment on WHA indicates a more favorable surface for initial cell adhesion, critical for bone regeneration.
Cell viability	Lower viability and proliferation compared to WHA	Higher viability and proliferation compared to BO	Higher cell viability and proliferation on WHA indicate better support for cell growth and bone regeneration.

In contrast, no significant differences in ALP activity were observed between WHA and BO, except after 5 days of incubation, when WHA showed significantly higher ALP activity. ALP activity is a marker of osteogenic differentiation and new bone formation, as it can be influenced by intrinsic properties of biomaterials (Zeng et al., 2012, Korovessis and Deligianni, 2002, Antonini et al., 2019), such as magnesium release (Mammoli et al., 2019, He et al., 2016). The higher ALP activity in WHA could be attributed to its higher magnesium release, as magnesium and zinc are key cofactors in ALP's enzymatic function (Lowe et al., 2024, Makris et al., 2023).

Building on these findings, it was crucial to explore the role of chemotaxis in the context of bone regeneration. Chemotaxis plays a critical role in recruiting macrophages to the regeneration area, where they initiate and regulate the healing process (Ridiandries et al., 2018). In this study, BO significantly attracted more macrophages than WHA. This initial lower chemotactic behavior presented in WHA might indicate a more balanced inflammatory response, avoiding excessive or prolonged inflammation and facilitating a quicker transition from the inflammatory phase (dominated by M1 macrophages) to the reparative phase (dominated by M2 macrophages). This immunomodulatory effect likely contributes to the favorable healing environment seen in vivo, supporting both osteogenesis, material integration and faster tissue remodeling (Gibon et al., 2016, Spiller et al., 2014).

Furthermore, the observed release of magnesium ions from WHA could also play a role in the chemotactic behavior. Magnesium has been shown to influence the polarization of macrophages toward the M2 phenotype, which is linked to anti-inflammatory and tissue-repair functions, as opposed to the pro-inflammatory and chemotactic M1 phenotype (Costantino et al., 2020). While the exact role of magnesium in this process is not fully understood, evidence suggests that it might play a significant part in the modulation of the inflammatory responses and macrophage activity (Belluci et al., 2011, Del Barrio et al., 2010, Liao et al., 2023, Weglicki et al., 1992).

Moreover, when assessing total protein adsorption, WHA adsorbed significantly more total serum proteins and albumin on its surface than BO over time (from 1 to 4 days and from 2 h to 24 h respectively). The adsorption of proteins might be related with the existence of a more hydrophilic surface in WHA, which tends to attract and bind more proteins from the surrounding environment (Lee et al., 2018). In the present study, the degree of hydrophilicity was analyzed by the capillarity test (Kammerhofer et al., 2018) which showed a high level of capillarity, suggesting that WHA has a strong affinity for blood absorption and a higher capacity to draw blood faster through its structure when compared to BO. Hydrophilicity plays a significant role in the bioactivity of bone regeneration since it influences the adsorption of biological molecules. Bone cells have shown a strong preference for hydrophilic substrates, and it is believed that hydrophilic materials offer better resistance to bacterial adhesion compared to hydrophobic

ones (Aronov et al., 2006, Popa et al., 2017, Maidaniuc et al., 2018). This is also supported by the results obtained for cell attachment, viability, and proliferation where WHA performed significantly better than BO. The attachment of osteogenic cells is essential for the regenerative outcomes of different biomaterials (Kwan et al., 2008). Hence, cells rarely get in direct contact with the biomaterial surface, they tend to create an interaction with the adsorbed proteins providing signals to the cells through the cell adhesion receptors (Tseng et al., 2013, Thomas et al., 1997). WHA adsorbing more albumin and total proteins, confirms that the protein-rich layer facilitates stronger initial cell adhesion and provides a conducive environment for cell growth, sustenance, and proliferation. Enhanced cell attachment and proliferation are critical for the successful integration of BHA into the bone tissue ensuring that the scaffold supports new tissue formation and integrates properly in the host bone.

The present study presents some limitations. While hydrophilicity was assessed qualitatively using capillarity tests, direct contact angle measurements were not performed. A quantitative assessment of hydrophilicity would provide more precise data on the material's surface properties. Specific cell lines (MC3T3, hBMSC, and RAW 264.7) were used to evaluate biological responses. These cell lines, while relevant, do not encompass the full diversity of cell types involved in bone regeneration. Moreover, the study evaluates biological responses over relatively short durations (up to 21 days for ion release and 7 days for cell viability/proliferation). Bone regeneration is a long process, and longer-term studies could provide more comprehensive insights into the material's performance over time. Additionally, although the chemotaxis assays provide valuable insights into macrophage recruitment, they do not evaluate polarization into M1 (pro-inflammatory) or M2 (anti-inflammatory) phenotypes. Evaluating the chemotactic behavior of both macrophage phenotypes would offer a more complete understanding of the immunomodulatory effects of WHA and BO that influence the healing process. Regarding ALP activity, this test was normalized to total protein content and measured under osteogenic induction over 21 days. This marker reflects only the early-to-intermediate stage of osteogenic commitment. A biomaterial-free (cells-only) osteogenic baseline was not included, and complementary markers of later differentiation and mineralization, such as osteocalcin expression, alizarin red staining, or osteogenic gene expression by qPCR would have strengthened the assessment of osteogenic potential. Lastly, in this study only two commercially available xenografts were tested, which may restrict the broader applicability of our findings. Two xenografts sharing the same origin but using distinct manufacturing processes were tested, thereby demonstrating how production refinements can optimize their physicochemical and biological properties. Investigations involving additional products could further validate and expand these results.

5. CONCLUSION

In conclusion, this study underscores the significant impact of manufacturing processes on the in vitro biological characteristics and subsequent in vivo performance of bovine hydroxyapatite (BHA) biomaterials. The comparative analysis between BO and WHA reveals distinct differences in their physicochemical properties and in vitro biological behaviors. The findings align well with previously observed in vivo outcomes, suggesting that optimizing the physicochemical properties through specific manufacturing processes can significantly enhance the clinical efficacy of BHA biomaterials in oral bone regeneration. Future studies should aim to explore long-term biological responses and the role of different cell types to further validate these findings and improve the understanding of the material's performance in clinical settings.

Authors:

Bruno De Carvalho, DDS, MSc, PhD Fellow, Head of Clinic, Department of Periodontology, Oro-Dental and Implant surgery, Dental biomaterial research Unit, University of Liège, Liège, Belgium; Sandy Anania, PhD, Department of Periodontology, Oro-Dental and Implant surgery, Dental biomaterial research Unit, University of Liège, Liège, Belgium; Dorien Van Hede, PhD, Department of Periodontology, Oro-Dental and Implant surgery, Dental biomaterial research Unit, University of Liège, Liège, Belgium; Emilie Dory, Ir, Chief Executive Officer, Wishbone SA, Liège, Belgium; Eric Rompen, DDS, MSc, PhD, Research consultant, Wishbone SA, Liège, Belgium; France Lambert, DDS, MSc, PhD, Professor and Head, Department of Periodontology, Oro-Dental and Implant surgery, Dental biomaterial research Unit, University of Liège, Liège, Belgium

Corresponding author:

France Lambert, DDS, MSc, PhD, Head of Service, Department of Periodontology, Oro-Dental and Implant surgery, Dental biomaterial research Unit,
Address: Avenue de l'Hôpital 1, 4000 Liège, Belgique.
Email: france.lambert@chuliege.be

Conflict of interest statement:

E.D. is currently employee at Wishbone SA., declaring having no financial arrangements or other interests in the activities of Wishbone that could affect her impartiality and/or influence the outcome of the study. F.L. and E.R. are founders and shareholders of Wishbone SA, and E.R. is a founder and shareholder of Wishbone Cy; their contribution to the study was limited and neither of them were involved in the analysis and interpretation of the results. B.D.C. declares no conflict of interest.

Author contribution statement:

E.D., F.L. and E.R. designed the study; B.D.C., S.A and D.V.H. analyzed the data. B.D.C. led the writing. All authors critically revised the manuscript and have given their approval for the final version to be published.

Acknowledgements:

This study was funded by Wishbone SA. The funders had no role in data collection/analysis or manuscript preparation. The authors thank the Center for Craniofacial Regeneration of the University of Pittsburgh for conducting the experimental part of the study.

REFERENCES

- 1 ABDELMONEIM, D., PORTER, G. C., COATES, D. E., DUNCAN, W. J., WADDELL, J. N., HAMMER, N. & LI, K. C. 2022. **The Effect of Low-Processing Temperature on the Physicochemical and Mechanical Properties of Bovine Hydroxyapatite Bone Substitutes.** *Materials* (Basel), 15.
- 2 ABOU FADEL, R., SAMARANI, R. & CHAKAR, C. 2018. **Guided bone regeneration in calvarial critical size bony defect using a double-layer resorbable collagen membrane covering a xenograft: a histological and histomorphometric study in rats.** *Oral Maxillofac Surg*, 22, 203–213.
- 3 ALBREKTSSON, T. & JOHANSSON, C. 2001. **Osteoinduction, osteoconduction and osseointegration.** *Eur Spine J*, 10 Suppl 2, S96–101.
- 4 ANTONINI, L. M., MENEZES, T. L., DOS SANTOS, A. G., JR., TAKIMI, A. S., VILLARINHO, D. J., DOS SANTOS, B. P., CAMASSOLA, M., MARCUZZO, J. S. & DE FRAGA MALFATTI, C. 2019. **Osteogenic differentiation of bone marrow-derived mesenchymal stem cells on anodized niobium surface.** *J Mater Sci Mater Med*, 30, 104.
- 5 AQUINO-MARTÍNEZ, R., ARTIGAS, N., GÁMEZ, B., ROSA, J. L. & VENTURA, F. 2017. **Extracellular calcium promotes bone formation from bone marrow mesenchymal stem cells by amplifying the effects of BMP-2 on SMAD signalling.** *PLoS One*, 12, e0178158.
- 6 ARONOV, D., ROSEN, R., RON, E. Z. & ROSENMAN, G. 2006. **Tunable hydroxyapatite wettability: Effect on adhesion of biological molecules.** *Process Biochemistry*, 41, 2367–2372.
- 7 BELLUCI, M. M., GIRO, G., DEL BARRIO, R. A. L., PEREIRA, R. M. R., MARCANTONIO, E., JR. & ORRICO, S. R. P. 2011. **Effects of magnesium intake deficiency on bone metabolism and bone tissue around osseointegrated implants.** *Clin Oral Implants Res*, 22, 716–721.

- 8** CARMAGNOLA, D., ADRIAENS, P. & BERGLUNDH, T. 2003. **Healing of human extraction sockets filled with Bio-Oss.** Clin Oral Implants Res, 14, 137–43.
- 9** COSTANTINO, M. D., SCHUSTER, A., HELMHOLZ, H., MEYER-RACHNER, A., WILLUMEIT-RÖMER, R. & LUTHRINGER-FEYERABEND, B. J. C. 2020. **Inflammatory response to magnesium-based biodegradable implant materials.** Acta Biomater, 101, 598–608.
- 10** DE CARVALHO, B., DORY, E., TRUS, C., PIRSON, J., GERMAIN, L., LECLOUX, G., LAMBERT, F. & ROMPEN, E. 2023. **Biological performance of a novel bovine hydroxyapatite in a guided bone regeneration model: A preclinical study in a mandibular defect in dogs.** Clin Implant Dent Relat Res.
- 11** DE CARVALHO, B., ROMPEN, E., LECLOUX, G., SCHUPBACH, P., DORY, E., ART, J. F. & LAMBERT, F. 2019. **Effect of Sintering on In Vivo Biological Performance of Chemically Deproteinized Bovine Hydroxyapatite.** Materials (Basel), 12.
- 12** DEL BARRIO, R. A., GIRO, G., BELLUCI, M. M., PEREIRA, R. M. & ORRICO, S. R. 2010. **Effect of severe dietary magnesium deficiency on systemic bone density and removal torque of osseointegrated implants.** Int J Oral Maxillofac Implants, 25, 1125–30.
- 13** FDA, U. 2021. Wishbone SA [Online]. Available: https://www.accessdata.fda.gov/cdrh_docs/pdf21/K211551.pdf [Accessed 2024].
- 14** GANI, M. A., BUDIATIN, A. S., SHINTA, D. W., ARDIANTO, C. & KHOTIB, J. 2023. **Bovine hydroxyapatite-based scaffold accelerated the inflammatory phase and bone growth in rats with bone defect.** J Appl Biomater Funct Mater, 21, 22808000221149193.
- 15** GIBON, E., LU, L. & GOODMAN, S. B. 2016. **Aging, inflammation, stem cells, and bone healing.** Stem Cell Res Ther, 7, 44.
- 16** HASSAN, M. N., ELTAWILA, A. M., MOHAMED-AHMED, S., AMIN, W. M., SULIMAN, S., KANDIL, S., YASSIN, M. A. & MUSTAFA, K. 2024. **Correlation between Ca Release and Osteoconduction by 3D-Printed Hydroxyapatite-Based Templates.** ACS Appl Mater Interfaces, 16, 28056–28069.

- 17 HE, L. Y., ZHANG, X. M., LIU, B., TIAN, Y. & MA, W. H. 2016. **Effect of magnesium ion on human osteoblast activity.** *Braz J Med Biol Res*, 49.
- 18 IELO, I., CALABRESE, G., DE LUCA, G. & CONOCI, S. 2022. **Recent Advances in Hydroxyapatite-Based Biocomposites for Bone Tissue Regeneration in Orthopedics.** *Int J Mol Sci*, 23.
- 19 KAMMERHOFER, J., FRIES, L., DUPAS, J., FORNY, L., HEINRICH, S. & PALZER, S. 2018. **Impact of hydrophobic surfaces on capillary wetting.** *Powder Technology*, 328, 367–374.
- 20 KLENKE, F. M., LIU, Y., YUAN, H., HUNZIKER, E. B., SIEBENROCK, K. A. & HOFSTETTER, W. 2008. **Impact of pore size on the vascularization and osseointegration of ceramic bone substitutes in vivo.** *J Biomed Mater Res A*, 85, 777–86.
- 21 KOROVESSIS, P. G. & DELIGIANNI, D. D. 2002. **Role of surface roughness of titanium versus hydroxyapatite on human bone marrow cells response.** *J Spinal Disord Tech*, 15, 175–83; discussion 183.
- 22 KWAN, M. D., SLATER, B. J., WAN, D. C. & LONGAKER, M. T. 2008. **Cell-based therapies for skeletal regenerative medicine.** *Hum Mol Genet*, 17, R93–8.
- 23 LAMBERT, F., BACEVIC, M., LAYROLLE, P., SCHÜPBACH, P., DRION, P. & ROMPEN, E. 2017. **Impact of biomaterial microtopography on bone regeneration: comparison of three hydroxyapatites.** *Clin Oral Implants Res*, 28, e201–e207.
- 24 LEE, J. E., BARK, C. W., QUY, H. V., SEO, S. J., LIM, J. H., KANG, S. A., LEE, Y., LEE, J. M., SUH, J. Y. & KIM, Y. G. 2018. **Effects of Enhanced Hydrophilic Titanium Dioxide-Coated Hydroxyapatite on Bone Regeneration in Rabbit Calvarial Defects.** *Int J Mol Sci*, 19.
- 25 LIAO, Z., FU, L., LI, P., WU, J., YUAN, X., NING, C., DING, Z., SUI, X., LIU, S. & GUO, Q. 2023. **Incorporation of Magnesium Ions into an Aptamer-Functionalized ECM Bioactive Scaffold for Articular Cartilage Regeneration.** *ACS Appl Mater Interfaces*, 15, 22944–22958.

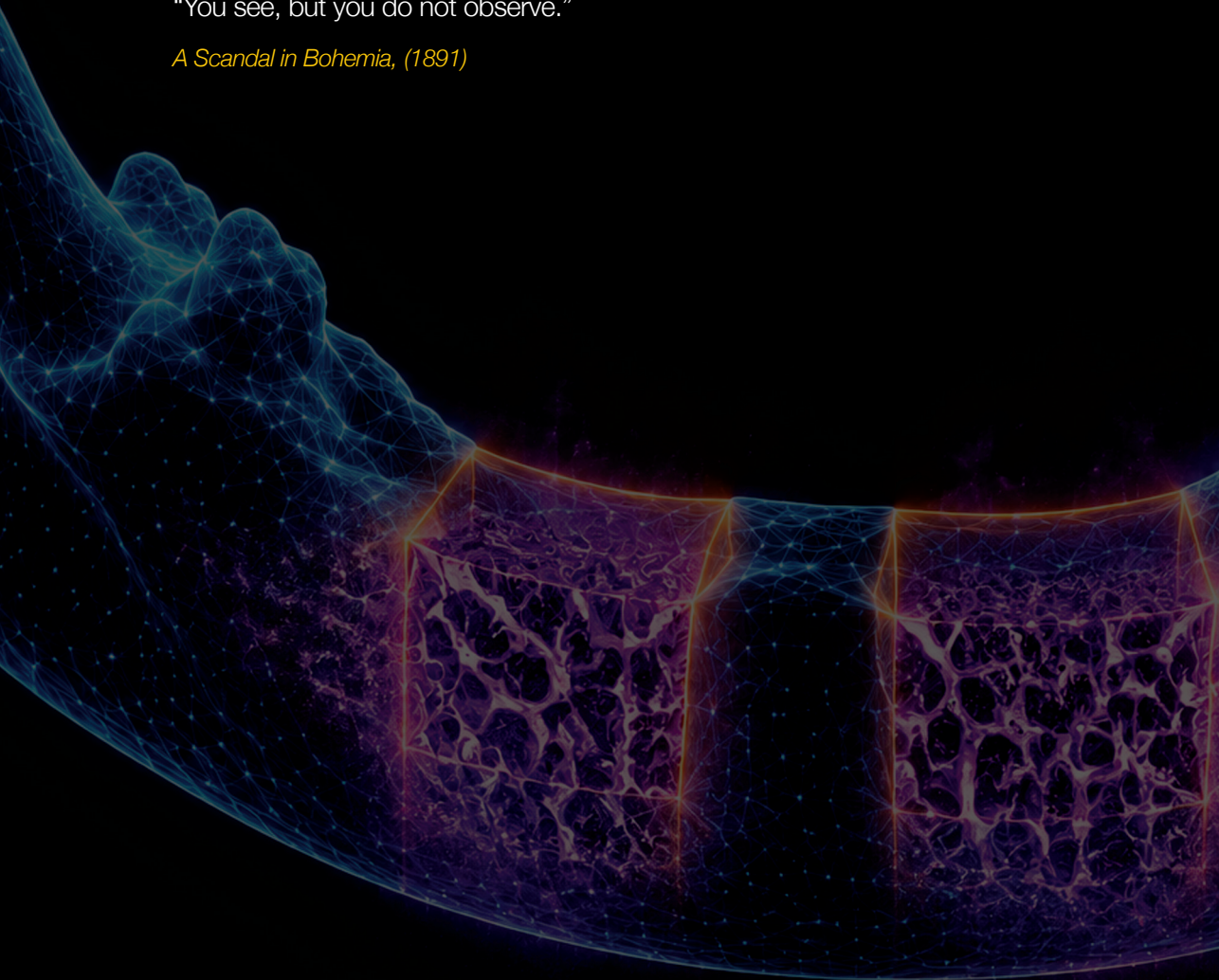
- 26 LOWE, D., SANVICTORES, T., ZUBAIR, M. & JOHN, S. 2024. **Alkaline Phosphatase**. StatPearls. Treasure Island (FL) ineligible companies.: StatPearls Publishing
- 27 Copyright © 2024, StatPearls Publishing LLC.
- 28 MAIDANIUC, A., MICULESCU, F., VOICU, S. I., ANDRONESCU, C., MICULESCU, M., MATEI, E., MOCANU, A. C., PENCEA, I., CSAKI, I., MACHEDON-PISU, T. & CIOCAN, L. T. 2018. **Induced wettability and surface-volume correlation of composition for bovine bone derived hydroxyapatite particles**. Applied Surface Science, 438, 158–166.
- 29 MAKRIS, K., MOUSA, C. & CAVALIER, E. 2023. Alkaline Phosphatases: Biochemistry, Functions, and Measurement. Calcif Tissue Int, 112, 233–242.
- 30 MAMMOLI, F., CASTIGLIONI, S., PARENTI, S., CAPPADONE, C., FARRUGGIA, G., IOTTI, S., DAVALLI, P., MAIER, J. A. M., GRANDE, A. & FRASSINETI, C. 2019. **Magnesium Is a Key Regulator of the Balance between Osteoclast and Osteoblast Differentiation in the Presence of Vitamin D₃**. Int J Mol Sci, 20.
- 31 MITRAGOTRI, S. & LAHANN, J. 2009. **Physical approaches to biomaterial design**. Nat Mater, 8, 15–23.
- 32 POPA, A. C., STAN, G. E., HUSANU, M. A., MERCIONIU, I., SANTOS, L. F., FERNANDES, H. R. & FERREIRA, J. 2017. **Bioglass implant-coating interactions in synthetic physiological fluids with varying degrees of biomimicry**. Int J Nanomedicine, 12, 683–707.
- 33 RIDIANDRIES, A., TAN, J. T. M. & BURSILL, C. A. 2018. **The Role of Chemokines in Wound Healing**. Int J Mol Sci, 19.
- 34 ROMPEN, E., LAMBERT, F., LECLOUX, G. & MONIOTTE, P. 2015. **Matériau de régénération osseuse et son procédé de fabrication**. patent WO 2015/049336 A1.
- 35 SADEGHIAN DEHKORD, E., DE CARVALHO, B., ERNST, M., ALBERT, A., LAMBERT, F. & GERIS, L. 2024. **Influence of physicochemical characteristics of calcium phosphate-based biomaterials in cranio-maxillofacial bone regeneration. A systematic literature review and meta-analysis of preclinical models**. Mater Today Bio, 26, 101100.

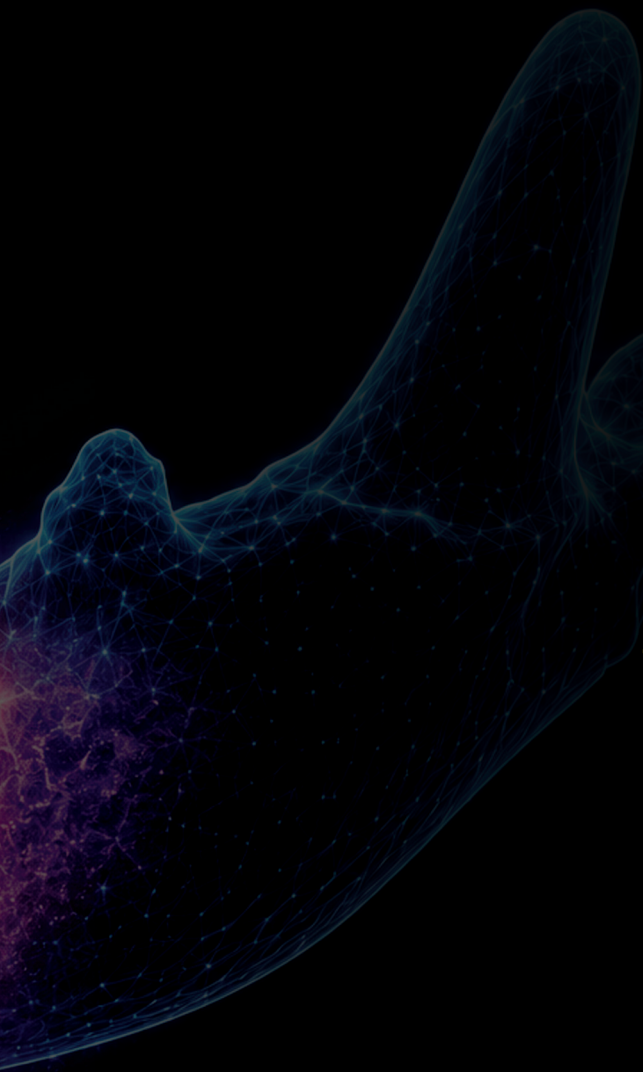
- 36 SERRANO MÉNDEZ, C. A., LANG, N. P., CANEVA, M., RAMÍREZ LEMUS, G., MORA SOLANO, G. & BOTTICELLI, D. 2017. **Comparison of allografts and xenografts used for alveolar ridge preservation. A clinical and histomorphometric RCT in humans.** *Clin Implant Dent Relat Res*, 19, 608–615.
- 37 SPILLER, K. L., ANFANG, R. R., SPILLER, K. J., NG, J., NAKAZAWA, K. R., DAULTON, J. W. & VUNJAK-NOVAKOVIC, G. 2014. **The role of macrophage phenotype in vascularization of tissue engineering scaffolds.** *Biomaterials*, 35, 4477–88.
- 38 SUN, R., LV, Y., NIU, Y., ZHAO, X.-H., CAO, D., TANG, J., SUN, X. & CHEN, K. 2017. **Physicochemical and biological properties of bovine-derived porous hydroxyapatite/collagen composite and its hydroxyapatite powders.** *Ceramics International*, 43, 16792–16798.
- 39 TADIC, D. & EPPLE, M. 2004. **A thorough physicochemical characterisation of 14 calcium phosphate-based bone substitution materials in comparison to natural bone.** *Biomaterials*, 25, 987–94.
- 40 THOMAS, C. H., MCFARLAND, C. D., JENKINS, M. L., REZANIA, A., STEELE, J. G. & HEALY, K. E. 1997. **The role of vitronectin in the attachment and spatial distribution of bone-derived cells on materials with patterned surface chemistry.** *J Biomed Mater Res*, 37, 81–93.
- 41 TSENG, L.-L., HO, C.-M., LIANG, W.-Z., HSIEH, Y.-D. & JAN, C.-R. 2013. **Comparison of efficacies of different bone substitutes adhered to osteoblasts with and without extracellular matrix proteins.** *Journal of Dental Sciences*, 8, 399–404.
- 42 WALTHERS, C. M., NAZEMI, A. K., PATEL, S. L., WU, B. M. & DUNN, J. C. 2014. **The effect of scaffold macroporosity on angiogenesis and cell survival in tissue-engineered smooth muscle.** *Biomaterials*, 35, 5129–37.
- 43 WEGGLICKI, W. B., PHILLIPS, T. M., FREEDMAN, A. M., CASSIDY, M. M. & DICKENS, B. F. 1992. **Magnesium-deficiency elevates circulating levels of inflammatory cytokines and endothelin.** *Mol Cell Biochem*, 110, 169–73.

ZENG, X. B., HU, H., XIE, L. Q., LAN, F., JIANG, W., WU, Y. & GU, Z. W. 2012. **Magnetic responsive hydroxyapatite composite scaffolds construction for bone defect repair.** *Int J Nanomedicine*, 7, 3365–78.

"You see, but you do not observe."

A Scandal in Bohemia, (1891)





5

METHODOLOGICAL
INNOVATION

Digital Twin Guided Optical Impression Analysis for Anatomically Accurate ROI Determination and Histologic Alignment in Bone Regeneration Models.

Bruno De Carvalho, DDS, MSc, PhD Fellow, Head of Clinic, Department of Periodontology, Oro-Dental and Implant surgery, Dental Biomaterial Research Unit, University of Liège, Liège, Belgium

Britta Knight, DVM, MSc, PhD, DVSc, DACVP, Charles River Laboratories, Senneville, QC, Canada

Diane Beaudry, MSc, Infusion, Parenteral Administration and Neurotoxicology, Charles River Laboratories, Senneville, QC, Canada

Geoffrey Lecloux, DDS, MSc, Head of Clinic, Department of Periodontology, Oro-Dental and Implant surgery, Dental Biomaterial Research Unit, University of Liège, Liège, Belgium

Eric Rompen, DDS, MSc, PhD, Research consultant, Wishbone SA, Liège, Belgium

Keywords:

Digital twin - Intraoral optical scanning - Histomorphometry - Region of interest

ABSTRACT

Objective: Accurate definition of the region of interest is essential for quantitative assessment of bone regeneration in preclinical defect models. Conventional histomorphometric analyses rely on retrospective identification of defect boundaries after healing has altered the original geometry. The present study introduces a digital twin-guided workflow based on intraoperative intraoral optical scanning (IOS) for reconstruction of the original defect envelope (ROI1) and spatial alignment with histologic section planes.

Methods: The methodology was implemented in a mandibular critical-size defect model in beagle dogs. A digital twin of the surgically created defect was reconstructed from intraoperative intraoral optical scans (IOS) and used to define the original defect region of interest (ROI1) prior to healing. Geometric fidelity of the workflow was evaluated by comparing IOS-derived and

histologic measurements of lingual wall thickness, assessing positional agreement between planned and actual section planes, and testing the geometric representativity of section-level ROI1 projections relative to IOS derived defect volume using correlation and regression analysis. The reconstructed baseline defect envelope (ROI1) was further compared with the post-healing compartment (ROI3) to characterize volumetric changes during regeneration.

Results: Defect geometry demonstrated high reproducibility across animals and timepoints, with coefficients of variation between 7% and 15% for all measured parameters. Section-level ROI1 projections showed strong geometric correspondence with reconstructed defect volume ($r = 0.934$; $R^2 = 0.873$), confirming their representativity of the three-dimensional baseline defect envelope. Spatial correspondence between planned and actual section planes was submillimetric on average (mean absolute deviation: 0.61 ± 0.56 mm; $r = 0.937$), confirming accurate transfer of the digital coordinate system to the physical sectioning workflow. Comparison between ROI1 and ROI3 revealed systematic contraction of the regenerated compartment relative to the original defect envelope across all healing periods.

Conclusion: The digital twin-guided IOS workflow enables prospective reconstruction of baseline defect geometry and precise spatial registration between three-dimensional surface datasets and histologic sections. This methodology improves the accuracy and reproducibility of quantitative regeneration analysis and provides a standardized framework for section-specific normalization of histomorphometric outcomes in preclinical mandibular defect models.

1. INTRODUCTION

Bone regeneration remains a central challenge in reconstructive oral and maxillofacial surgery, particularly in preclinical translational models where reliable quantitative measurements are essential to validate novel biomaterials and regenerative strategies prior to clinical translation (Sadeghian Dehkord et al., 2024, Shanbhag et al., 2024, Hooijmans et al., 2014). Histomorphometry and micro-computed tomography (micro-CT) are widely used as reference techniques for evaluating bone formation. Micro-CT enables three-dimensional evaluation, but quantitative outcomes remain sensitive to segmentation strategies, threshold selection, and volume-of-interest definition, which may influence measurements of newly formed bone (Donnelly, 2011, Park et al., 2011). Bone histomorphometry is a quantitative technique used to evaluate bone formation, microarchitecture and remodeling by analyzing structural and cellular features in histological sections (Malhan et al., 2018). However, both methods rely on the definition of a baseline region of interest that is typically established after healing has already

progressed, when the original geometry of the surgical defect can no longer be directly identified. Most studies quantify new bone formation within a predefined defect area, but the method used to define that area varies widely and is very often poorly described (Rogova et al., 2024). As the original defect progressively heals through remodeling and integration of newly formed tissue, retrospective identification of the initial defect boundaries, and therefore reconstruction of the baseline defect area required for standardized histomorphometric assessment, remains a major limitation that may reduce comparability across experimental models and studies. (Beitlitum et al., 2024). Contrarywise, histological processing further limits analysis through destructive sectioning and two-dimensional sampling of a restricted portion of the regenerated tissue volume and wrong orientation of the samples (Liu et al., 2021, Vandeweghe et al., 2013).

In this context, intraoral optical scanning (IOS) technologies have emerged as valuable tools for three-dimensional documentation. Initially developed for clinical applications in restorative, implant dentistry and periodontology, IOS provides high-resolution surface data capable of reconstructing soft- and hard-tissue contours with sub-millimeter accuracy (Benic et al., 2015). The advancements in the precision of these scanners enabled the emergence of methods that evaluate soft tissue volume changes in different clinical time points by techniques of superimposition of the 3D images generated (Lee et al., 2020, Tavelli et al., 2021). Although IOS technologies are widely used to capture dental and soft-tissue morphology in restorative and implant dentistry, their application for direct acquisition of exposed bone surfaces remains uncommon. Most IOS workflows rely on enamel or mucosal landmarks as reference structures, whereas intraoperative scanning of surgically created bone defects has, to the best of our knowledge, never been explored in experimental regeneration models. Extending IOS acquisition to the level of the defect walls enables direct three-dimensional documentation of the original defect geometry prior to healing, improving the reproducibility and accuracy of preclinical bone regeneration analysis. Three regions of interest were defined for spatial normalization: ROI1 represented the original defect envelope reconstructed from intraoperative IOS prior to healing; ROI2 corresponded to regenerated tissues within the original defect boundaries; and ROI3 described the post-healing compartment identified histologically at the endpoint. This framework enabled comparison between baseline defect geometry and the effective regenerated compartment. Building upon this rationale, the present study aimed to refine the methodological framework for volumetric bone regeneration assessment using IOS technology, based on our previous publication (De Carvalho et al., 2023). A digital workflow was developed that integrates pre-defect and post-defect IOS datasets to reconstruct, for each surgical defect, a digital twin of its original geometry. Within the context of this work, a digital twin is defined as a spatially registered, geometry-preserving virtual replica of a surgical defect,

generated from intraoperative optical scans and permanently linked to its physical counterpart through stable anatomical landmarks. By capturing the defect geometry immediately after surgery and before healing begins, the digital twin preserves the true baseline anatomy as a persistent digital reference that can be repeatedly co-registered with histological sections throughout the analysis. Unlike digital twins in engineering and systems modelling, which are continuously updated with real-time data to simulate and predict the behaviour of dynamic systems, the model proposed here represents a static digital twin. Its purpose is not to predict biological events or evolve over time, but to preserve the original anatomy with high spatial fidelity, providing a stable and reproducible baseline (ROI1).

2. METHODS

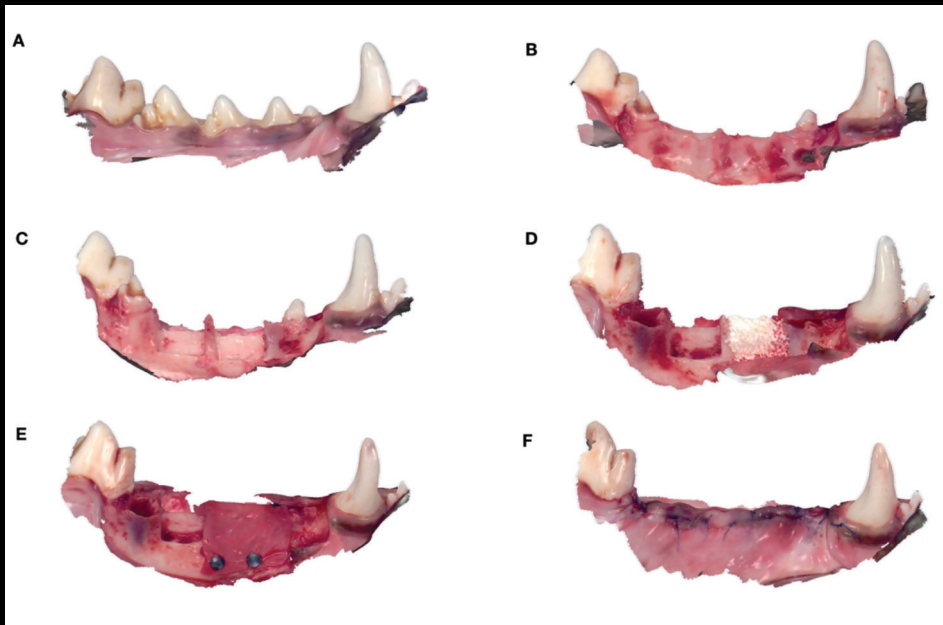
2.1. Preclinical Model and Surgical Protocol

The digital twin-guided ROI1 methodology was implemented on specimens obtained from a previously published preclinical guided bone regeneration study in a standardized mandibular defect model in dogs (De Carvalho et al., 2023). Briefly, twenty-four beagle dogs were enrolled and allocated to three euthanasia timepoints (4, 12, and 26 weeks), with eight animals per cohort. For each animal, four independent critical-size wall defects were created in the mandible, two per hemimandible. After extraction of the premolars on each side, two standardized box-shaped defects were prepared in the edentulous ridge using a rotary bone saw with approximately 10.0 mm in the mesio-distal direction, 8.0 mm in height (corono-apical), and 6.0 mm in bucco-lingual width, achieved by complete removal of the buccal cortical plate and partial removal of the lingual plate. All preparations followed the same geometric template and were performed by experienced surgeons. Within each animal, the four defects were randomly allocated to different regenerative protocols (two biomaterial-filled and two sham defects, with or without a collagen membrane), ensuring that each treatment condition was represented once per mandible. In defects assigned to regenerative treatment, the graft material was covered with a resorbable collagen membrane stabilized with titanium pins. Sham defects were either left empty or covered by a collagen membrane. Mucoperiosteal flaps were repositioned and closed with non-resorbable sutures, and all animals received standardized peri-operative antibiotic prophylaxis and postoperative analgesia. Throughout the healing period, animals were monitored daily for general health, wound integrity, and signs of local complications. The present methodological work represents a secondary, in-depth analysis of this established

Figure 1

Sequential intraoperative IOS reconstructions illustrating the surgical workflow of the standardized mandibular defect model.

(A) Baseline mandibular anatomy before extraction. (B) Edentulous ridge following tooth extraction. (C) Creation of standardized critical-size box-shaped defects. (D) Placement of particulate biomaterial within the defect compartment. (E) Collagen membrane fixation with titanium pins. (F) Flap closure and sutured wound stabilization. The sequential IOS-derived reconstructions are presented for illustrative purposes to demonstrate the surgical and regenerative workflow; only pre-defect and postdefect IOS datasets were used for quantitative digital twin reconstruction and ROI1 determination.



model, focusing specifically on the geometric reconstruction of the original defects and their correspondence with histologic sections. This standardized surgical protocol provided the experimental framework in which the intraoral optical scanning (IOS) based digital twin was constructed and later used to define the original defect region of interest (ROI1) for each histologic section. The sequential surgical workflow, including tooth extraction, defect preparation, biomaterial placement, membrane stabilization when applicable and flap closure, is illustrated in Fig. 1.

2.2. Intraoperative IOS Acquisition

IOS was used to establish a precise digital twin of the surgically created defect prior to the onset of healing. Surface scans of the mandibular ridge were acquired intraoperatively, after dental extraction and immediately after creation of the standardized critical-size defect using a structured-light scanner (Trios, 3Shape, Copenhagen, Denmark), which enables high-resolution surface acquisition through projection and detection of phase-shifted light patterns. Soft-tissue retraction and bleeding control were maintained throughout scanning to ensure accurate visualization of the exposed bone surfaces. Both the canine and molar adjacent to the defect area were systematically included in each IOS, to be used as anatomical landmarks. The resulting datasets were exported as STL files without smoothing or mesh repair.

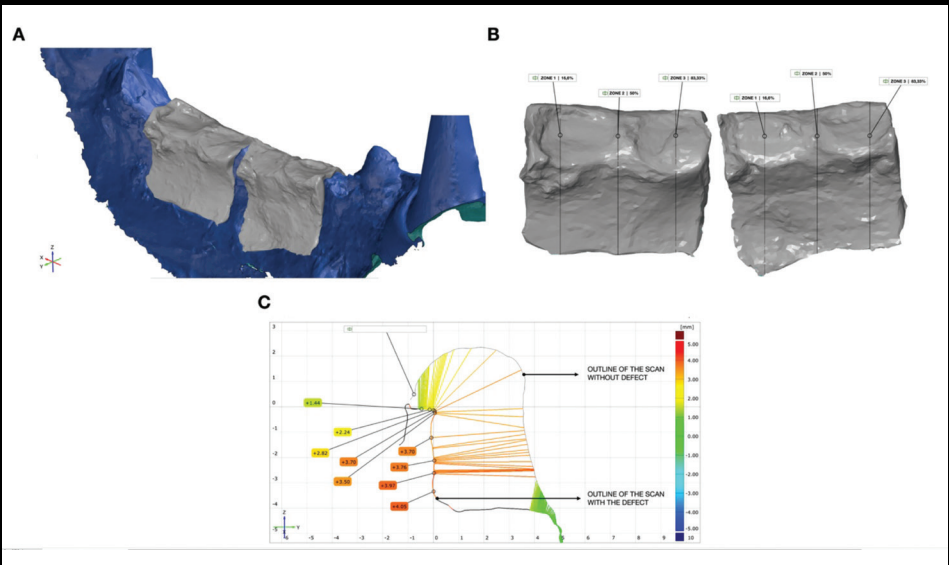
2.3. Digital Twin–Guided ROI1 Methodology

The pre- and post-defect surface scans were imported into metrology software (GOM Inspect Professional, GOM GmbH, Braunschweig, Germany) and aligned using intact anatomical landmarks (canine and molar crown surfaces). Once registered within a common coordinate system, subtraction of the post-defect scan from the pre-defect scan generated an animal-specific three-dimensional reconstruction of the removed bone volume (Fig. 2A). This subtraction model represented the STL digital twin of the original defect geometry and was preserved as a reference dataset for subsequent spatial registration with histological sections. Following subtraction, the reconstructed defect cavities were isolated from surrounding anatomical structures by manual mesh editing within the metrology software environment. Duplicate meshes of the defect regions were combined and non-relevant geometry removed to retain only the defect envelopes (Fig. 2B). Open mesh boundaries were subsequently closed to generate continuous surfaces, enabling reliable computation of defect perimeter, surface area, and enclosed volume prior to extraction of section-level ROI1 projections (Fig. 2C). To ensure spatial correspondence between the digital twin and histologic sections, the mesio–distal location of each cutting plane was predefined using linear measurements derived from the STL-based defect geometry. A fixed anatomical landmark corresponding to the center of the alveolar ridge between the two standardized defects, defined as the “bone peak” (Fig. 3A), was used as the reference origin for all measurements. Using this coordinate system, the mesio–distal distance from the bone peak to the geometric center of each defect was determined from the digital twin and provided to the histology laboratory as target sampling locations. In addition to the central section corresponding to the geometric midpoint of each defect, two supplementary section planes were defined at standardized proportional offsets along the defect axis to enable

Figure 2

Digital twin reconstruction and definition of standardized section planes for ROI1 determination.

(A) Three-dimensional reconstruction of the original defect geometry generated by subtraction and superimposition of pre-defect and post-defect IOS-derived STL datasets. The reconstructed defect envelope is isolated from surrounding anatomical structures following mesh alignment in GOM Inspect. (B) Definition of standardized sampling zones and target section planes along the mesio-distal axis of the reconstructed defect envelope. (C) Representative cross-sectional comparison between IOS-derived contours before and after defect preparation following STL superimposition. Colored deviation vectors illustrate geometric displacement generated by defect creation and used for reconstruction of the baseline defect envelope.



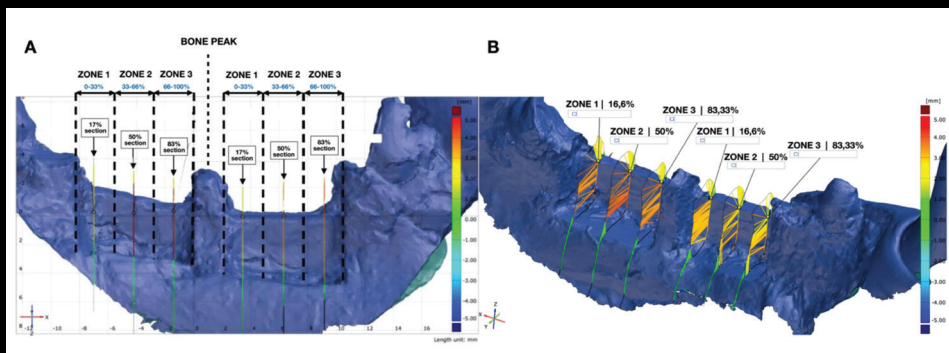
reproducible sampling across the mesial, central, and distal thirds of the reconstructed defect envelope (Fig. 3).

2.4. Sampling Zones and Histologic Slide Selection

Within the digital twin, the reconstructed defect was subdivided along its mesio-distal axis into three standardized zones — a mesial third (Zone 1), a central third (Zone 2), and a distal third (Zone 3) — and a target sampling location was defined within each zone using the bone peak landmark as the reference origin (Fig. 3). This zone-based scheme ensured consistent spatial representation of the mesial, central, and distal regions of each defect across specimens. These target locations served as a guide for histologic slide selection and did not direct the

Figure 3

Standardized spatial definition of histologic sampling planes within the reconstructed defect envelope. (A) Division of the reconstructed defect into mesial (Zone 1), central (Zone 2), and distal (Zone 3) thirds using the bone peak landmark as the reference origin, with target sampling locations positioned at approximately 17%, 50%, and 83% of the mesio–distal defect length. (B) Three-dimensional visualization of the predefined virtual section planes used for spatial registration between histologic sections and the IOS-derived digital twin.



sectioning itself. Following specimen embedding in methyl methacrylate, each defect was serially sectioned along its entire mesio–distal length, producing bucco–lingual ground sections (20–40 μm), and the distance of every section from the bone peak landmark was recorded. The unstained slides were examined and, for each zone, the slide of acceptable histological quality lying closest to the target location was selected for analysis. Two slides were retained per zone; the central slides were stained with Stevenel's Blue and the mesial and distal slides with haematoxylin and eosin for histomorphometric and histopathological evaluation. The actual measured position of each selected slide subsequently served as the basis for the digital twin–based ROI1 determination described in the following section.

2.5. Defect homogeneity assessment

To verify reproducibility of the standardized mandibular defect model, geometric parameters of the surgically created defects were extracted from the STL-based digital twin datasets. Measurements included anterior wall height, posterior wall height, lingual wall height, mesio–distal defect width, and distance from the bone peak landmark to the geometric center of each defect. Descriptive statistics and coefficients of variation were calculated across animals and time points to evaluate consistency of defect geometry prior to regenerative analysis.

2.6. Geometric representativity of section-level ROI1 projections

To evaluate whether section-level ROI1 projections were representative of the reconstructed three-dimensional defect envelope, the relationship between mean ROI1 cross-sectional area (ROI1 mean values mm²) and STL-derived defect volume (Volume mm³) was assessed using Pearson correlation and ordinary least squares regression. Observations containing missing values in either variable were excluded prior to analysis. Regression slope estimates were interpreted relative to the expected mesio–distal extent of the experimental defect to assess geometric consistency between section-based ROI1 projections and volumetric reconstruction. Where cohort information was available, an interaction model (Volume ~ ROI1_mean × Time point) was additionally used to evaluate whether this relationship differed across healing periods.

2.7. Digital Twin–Based Determination of ROI1 for Histomorphometric Analysis

Following specimen harvesting, fixation, and embedding in methyl methacrylate (MMA), grinded bucco–lingual sections (20–40 µm) were obtained along the entire length of the defect, and select slides were chosen for histomorphometric analysis using the predefined cutting positions as a guide and a quality control check by a veterinary pathologist. The recorded distance between the bone peak and the slide selected for analysis was used to create a virtual slicing plane at the same mesio–distal coordinate (relative to the bone peak) within the digital twin model that was reopened in GOM Inspect. Both the IOS-derived digital twin and the physical specimens were referenced to the same anatomical landmark ('bone peak'). Each histologic section could be mapped to an exact mesio–distal coordinate within the reconstructed defect geometry, allowing extraction of anatomically matched ROI1 cross-sections. The resulting projection defined the baseline region of interest (ROI1) corresponding to the true pre-healing defect area for that section and served as the reference for calculation of regenerated tissue measurements. In the original experimental dataset associated with this model (De Carvalho et al., 2023), histomorphometric tissue fractions were calculated relative to the baseline defect envelope reconstructed from intraoperative IOS (ROI1), allowing calculation of regenerated tissue areas to the true pre-healing geometry rather than to the post-healing augmented compartment.

2.8. Validation of IOS–Histology Geometric Correspondence

To assess the geometric fidelity of the digital twin, lingual wall thickness was measured on both the IOS-derived models and the corresponding pre-healing histologic sections at matched

anatomical locations. Measurements were obtained at three predefined positions along the mesio–distal axis of each defect (mesial, central, and distal planes) to ensure representative sampling of wall thickness and to minimize sectioning-related positional bias.

2.9. Spatial correspondence between planned and actual section planes

To verify the spatial correspondence between STL-derived ROI section positions and physical histologic section planes, the mesio–distal location of each section relative to the bone peak reference landmark was compared between planned digital coordinates and observed histologic slide positions. Observations containing missing values in either variable were excluded prior to analysis. Positional deviation was calculated as the difference between histologic and planned section coordinates, and absolute positional deviation as the absolute value of this difference. Agreement between planned and observed section locations was assessed using descriptive statistics and Pearson correlation analysis.

2.10. Regenerative envelope contraction and expansion classification

The volumetric behavior of the regenerated compartment was assessed by comparing ROI3 to ROI1 using:

- Filling ratio: $\text{ROI3}/\text{ROI1} \times 100$
- Relative change: $\text{ROI3} - \text{ROI1}$

Defects were categorized according to predefined thresholds:

- **Envelope Contraction:** $\text{ROI3} < 80\%$ of ROI1
- **Envelope Preservation:** $80\% \leq \text{ROI3} \leq 100\%$
- **Envelope Expansion:** $\text{ROI3} > 100\%$ of ROI1

These thresholds were applied uniformly across all cohorts.

2.11. Histologic composition across ROI3/ROI1 collapse categories

To investigate whether preservation of the original defect envelope was associated with differences in regenerated tissue composition, histologic fractions of mineralized tissue, residual biomaterial, and soft tissue within ROI3 were analyzed according to collapse categories defined by the ROI3/ROI1 ratio. Defects were classified as severe collapse ($<80\%$), moderate collapse ($80\text{--}100\%$), or overfilling ($>100\%$) relative to the baseline defect envelope. Tissue composition values were extracted from the histomorphometric dataset and summarized descriptively

within each category. Because defects from multiple regenerative conditions (biomaterial-filled, membrane-only, and untreated defects) and healing periods were included, this analysis was intended to characterize spatial patterns of compartment composition rather than treatment- or time-dependent effects.

2.12. Statistical Analysis

All analyses were descriptive. Continuous variables are presented as means, standard deviations, and ranges. Agreement between IOS and histology derived measurements was assessed using correlation and Bland–Altman methods. Measurements affected by sectioning artefacts that prevented reliable geometric correspondence between IOS, and histologic planes were excluded from agreement analysis prior to calculation of accuracy metrics. No hypothesis-testing was performed to compare treatment groups, as the objective of the study was methodological validation rather than evaluation of regenerative efficacy. Statistical analyses were limited to agreement testing, correlation analysis, and regression modeling to assess geometric correspondence between digital and histologic measurements.

3. RESULTS

3.1. Defect Homogeneity Across Animals

A total of 96 standardized defects were surgically created across all timepoints (32 per cohort). Due to incomplete or corrupted IOS surface data (STL errors), 5 defects could not be completely reconstructed digitally and were excluded from geometric analysis. Therefore, 91 mandibular defects with complete morphometric data were included in the assessment of defect homogeneity (31 defects at 4 weeks, 32 at 12 weeks, and 28 at 16 weeks). Defect geometry was highly consistent across animals and time points. The posterior wall height averaged 6.62 ± 0.80 mm at 4 weeks, 7.20 ± 0.75 mm at 12 weeks, and 7.22 ± 0.98 mm at 16 weeks, while the anterior wall height averaged 5.82 ± 0.88 mm, 6.56 ± 0.87 mm, and 6.67 ± 0.87 mm, respectively. The defect width was remarkably homogeneous, with values of 8.72 ± 0.67 mm (4 weeks), 8.90 ± 0.67 mm (12 weeks), and 8.59 ± 0.58 mm (16 weeks), corresponding to a coefficient of variation below 8% across cohorts. Similarly, the lingual wall height showed low dispersion, measuring 4.02 ± 0.47 mm, 4.16 ± 0.63 mm, and 4.03 ± 0.59 mm at 4, 12, and 16 weeks, respectively. The distance from the “bone peak” to the defect center also remained stable, with means of 5.32 ± 0.41 mm, 5.67 ± 0.45 mm, and 5.35 ± 0.49 mm. Across all

parameters, the coefficients of variation ranged between 7 % and 15 %, confirming that the surgically created defects exhibited a high degree of geometric reproducibility in the coronal, mesio-distal, and bucco-lingual dimensions (Fig. 4). This homogeneity validates the reliability of the defect model and ensures that subsequent differences in regenerative outcomes can be confidently attributed to treatment effects rather than variability in the initial defect anatomy.

3.2. Geometric representativity of ROI1 cross-sectional projections

After exclusion of incomplete observations, 88 defect-level measurements were available for analysis. ROI1 mean values (mm^2) showed a strong positive correlation with STL-derived defect volume (mm^3) ($r = 0.934$, $p < 0.001$; 95 % CI: 0.901–0.957). Linear regression yielded the equation $\text{Volume mm}^3 = -5.57 + 8.59 \times \text{ROI1 mean}$ with an R^2 of 0.873, indicating that ROI1 mean area accounted for 87.3 % of the variance in reconstructed defect volume. Notably, the regression slope (8.59 mm) closely approximated the expected mesio-distal extent of the experimental defect, supporting the geometric representativity of section-level ROI1 projections as descriptors of the three-dimensional baseline defect envelope. No significant interaction between ROI1 mean values and time point was observed ($p = 0.322$), confirming stability of this relationship across healing cohorts (Fig. 5).

3.3. Geometric accuracy of IOS-derived wall thickness (baseline validation).

To validate the geometric fidelity of the IOS-derived digital twin at baseline, lingual wall thickness was measured on both the IOS models and the corresponding histologic sections obtained from the same anatomical locations in cadaveric mandibles that were used to create baseline assessments of unhealed defects. A total of 18 paired measurements were obtained. Three measurements affected by sectioning artefacts were excluded, leaving 15 paired IOS–histologic measurements for analysis. After averaging mesial, central and distal sections per defect, the mean difference between histologic and IOS wall thickness (Histo – IOS) was 0.06 ± 0.09 mm, with a mean absolute difference of 0.07 ± 0.08 mm and a mean relative error of 5.8 ± 6.8 %. Individual relative errors ranged from 0.2 % to 17.5 %. IOS and histologic measurements were strongly correlated ($r = 0.95$), and Bland–Altman analysis showed a small positive bias, with 95 % limits of agreement between -0.12 mm and $+0.24$ mm (Fig. 6).

Figure 4

Defect width homogeneity across timepoints.
Mesio-distal defect width derived from IOS-based digital twin reconstructions at 4, 12, and 26 weeks, confirming reproducible baseline geometry of the standardized mandibular defect model.

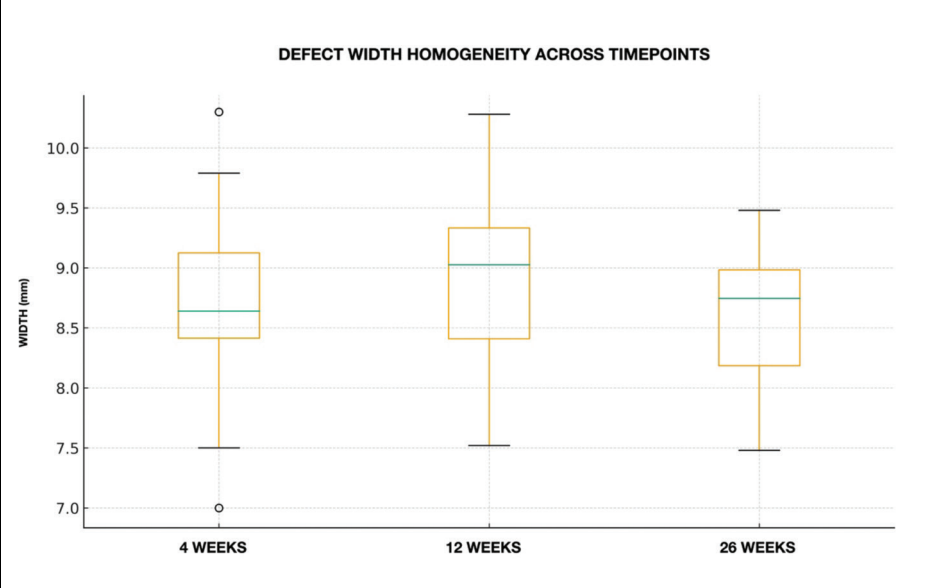


Figure 5

Geometric relationship between mean ROI1 cross-sectional area and reconstructed defect volume.
Scatter plot showing the association between ROI1_mean (mm²) and STL-derived defect volume (mm³) across specimens, color-coded by healing timepoint. The regression line illustrates the pooled linear relationship, supporting the geometric representativity of ROI1_mean as a section-level descriptor of the three-dimensional defect envelope.

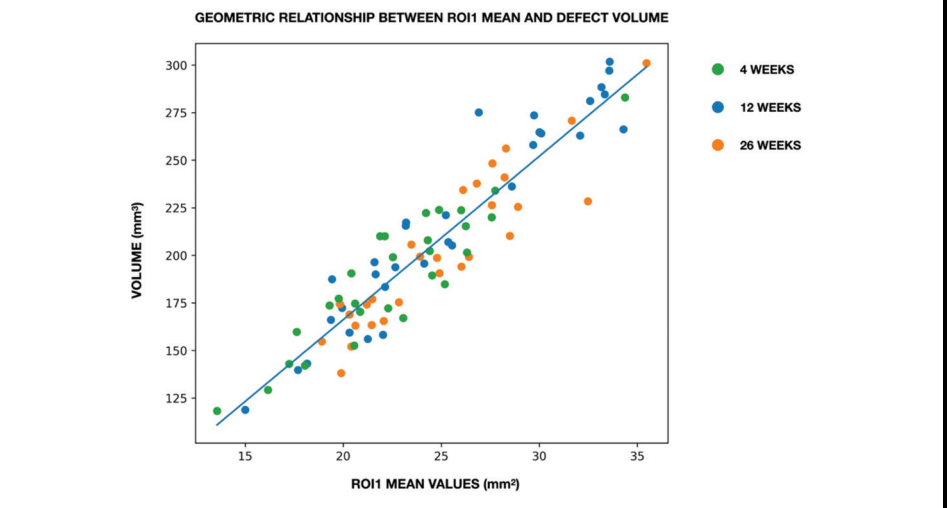
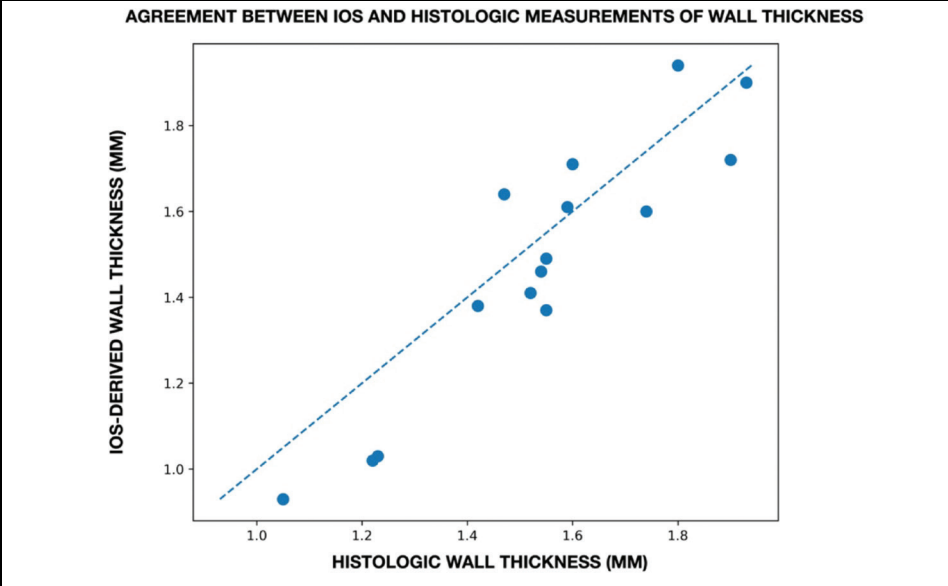


Figure 6

Agreement between IOS-derived and histologic measurements of lingual wall thickness at baseline. Scatter plot of paired measurements obtained at matched anatomical locations after exclusion of three measurements affected by sectioning artefacts (15/18 retained pairs). The dashed identity line represents perfect agreement between modalities. The close clustering of measurements along this line confirms strong geometric correspondence between IOS and histologic measurements.



3.4. Spatial correspondence between planned and actual section planes

Across 270 valid paired measurements, the mean positional deviation between planned STL-derived ROI section positions and actual histologic slide locations relative to the bone peak landmark was -0.35 ± 0.74 mm, with a mean absolute positional error of 0.61 ± 0.56 mm (range: -2.56 to 2.05 mm). Planned and observed section coordinates showed a strong linear relationship ($r = 0.937$; $R^2 = 0.877$), confirming accurate spatial transfer of the digital coordinate system to the physical sectioning workflow. These findings support the geometric reliability of the digital twin framework for section-level ROI1 determination.

3.5. Expansion and contraction patterns relative to the original defect (ROI1)

Using the IOS-derived digital twin as geometric reference, the original defect area (ROI1) was compared with the post-healing compartment at the histologic endpoint (ROI3) for each grafted

defect. After averaging across sections, 32 defects per timepoint (4, 12, and 26 weeks; 96 defects in total) were available for analysis. At 4 weeks, the mean ROI1 and ROI3 areas were $22.89 \pm 4.01 \text{ mm}^2$ and $15.92 \pm 8.83 \text{ mm}^2$, respectively, corresponding to a mean filling of the defect ($\text{ROI3}/\text{ROI1} \times 100$) of $70.8 \pm 39.8 \%$ and a relative change ($\text{ROI3}-\text{ROI1}$) of $-29.2 \pm 39.7 \%$. At 12 weeks, ROI1 and ROI3 averaged $25.48 \pm 5.56 \text{ mm}^2$ and $16.26 \pm 9.96 \text{ mm}^2$, with a mean filling of $63.9 \pm 36.6 \%$ and a relative change of $-36.2 \pm 36.5 \%$. At 26 weeks, ROI1 and ROI3 were $25.01 \pm 4.02 \text{ mm}^2$ and $14.04 \pm 7.35 \text{ mm}^2$, with a filling of $57.7 \pm 33.0 \%$ and a relative change of $-42.4 \pm 32.9 \%$ (Table 1). When defects were categorized according to the ROI3/ROI1 ratio, the majority showed contraction of the post-healing compartment relative to the original defect envelope ($<80 \%$ of ROI1), representing 19/32 defects at 4 weeks, 20/32 defects at 12 weeks, and 26/32 defects at 26 weeks. In contrast, 3, 4, and 2 defects at 4, 12, and 26 weeks, respectively, maintained a compartment size within 80–100 % of the original geometry, whereas expansion beyond the initial defect boundaries ($>100 \%$) was observed in 10, 8, and 4 defects, respectively.

3.6. Histologic composition across ROI3/ROI1 contraction categories

To evaluate whether the geometric behavior captured by the digital twin corresponded to differences in tissue composition, the histologic content of the post-healing compartment (ROI3) was analyzed across the three envelope-behavior categories defined by the ROI3/ROI1 ratio: envelope contraction ($<80 \%$; $n = 65$), envelope preservation (80–100 %; $n = 9$), and envelope expansion ($>100 \%$; $n = 22$). Because these categories pooled defects from multiple regenerative conditions (biomaterial-filled, membrane-covered sham, and untreated defects) and all healing periods, the analysis describes spatial characteristics of the post-healing compartment rather than treatment or time-dependent effects. Tissue contents are expressed as a percentage of the original defect envelope (ROI1) to allow comparison on a common reference.

Absolute areas are also reported in Table 1. Newly formed bone, expressed relative to the original defect envelope, remained within a narrow range across the three categories (15.9 %, 17.6 %, and 19.6 % of ROI1 for contracted, preserved, and expanded defects, respectively). Residual biomaterial and soft tissue, by contrast, increased markedly from contraction to expansion. Residual biomaterial increased from 4.6 % of ROI1 in contracted defects to 24.2 % and 38.0 % in preserved and expanded defects, respectively, while soft tissue content increased from 21.7 % to 48.0 % and 60.7 %. Expanded compartments were therefore characterized not by greater bone formation but by a substantially larger volume of residual

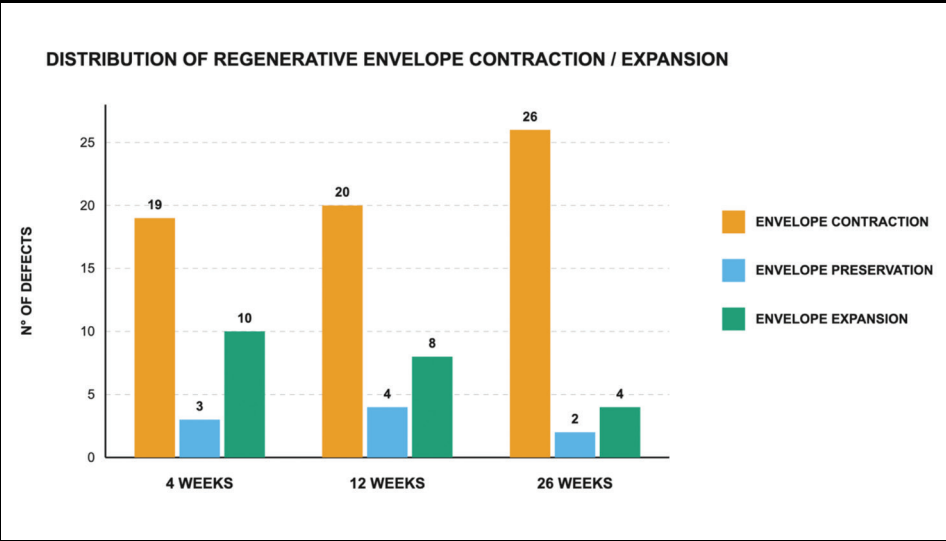
Table 1

Histologic composition of the post-healing compartment (ROI3) across envelope-behaviour categories. Values are presented as mean $\pm$ standard deviation. ROI1 represents the original defect envelope reconstructed from intraoperative IOS-derived digital twin analysis, whereas ROI3 corresponds to the post-healing submembrane compartment identified histologically at the endpoint. Filling ratio (%) was calculated as $ROI3/ROI1 \times 100$.

Category	n	ROI1 (mm ²)	ROI3 (mm ²)	Filling ratio (%)
Envelope contraction (<80%)	65	24.68 $\pm$ 4.56	10.33 $\pm$ 4.54	42.20
Envelope preservation (80–100%)	9	27.18 $\pm$ 5.14	24.47 $\pm$ 5.36	89.90
Envelope expansion (>100%)	22	22.72 $\pm$ 4.03	26.68 $\pm$ 4.53	118.50

Figure 7

Distribution of regenerative envelope contraction and expansion relative to the original defect geometry. Frequency of envelope contraction (<80 % of ROI1), envelope moderation (80–100 % of ROI1), and expansion (>100 % of ROI1) across healing timepoints (4, 12, and 26 weeks). Most defects showed contraction of the regenerated compartment relative to the IOS-derived baseline defect envelope (ROI1).



biomaterial and soft tissue occupying the space beyond the original defect boundary. This pattern is consistent with overfilling rather than enhanced regeneration.

4. DISCUSSION

Accurate definition of the region of interest remains a fundamental methodological requirement for quantitative assessment of bone regeneration in preclinical defect models. Conventional histomorphometric approaches typically define the analyzed compartment retrospectively within histologic sections after healing has already modified the original defect geometry (Rogova et al., 2024). As a consequence, measurements of regenerated tissue frequently depend on section orientation, sampling location, and operator-dependent identification of defect boundaries rather than on the true baseline anatomy (Bonucci et al., 1990, Lopez-Heredia et al., 2012, Overgaard et al., 2000). Previous clinical and experimental histomorphometric studies have similarly relied on section-based identification of regenerated compartments without access to the original defect configuration, highlighting the potential influence of biopsy geometry on outcome interpretation (Cucchi et al., 2019). These limitations are further compounded by the intrinsic spatial heterogeneity of bone repair tissues. Unlike metabolic bone remodeling, biomaterial-filled regenerating defects contain variable proportions of woven bone, residual biomaterial, connective tissue, and marrow-like spaces that evolve dynamically during healing. Histological methods originally developed for metabolic bone disease do not adequately account for the structural variability characteristic of bone repair environments (Gerstenfeld et al., 2005). As emphasized in experimental regeneration models, tissue composition differs substantially between periosteal, central, and host-bone interface regions within the same defect, demonstrating that regeneration outcomes are strongly influenced by the spatial position of the analyzed region (Ghiacci et al., 2016). Together, these observations underscore the importance of standardized approaches capable of preserving the original defect geometry during quantitative analysis.

Another important methodological limitation arises from the dependence of histomorphometric measurements on sectioning orientation. Cross-sectional defect dimensions may vary substantially depending on whether sections are obtained along longitudinal or transverse planes, introducing variability that complicates comparison between specimens and studies. In addition, sampling strategies restricted to a limited number of central sections may not adequately represent the entire regenerated compartment, further increasing statistical uncertainty in quantitative assessments of healing (Gerstenfeld et al., 2005, Overgaard et al., 2000). These

considerations highlight the need for spatially registered methodologies capable of linking histologic measurements to the original three-dimensional defect configuration.

The present study addresses these limitations through implementation of an intraoperative IOS workflow that preserves the geometry of the surgically created defect as a digital twin prior to the onset of healing. By integrating pre-defect and post-defect IOS datasets with metrology-based surface alignment, the original defect envelope could be reconstructed with submillimetric accuracy and subsequently matched to individual histologic section planes. This approach enabled prospective definition of the baseline region of interest (ROI1) rather than retrospective approximation based on post-healing contours, thereby improving the reproducibility and interpretability of histomorphometric outcome measurements.

Geometric validation of the digital twin demonstrated a high degree of correspondence between IOS-derived and histologically measured wall thickness values, supporting the reliability of structured-light surface acquisition for intraoperative documentation of exposed bone morphology. In addition, the standardized mandibular defect model exhibited low dispersion across corono-apical, mesio-distal, and bucco-lingual dimensions, confirming that the surgical template provided a consistent framework for evaluation of spatially registered regeneration outcomes. These findings indicate that IOS-derived digital reconstruction can serve as a stable geometric reference for section-specific normalization of regenerated tissue measurements. The strong linear relationship observed between ROI1 mean values of the cross-sectional areas and STL-derived defect volume further confirms that section-level ROI1 projections are not only anatomically accurate but also geometrically scalable descriptors of defect volume providing a geometrically representative estimate of the reconstructed three-dimensional defect envelope, supporting their use as a spatial framework for histomorphometric outcome assessment.

Beyond geometric validation, comparison between the original defect area (ROI1) and the post-healing augmented compartment (ROI3) revealed systematic contraction of the regenerated space relative to the initial defect envelope across all healing periods. Since conventional histologic analysis typically relies on the post-healing contour as the measurement boundary, such contraction would remain undetected without access to baseline defect geometry. Preservation of the original defect configuration therefore allows quantification of space-maintenance behavior independently of tissue composition and provides a more accurate estimate of the effective regenerative compartment. This advantage is particularly relevant in defects exhibiting pronounced collapse, such as untreated defects that typically serve as control groups or defects covered with a membrane alone. When the region of interest is defined retrospectively from the post-healing contour, contraction of the defect envelope results in underestimation of the original defect size and may artificially increase the apparent

proportion of regenerated tissues, thereby masking differences between control and biomaterial-filled defects.

In this study, collapse categories were derived from pooled defects representing multiple regenerative conditions, including biomaterial-filled, membrane-only, and untreated defects. The present analysis was not intended to compare treatment efficacy but rather to evaluate how preservation of the original defect envelope relates to tissue composition within the reconstructed regenerative compartment. Essentially, the geometric behavior captured through ROI1 based analysis was associated with biologically meaningful differences in histologic composition. Defects maintaining dimensions closer to the original envelope exhibited higher fractions of mineralized tissue, whereas severe contraction was associated with reduced bone formation and near-empty post-healing compartments. In contrast, expansion beyond the original defect boundaries corresponded to increased proportions of residual biomaterial and soft tissue, suggesting overfilling of the defect rather than effective regeneration within the intended defect space. These observations support previous experimental evidence demonstrating that spatial structure and distribution of tissues within the defect strongly influences regenerative and healing outcomes, (Ghiacci et al., 2016, Schulze et al., 2023) and indicate that preservation of defect geometry represents a relevant determinant of osteogenesis in guided bone regeneration models. Although radiographic techniques such as micro-computed tomography provide valuable three-dimensional information on mineralized tissue formation during healing, they do not allow direct reconstruction of the original defect onfiguration once remodeling has begun. In contrast, intraoperative IOS enables immediate acquisition of exposed bone surface morphology at the time of defect preparation and therefore preserves the true baseline anatomical envelope. Integration of this dataset with histologic sectioning coordinates establishes spatial correspondence between virtual defect geometry and physical tissue sections, addressing a limitation previously identified in histomorphometric analyses of bone repair that require alignment with radiological and structural measurements of healing tissues (Gerstenfeld et al., 2005).

Certain limitations in this study should be considered. IOS captures surface geometry rather than internal mineralized structures and therefore cannot replace micro-computed tomography for assessment of trabecular architecture or mineral density. In addition, accurate reconstruction of the digital twin depends on adequate intraoperative visualization of the defect surface, proper bleeding control and the availability of stable anatomical landmarks for mesh alignment. Further validation across additional defect configurations and species models would be valuable to confirm the general applicability of this workflow. Within these limitations, the digital twin-guided ROI1 methodology represents a reproducible strategy for preserving baseline defect geometry and integrating three-dimensional surface acquisition with histomorphometric analysis. By enabling spatially registered calculation of regenerated tissue measurements relative to the

original defect envelope, this approach improves the accuracy of quantitative outcome assessment and provides a methodological framework for more standardized evaluation of bone regeneration in preclinical mandibular defect models.

To the best of our knowledge, this study represents the first implementation of an intraoperative intraoral optical scanning workflow specifically designed to preserve the original geometry of surgically created bone defects as a digital twin for subsequent histomorphometric calculations. While IOS has been widely applied for longitudinal assessment of soft tissue contour changes and surface dimensional alterations following extraction or implant therapy, its use for reconstruction of baseline defect geometry immediately after defect preparation has not previously been described in preclinical bone regeneration models. By enabling prospective definition of ROI1 prior to the onset of healing, the present methodology provides a spatially registered reference framework that addresses a fundamental limitation of conventional histologic outcome assessment.

5. CONCLUSION

The digital twin-guided IOS workflow presented in this study enables prospective reconstruction of the original defect geometry and precise spatial alignment between three-dimensional surface datasets and histological section planes, allowing anatomically accurate definition of ROI1 for quantitative regeneration analysis. This approach overcomes one of the limitations of conventional histomorphometric methodologies that rely on retrospective estimation of defect boundaries after tissue remodeling has occurred. The strong agreement between IOS-derived measurements and histologic references further supports the geometric reliability of the proposed framework and confirms its suitability for section-specific normalization of regenerated tissue outcomes. Beyond improving measurement accuracy, the methodology enables detection of biologically meaningful variations in defect space maintenance that remain inaccessible using traditional analysis workflows. Overall, the digital twin-guided strategy establishes a reproducible, spatially consistent framework registration for quantitative evaluation of bone regeneration and with potential to improve standardization of outcomes in preclinical bone regeneration models.

Corresponding author:

Bruno De Carvalho, DDS, MSc, PhD Fellow, Head of Clinic, Department of Periodontology,
Oro-Dental and Implant surgery, Dental biomaterial research Unit,
Address: Avenue de l'Hôpital 1, 4000 Liège, Belgique.
Email: bruno.decarvalho@chuliege.be

REFERENCES

- 1 BEITLITUM, I., RAYYAN, F., POKHOJAEV, A., TAL, H. & SARIG, R. 2024. **A novel micro-CT analysis for evaluating the regenerative potential of bone augmentation xenografts in rabbit calvarias.** *Sci Rep*, 14, 4321.
- 2 BENIC, G. I., ELMASRY, M. & HÄMMERLE, C. H. 2015. **Novel digital imaging techniques to assess the outcome in oral rehabilitation with dental implants: a narrative review.** *Clin Oral Implants Res*, 26 Suppl 11, 86–96.
- 3 BONUCCI, E., BALLANTI, P., DELLA ROCCA, C., MILANI, S., LO CASCIO, V. & IMBIMBO, B. 1990. **Technical variability of bone histomorphometric measurements.** *Bone Miner*, 11, 177–86.
- 4 CUCCHI, A., SARTORI, M., PARRILLI, A., ALDINI, N. N., VIGNUDELLI, E. & CORINALDESI, G. 2019. **Histological and histomorphometric analysis of bone tissue after guided bone regeneration with non-resorbable membranes vs resorbable membranes and titanium mesh.** *Clin Implant Dent Relat Res*, 21, 693–701.
- 5 DE CARVALHO, B., DORY, E., TRUS, C., PIRSON, J., GERMAIN, L., LECLOUX, G., LAMBERT, F. & ROMPEN, E. 2023. **Biological performance of a novel bovine hydroxyapatite in a guided bone regeneration model: A preclinical study in a mandibular defect in dogs.** *Clin Implant Dent Relat Res*.
- 6 DONNELLY, E. 2011. **Methods for assessing bone quality: a review.** *Clin Orthop Relat Res*, 469, 2128–38.
- 7 GERSTENFELD, L. C., WRONSKI, T. J., HOLLINGER, J. O. & EINHORN, T. A. 2005. **Application of histomorphometric methods to the study of bone repair.** *J Bone Miner Res*, 20, 1715–22.
- 8 GHIACCI, G., GRAIANI, G., RAVANETTI, F., LUMETTI, S., MANFREDI, E., GALLI, C., CACCHIOLI, A., MACALUSO, G. M. & SALA, R. 2016. **«Over-inlay» block graft and differential morphometry: a novel block graft model to study bone regeneration and host-to-graft interfaces in rats.** *J Periodontal Implant Sci*, 46, 220–33.

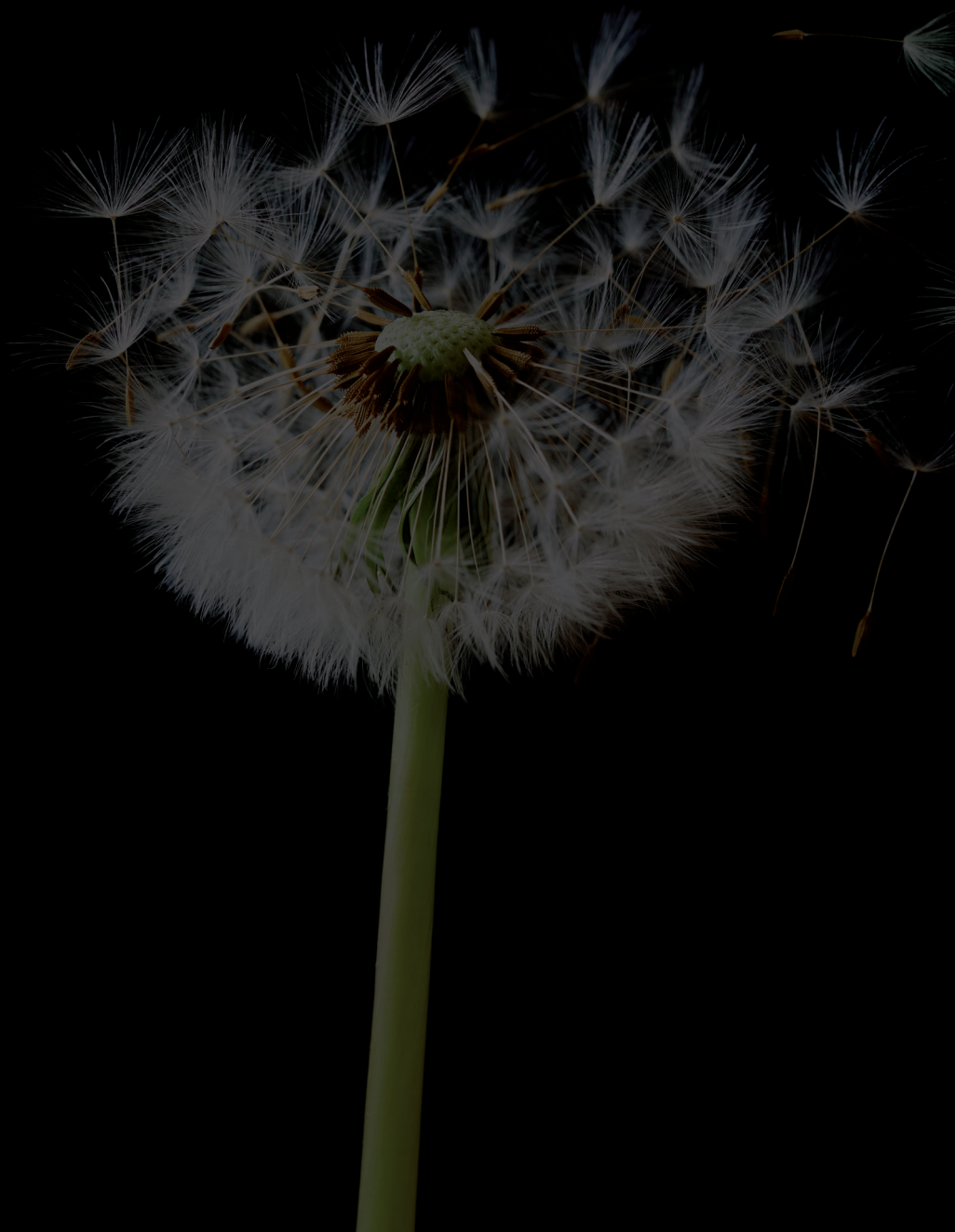
- 9 HOOIJMANS, C. R., INTHOUT, J., RITSKES-HOITINGA, M. & ROVERS, M. M. 2014. **Meta-analyses of animal studies: an introduction of a valuable instrument to further improve healthcare.** *Ilar j*, 55, 418–26.
- 10 LEE, J. S., JEON, Y. S., STRAUSS, F. J., JUNG, H. I. & GRUBER, R. 2020. **Digital scanning is more accurate than using a periodontal probe to measure the keratinized tissue width.** *Sci Rep*, 10, 3665.
- 11 LIU, J. T. C., GLASER, A. K., BERA, K., TRUE, L. D., REDER, N. P., ELICEIRI, K. W. & MADABHUSHI, A. 2021. **Harnessing non-destructive 3D pathology.** *Nat Biomed Eng*, 5, 203–218.
- 12 LOPEZ-HEREDIA, M. A., BONGIO, M., CUIJPERS, V. M., VAN DIJK, N. W., VAN DEN BEUCKEN, J. J., WOLKE, J. G. & JANSEN, J. A. 2012. **Bone formation analysis: effect of quantification procedures on the study outcome.** *Tissue Eng Part C Methods*, 18, 369–73.
- 13 MALHAN, D., MUELKE, M., ROSCH, S., SCHAEFER, A. B., MERBOTH, F., WEISWEILER, D., HEISS, C., ARGANDA-CARRERAS, I. & EL KHASSAWNA, T. 2018. **An Optimized Approach to Perform Bone Histomorphometry.** *Front Endocrinol (Lausanne)*, 9, 666.
- 14 OVERGAARD, S., SØBALLE, K., JØRGEN, H. & GUNDERSEN, G. 2000. **Efficiency of systematic sampling in histomorphometric bone research illustrated by hydroxyapatite-coated implants: optimizing the stereological vertical-section design.** *J Orthop Res*, 18, 313–21.
- 15 PARK, S. Y., KIM, K. H., KOO, K. T., LEE, K. W., LEE, Y. M., CHUNG, C. P. & SEOL, Y. J. 2011. **The evaluation of the correlation between histomorphometric analysis and micro-computed tomography analysis in AdBMP-2 induced bone regeneration in rat calvarial defects.** *J Periodontal Implant Sci*, 41, 218–26.
- 16 ROGOVA, V. V., PEEV, S., YOTSOVA, R., GEROVA-VATSOVA, T. & PARUSHEV, I. 2024. **Histomorphometric Assessment of Non-Decalcified Plastic-Embedded Specimens for Evaluation of Bone Regeneration Using Bone Substitute Materials-A Systematic Review.** *Materials (Basel)*, 18.

- 17 SADEGHIAN DEHKORD, E., DE CARVALHO, B., ERNST, M., ALBERT, A., LAMBERT, F. & GERIS, L. 2024. **Influence of physicochemical characteristics of calcium-phosphate-based biomaterials in cranio-maxillofacial bone regeneration. A systematic literature review and meta-analysis of preclinical models.** *Mater Today Bio*, 26, 101100.
- 18 SCHULZE, F., LANG, A., SCHOON, J., WASSILEW, G. I. & REICHERT, J. 2023. **Scaffold Guided Bone Regeneration for the Treatment of Large Segmental Defects in Long Bones.** *Biomedicines*, 11.
- 19 SHANBHAG, S., KAMPLEITNER, C., SANZ-ESPORRIN, J., LIE, S. A., GRUBER, R., MUSTAFA, K. & SANZ, M. 2024. **Regeneration of alveolar bone defects in the experimental pig model: A systematic review and meta-analysis.** *Clin Oral Implants Res*, 35, 467–486.
- 20 TAVELLI, L., BAROOTCHI, S., MAJZOUB, J., SIQUEIRA, R., MENDONÇA, G. & WANG, H. L. 2021. **Volumetric changes at implant sites: A systematic appraisal of traditional methods and optical scanning-based digital technologies.** *J Clin Periodontol*, 48, 315–334.
- 21 VANDEWEGHE, S., COELHO, P. G., VANHOVE, C., WENNERBERG, A. & JIMBO, R. 2013. **Utilizing micro-computed tomography to evaluate bone structure surrounding dental implants: a comparison with histomorphometry.** *J Biomed Mater Res B Appl Biomater*, 101, 1259–66.



"You mustn't be afraid to dream a little bigger, darling."

Inception (2010)



A large, white, stylized number '6' is the central focus. To its left, several dandelion seed heads are visible, some in sharp focus and others blurred, creating a sense of depth. The background is a solid black.

6

NEXT-GENERATION
BIOMATERIAL
CONCEPTS

Next-generation biomaterials for alveolar bone regeneration: a structurally instructive, multiscale design paradigm.

Bruno De Carvalho, DDS, MSc, PhD Fellow, Head of Clinic, Department of Periodontology, Oro-Dental and Implant surgery, Dental Biomaterial Research Unit, University of Liège, Liège, Belgium

Margaryta Ivanets, Dental Biomaterials Research Unit, Biomechanics Research Unit (Biomech), Liège, Belgium

France Lambert, DDS, MSc, PhD, Professor and Head, Department of Periodontology, Oro-Dental and Implant surgery, Dental Biomaterial Research Unit, University of Liège, Liège, Belgium

Liesbet Geris, GIGA In Silico Medicine, Biomechanics Research Unit (Biomech), University of Liège, Belgium

Dorien Van Hede, PhD, Department of Periodontology, Oro-Dental and Implant surgery, Dental Biomaterial Research Unit, Liège, Belgium

Keywords:

Alveolar bone regeneration - Multiscale biomaterials - Scaffold design - Bioinstructive materials

ABSTRACT

Alveolar bone defects, particularly large or non-contained ones, remain a major challenge in implant dentistry and oral and maxillofacial surgery. Designing scaffolds capable of regenerating such defects requires the simultaneous integration of biological, structural, and mechanical properties across length scales spanning atomic-level surface chemistry to macroscopic architecture, raising trade-offs that cannot be resolved by optimizing any single design parameter in isolation. This narrative review examines how the structural properties of biomaterials at the pico-, nano-, micro-, and macroscale influence bone regeneration in oral and maxillofacial applications, identifies points of consensus and remaining debate at each scale, and outlines emerging directions in structurally instructive scaffold design. Beyond scale-by-scale optimization, hierarchical multiscale-hybrid architectures and dynamic, stimuli-responsive biomaterials are increasingly recognized as essential to meet the competing requirements of vascularization,

mechanical stability, and time-evolving tissue remodeling. Computational and AI-driven materials design, combined with patient-specific biological profiling, may enable the next generation of biomaterials capable of dynamically interacting with their biological environment. Realizing this potential will require integrating multiscale structural design with dynamic biomaterial behavior, supported by computational tools that can navigate the resulting multidimensional design space.

1. INTRODUCTION

Alveolar bone defects are extremely common and remain a major challenge in implant dentistry, particularly in cases involving large defects, compromised healing environments, or insufficient native bone volume. The need for alveolar ridge augmentation is continuously increasing, driven by an aging population and a growing access to advanced healthcare. As bone represents the second most transplanted tissue after blood, developing new bone grafts and bone tissue engineering solutions is extensively investigated in the literature.

Autologous bone is still considered as the current gold standard material for bone regeneration (Giannoudis et al., 2011, Grado et al., 2018) because of its histocompatibility, non-immunogenicity and ideal composition to support bone regeneration (Amini et al., 2012). However, this treatment strategy involves a second surgical site, associated with several complications and a certain degree of donor site morbidity. Allografts represent an intermediate solution between autografts and synthetic substitutes, avoiding donor site morbidity while retaining a bone-derived matrix. However, concerns regarding disease transmission, variable osteogenic potential, and reduced biological activity have limited their use, particularly in intra-oral bone regeneration. In this context, alveolar bone regeneration increasingly relies on alternative bone substitutes, primarily calcium phosphate-based biomaterials. Despite their advantages, available biomaterials still present drawbacks, leaving room for improvement (Ashfaq et al., 2024, Wang and Yeung, 2017).

The Consensus report of group 2 of the 15th European Workshop on Periodontology on Bone Regeneration (Sanz et al., 2019) reported the following specific requirements for bone substitutes materials : (1) biocompatibility, (2) adequate porosity, (3) osteoconductivity / osteoinductivity, (4) favorable surface properties, (5) biodegradability, (6) appropriate mechanical properties, (7) angiogenicity, (8) handleability and finally (9) manufacturability. Additionally, beyond their biological and structural specifications, biomaterials for alveolar bone regeneration must comply with updated regulatory requirements to reach the market and be cost-effective (Haugen et al., 2019).

The use of skeletal progenitor cells (SPCs) represents a significant potential in the field of regenerative medicine (Dulak et al., 2015, Humes, 2005, Samsonraj et al., 2017, Seo and Na, 2011). However, cell-based approaches remain expensive, are constrained by the limited availability of GMP-compliant manufacturing platforms and face regulatory barriers that have so far restricted clinical translation (Hoang et al., 2025, Ma et al., 2024). Similarly, the development of bioactive factor-based biomaterials, delivering growth factors, cytokines, or small molecules, faces its own challenges, including high production costs, limited spatiotemporal control over release, and uncertainty regarding supraphysiological dosing (Mariner et al., 2013, Wang et al., 2018, Zhujiang and Kim, 2016). Although some of these strategies (e.g., rhBMP-2, platelet-rich preparations) have reached clinical use in oral and maxillofacial applications, the scientific evidence supporting their efficacy for alveolar bone regeneration remains heterogeneous (Ramly et al., 2019, Siawasch et al., 2025).

An alternative bone regenerative strategy relies on the use of structurally instructive biomaterials to mobilize endogenous cells and facilitate the body's innate self-repairing ability. This involves tailoring the biomaterial structure at the multiple scales. Since bone graft substitutes primarily serve as a physical support for osteogenic cell colonization and bone matrix deposition, the modifications of the biomaterial structures (pico-, nano- and micro- topographies, micro-porosities, interconnectivity, geometry and macro-design) may influence the bone regenerative process. While most currently available biomaterials primarily focus on chemical composition, the role of structural design across multiple scales remains comparatively less explored. A large body of recent evidence supporting this hypothesis will be discussed in the present review.

Bone tissue is a hierarchical structure ranging from atomic interactions to complex three-dimensional trabecular architectures. Osteogenic cells reside within a dynamic extracellular matrix (ECM) characterized by a wide range of structural elements ranging from the nano- to microscale. These structural elements have a strong effect on cell adhesion, migration, and differentiation during bone remodeling and regeneration. These observations have encouraged a classical biomimetic design strategy in which biomaterials are engineered to replicate the structural features of the native ECM in order to enhance their regenerative performance (Cramer and Badylak, 2020, Simmons et al., 2020). This contrasts with developmental engineering approaches, which aim to recapitulate the biological processes underlying tissue formation and typically rely on cell-based platforms. Such strategies are beyond the scope of the present review, which focuses on structurally instructive scaffold design.

In addition to local micro- and nanoscale cues, successful bone regeneration also requires an appropriate three-dimensional environment that allows cell infiltration, extracellular matrix deposition, nutrient diffusion, and vascularization. Adequate structural space within biomaterials

(macroscopic scale) is therefore essential to support the formation of a functional and well-vascularized bone tissue (Abbasi et al., 2020a).

Considering the hierarchical nature of bone tissue, biomaterial design should also be approached from a multiscale perspective (Figure 1). From the atomic interactions at the picometer scale to the geometry at the macro scale, the aim of this review is to provide a comprehensive (though non-exhaustive) overview on how each dimension plays a vital role in enhancing the biological performance of biomaterials used for alveolar bone regeneration .

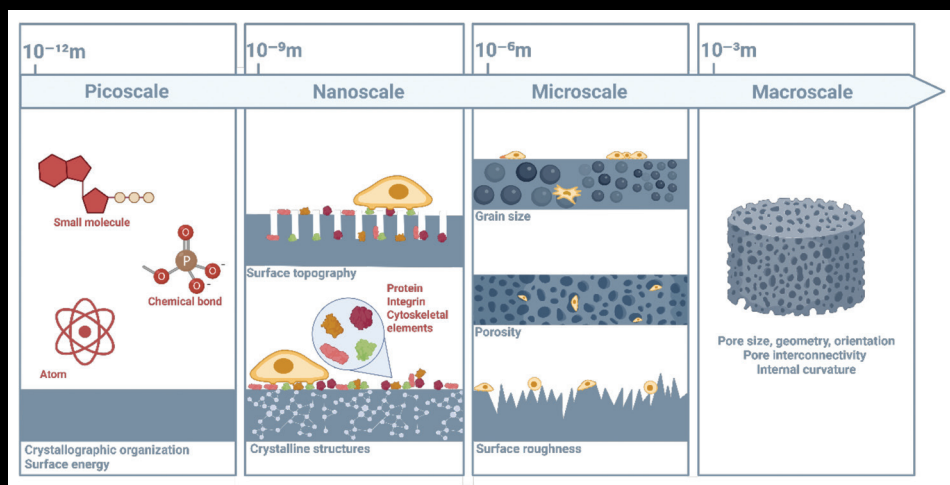
1.1. Scope and methodology

The aims of this narrative review are threefold: (i) to summarize the structural design principles operating at the pico-, nano-, micro-, and macroscale that influence biomaterial performance for alveolar bone regeneration; (ii) to identify points of consensus and remaining debate at each scale; and (iii) to outline emerging directions in smart, structurally instructive, and patient-specific scaffold design.

Literature was identified through targeted searches in PubMed, Scopus, and Web of Science, complemented by citation tracking from key reviews and consensus reports. Studies were

Figure 1

Schematic representation of the physicochemical and structural features of biomaterials across multiple length scales, ranging from atomic and molecular organization to pore architecture.



selected for inclusion when they (a) addressed structural or topographical design at one or more scales relevant to bone regeneration, and (b) provided either mechanistic insight, *in vitro* or *in vivo* data, or clinical evidence applicable to oral and maxillofacial applications. The review does not aim to provide an exhaustive systematic analysis; rather, examples were chosen to illustrate the dominant structural–biological relationships reported in the recent literature, with both established findings and points of current debate discussed where relevant. Cell-based therapies and growth-factor delivery strategies were considered outside the scope of this structurally focused review and are only briefly mentioned for context.

2. FOCUS ON BIOMATERIAL STRUCTURAL ASPECTS

2.1. From a picoscale perspective

The picometer scale (10^{-12} m) corresponds to atomic-level structural elements that determine the fundamental physicochemical properties of biomaterials, including crystallographic organization, surface energy, and chemical bonding. Although biological processes are generally studied at larger scales, atomic-level characteristics strongly influence the initial interactions occurring at the biomaterial–tissue interface (Mostafavi et al., 2018).

While research on the biological implications of the picometer scale remains scarce, picometer-based technologies are thought to revolutionize biomaterials and their biomedical applications (Pashazadeh-Panahi and Hasanzadeh, 2019, Qasim et al., 2020). As many biological processes ultimately depend on atomic-level interactions, controlling biomaterial properties at this scale may open new possibilities to improve biological integration, tissue regeneration, and material performance (Prathyusha et al., 2018). Regarding biomaterials for bone regeneration, altering electron distributions around atoms could enhance surface free energy and specific surface area, promoting tissue growth and influencing many cellular functions including cell movement, adhesion, growth, and ECM formation (Alpaslan and Webster, 2014, Prathyusha et al., 2018). For instance, human fibroblasts have demonstrated enhanced spreading on surfaces with higher surface energy, regardless of serum protein presence (Schakenraad et al., 1986). Similarly, *in vivo* studies by Baier et al. confirmed improved tissue incorporation on biomaterials with high surface energy (Baier et al., 1984). Surface energy, mediated by the chemical bonds on a biomaterial surface, determines the initial adsorption of proteins and other molecules, their orientation, and bioactivity, which in turn influences subsequent cell behavior such as adhesion and differentiation (Figure 2) (Rahmati et al., 2020). In contrast to the more intuitive fabrication

Table 1

Structural modifications of bone-regenerative biomaterials across hierarchical length scales.

For each scale, the principal structural features, representative parameters, and associated biological effects.

SCALE	STRUCTURAL FEATURES / MODIFICATIONS	REPRESENTATIVE PARAMETERS & EXAMPLES	BIOLOGICAL / REGENERATIVE EFFECT
Picoscale (10^{-12} m, atomic level)	Crystallographic organization, atomic composition, surface energy; ionic doping of the lattice.	Incorporation of Ca^{2+} , Cu^{2+} , Sr^{2+} , Mg^{2+} , Zn^{2+} , B^{3+} ; surface energy / wettability (contact angle); degree of crystallinity.	Governs initial protein adsorption, orientation and bioactivity at the biomaterial- tissue interface; influences angiogenesis, bone immunoregulation and antimicrobial environment.
Nanoscale (1–100 nm)	Surface nano-topography: nanotubes, nanopatterns (nanogrooves, nanogrids, nanopits), nanopillars, nanocrystalline coatings.	Nanotube $\varnothing$ 25–35 nm; nanopits 80–330 nm ($\approx$ 220 nm most osteogenic); nanocrystalline HA vs micron plasma-sprayed HA coatings.	Matches the dimensions of focal adhesions, integrin clusters and ECM fibrils, regulates cell recruitment/ anchorage, lineage specification and matrix mineralization; strongest osteogenic responses.
Microscale (100 nm – 100 μm)	Surface roughness, microporosity, grain size, micropatterns (adhesive islands, grooves, pits), microfibers	Intermediate roughness Ra 1–2 μm optimal; grain size 1 μm (bone formation) vs 3 μm (none); micropits 20–50 μm ; microgrooves 40 μm pits / 50 μm grooves.	Modulates cell adhesion, proliferation and differentiation via cytoskeletal tension and actomyosin contractility, osteogenic differentiation and bone neof ormation.
Macroscale (> 100 μm – mm)	Internal scaffold architecture: porosity and 3D spatial organization	Pore size, pore geometry, interconnectivity, pore orientation; cortical-to- cancellous porosity gradients.	Enables vascular invasion and tissue maturation while ensuring mechanical stability; supports bone ingrowth and integration.

strategies available at the nano-, micro-, and macroscale, direct manipulation of biomaterial properties at the picoscale relies on a more limited toolbox. Commonly applied techniques include plasma surface treatments, crystallographic and polymorphic modifications, controlled hydrothermal synthesis, ionic doping, and surface functionalization through chemical grafting (Gentleman and Gentleman, 2014, Gentleman and Ruud, 2010, Andrés et al., 2017, Hu et al., 2023, Szterner et al., 2023). In parallel, ions also play essential roles in bone regeneration, serving primarily as enzyme cofactors that shape various signaling pathways. However, many bone substitute materials, especially synthetic grafts, lack essential ions, which may contribute to their limited clinical effectiveness. Therefore, incorporating ions such as Ca^{2+} , Cu^{2+} , Sr^{2+} , Mg^{2+} , Zn^{2+} , and B^{3+} into biomaterials at appropriate concentrations has been shown to aid bone defect regeneration, stimulate angiogenesis, enhance bone immunoregulation, foster an antimicrobial environment through diverse mechanisms and modify crystal lattice features (Zhao et al., 2024). It is therefore crucial to understand how ions interact with cells *in vitro* and determine the appropriate doses for safe and effective *in vivo* use at precisely the right stages (Bosch-Rué et al., 2020, Sun et al., 1997). Although direct manipulation of biomaterials at the picometer scale remains challenging and comparatively less explored in bone regeneration research, growing evidence indicates that atomic-level modifications may significantly influence biological performance through their effects on surface physicochemical properties.

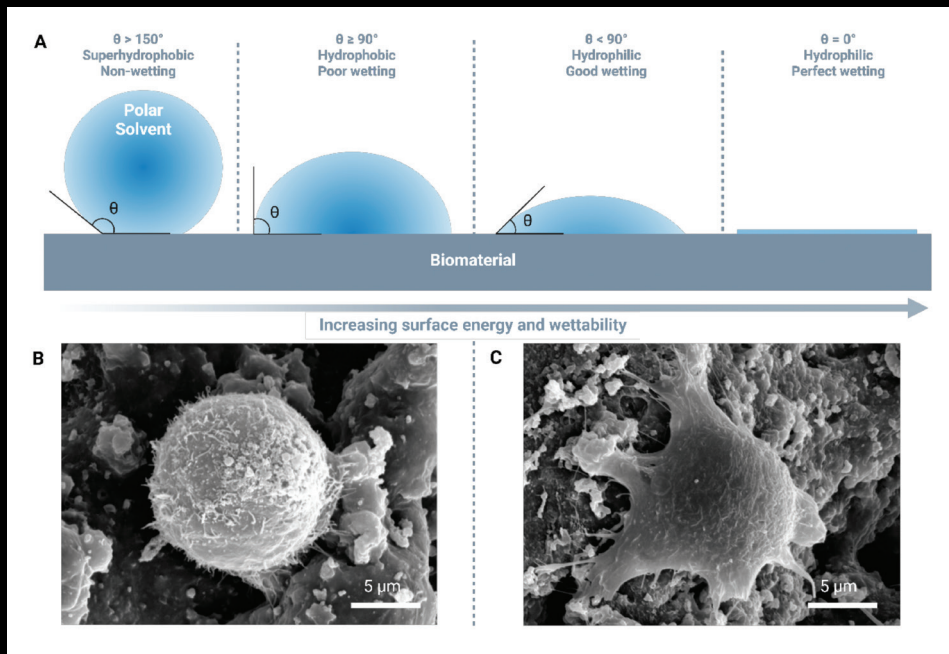
2.2. From a nanoscale perspective

The nanometer scale (10^{-9} m) refers to structures with dimensions ranging from 1 to 100 nm. At this scale, surface topography plays a critical role in regulating cell–material interactions, as many biological components involved in adhesion and signaling, including proteins, integrins, and cytoskeletal elements that operate in this dimensional range (Unadkat et al., 2011). Osteogenic cell behaviors influenced by surface nano-topography include cell recruitment and anchorage, lineage specification, and matrix mineralization. These processes ultimately contribute to osseointegration. In addition, these features may display antibacterial and immunomodulators effects (Elbourne et al., 2017, Gui et al., 2018, Nguyen et al., 2020). Advances in nanofabrication techniques, discussed below, have therefore enabled the development of a wide variety of nanoscale architectures designed to improve the regenerative performance of bone substitutes and the osseointegration of dental implants (Wang et al., 2018).

Nanotubes-associated biomaterials have been studied for diverse regenerative applications and are known to influence the cell behavior *in vitro* and *in vivo*. For example, Yavari et al. demonstrated that anodization-heat treatment of porous titanium surfaces generated regularly

Figure 2

(A) Schematic representation of biomaterial wettability and surface energy, illustrated by contact angle variations, and their influence on cell–material interactions. (B) Representative scanning electron microscopy (SEM) image of a fibroblast seeded on a superhydrophobic surface, exhibiting a rounded morphology with limited spreading, consistent with reduced cell–surface interactions. (C) Representative SEM image of a fibroblast cultured on a hydrophilic surface, displaying a spread morphology with cytoplasmic extensions, indicative of enhanced cell–surface interactions and favorable adhesion. SEM images in (B) and (C) are unpublished results.

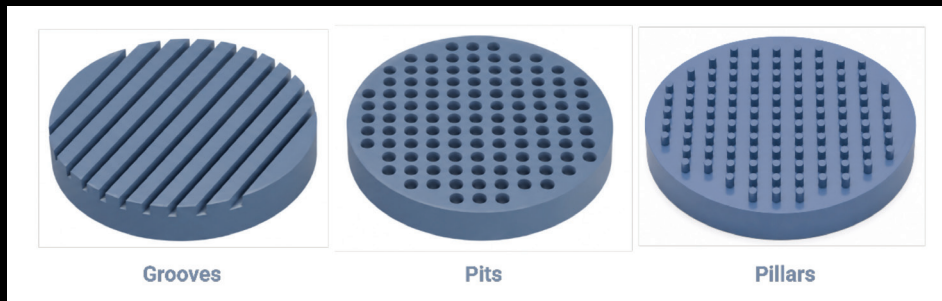


ordered nanotubes with diameters ranging from 25 to 35 nm, which significantly improved cellular proliferation and the expression of osteogenic markers (Amin Yavari et al., 2014). Hybrid nanoscale systems have also been developed to combine multiple nanoscale features. Patel et al. designed a bimodal nanoscale architecture consisting of electrospun polycaprolactone nanofibers (~500 nm) coated with carbon nanotubes (~25 nm), creating a nanofibrous–nanotube interface that enhanced angiogenic marker expression, stimulated bone formation, and reduced pro-inflammatory signaling in vivo (Patel et al., 2020). Importantly, modifications of the nanotube's conformation influence the cellular behavior and thus the bone regenerative performances (Necula et al., 2019, Wang et al., 2020, Zhou et al., 2019).

Another class of nanoscale features involves nanopatterned surfaces (Figure 3), including nanogrooves, nanogrids, and nanopits, which provide spatial cues that guide cell orientation,

Figure 3

Representative examples of engineered surface architectures, including grooves, pits, and pillars, commonly used to modulate cell–material interactions. Similar structural features can be observed across multiple length scales, including the nano-, micro-, and macroscale.



cytoskeletal organization, and stem cell fate. For instance, Yang et al. showed that hybrid micro- and nano-patterned surfaces could regulate hMSC functions in vitro: when nanogrooves were oriented parallel to 100 μm -spaced micro-stripes, this configuration favored osteogenic differentiation compared with orthogonally oriented nanogrooves, indicating that nanogroove orientation regulates hMSC fate towards an osteogenic lineage (Yang et al., 2018). The geometry and depth of these features are also critical: Davison et al. produced polycaprolactone discs containing nanopits with depths of 80, 220, and 330 nm and demonstrated that pits with a depth of approximately 220 nm most effectively stimulated osteogenic differentiation of mesenchymal stem cells in vitro (Davison et al., 2016).

Nanopillars constitute another nanoscale architecture capable of modulating cell behavior. The effect of nanopillars on osteogenesis was, for example, investigated through the fabrication of silk fibroin films treated by air-plasma, inducing the formation of nanopillar structures at the film surface which in turn promoted bone-like nano-hydroxylapatite formation (Zhang et al., 2019). Seeded with MSCs, these mineralized silk films efficiently stimulated bone neoformation in a subcutaneously implantation rat model.

Finally, nanocrystalline forms composed of calcium phosphate have shown a great potential for bone tissue regeneration. When used as a coating on titanium implants, HA nanocrystals could dramatically increase osteoblast adhesion, as compared with the one with traditionally used plasma-sprayed micron-sized HA coatings (Sato et al., 2006, Thian et al., 2006). Moreover, numerous studies demonstrated the positive impact of calcium phosphate-based nanocrystals or nanoparticles on osteogenic differentiation (Bernhardt et al., 2012, Hesarakı et al., 2014, Khajuria et al., 2018, Kołbuk et al., 2020) and these nano- composite materials for bone tissue

engineering have been discussed in a recently published literature review (Levingstone et al., 2019). Across nanotubes, nanopatterns, nanopillars and nanocrystalline coatings, the nanoscale features matching the dimensions of cell-adhesive structures (focal adhesions, integrin clusters, ECM fibrils) generate the strongest osteogenic responses, with effect on long-term bone formation depending on the underlying material and on the co-occurrence of features at other scales (Guadarrama Bello et al., 2017, Wähnert et al., 2021)

2.3. FROM A MICROSCALE PERSPECTIVE

At the microscale (10^{-6} m), structural features such as surface roughness, microporosity, grain size, and defined microgeometries operate at the same characteristic length as cells themselves and primarily influence cell adhesion, spreading, migration, and proliferation, ultimately affecting bone regenerative performance and osseointegration (Chen et al., 2018, Du et al., 2019). These microscale topographies span approximately 100 nm to 100 μ m. One of the most extensively investigated parameters at this scale is microroughness. Surface roughness has been shown to modulate cell adhesion, proliferation, and differentiation, although these effects depend on multiple factors including the underlying material, the cell type, and the specific roughness range investigated. For instance, Deligianni et al. showed that MSC ALP activity was not influenced by HA surface roughness ranging from 0.73 to 4.68 μ m, whereas it improved with increased titanium-alloy roughness (Deligianni et al., 2001a, Deligianni et al., 2001b). Similarly, Andrukhov et al. produced a set of titanium surfaces displaying different levels of surface roughness and showed in vitro that MG-63 osteoblastic cells proliferated and differentiated preferentially when cultured on surfaces with intermediate roughness (Ra of 1 and 2 μ m) compared to smoother ($\sim$ 0 μ m) or harboring higher roughness (4 μ m) surfaces (Andrukhov et al., 2016).

Although in vitro studies allow to screen the impact of biomaterial surface topographies on cellular behavior, in vivo experiments [LG27] are required to determine their bone regenerative and osseointegration performances. Given the wide range of microscale parameters reported in the literature, the examples discussed here were selected based on their relevance to oral and maxillofacial applications, with a focus on the most extensively studied materials and microstructural features in this context. Lambert et al. (2017) compared three commercially available HA-based biomaterials with different surface topographies in a sinus lift rabbit model and assessed their capacity to support bone neoformation and osteoconductivity (Lambert et al., 2017). The natural-derived hydroxyapatite, which displayed a greater specific surface area, achieved higher bone regenerative performance than the synthetic hydroxyapatite tested. In a separate study, De Carvalho et al. (2019) examined how the sintering temperature of bovine hydroxyapatite modulates this surface-topography effect, progressively increasing the sintering

temperature reduced specific surface area, altered surface topography, and impaired bone regenerative performance (De Carvalho et al., 2019). Beyond surface topography, microscale structural characteristics such as grain size also influence the biological performance of calcium phosphate biomaterials. Zhang et al. produced two β -TCP biomaterials with similar chemistry and overall microporosity but different surface microstructural dimensions, characterized by grain sizes of approximately 1 μm and 3 μm . The authors demonstrated that the material with the smaller microstructure (1 μm) promoted ectopic bone formation after implantation in dogs, whereas no bone formation occurred in the material with the larger features (3 μm) (Zhang et al., 2014).

In addition to randomly distributed surface roughness, researchers have also explored micro patterned geometries designed to control cellular organization and differentiation. McBeath et al. used microcontact printing to generate fibronectin-coated adhesive islands of two sizes (1024 μm^2 and 10,000 μm^2) on Pluronic-blocked PDMS substrates, ensuring that single hMSCs were confined to islands of defined geometry while the amount of available fibronectin per cell was held constant. They demonstrated that cells confined to larger islands, which adopted a more spread morphology, preferentially committed to an osteoblastic fate, whereas cells on smaller islands favored adipogenesis (McBeath et al., 2004). When varying the island shape and thus the subcellular curvature, Kilian et al. showed that variations in microgeometric shape influence cytoskeletal tension through actomyosin contractility, thereby promoting osteogenesis (Kilian et al., 2010). Similar approaches have been applied to calcium phosphate biomaterials. Zhao et al. fabricated hydroxyapatite ceramics with ordered quadrate micro patterns (~24, 55, and 110 μm) and observed that osteogenic gene expression decreased with increasing micropattern size (Zhao et al., 2015). Using a calcium phosphate/gelatin composite scaffold, Nadeem et al. discovered that a microgroove pattern with surfaces comprising 40 μm pits and 50 μm grooves induced optimal osteogenic differentiation in vitro as well as higher amount of newformed bone upon implantation in vivo (Nadeem et al., 2015). Several studies have attempted to reproduce biomimetic microstructures inspired by natural bone architecture. Using electrospun polystyrene microfibers (1–4.5 μm in diameter), Terranova et al. (2016) investigated in vitro the influence of microfiber organization on osteoblast-like (MC3T3) cell behavior and showed that cells became elongated along the fiber axis only on aligned scaffolds, while aligned-fiber substrates supported cell proliferation independently of fiber diameter (Terranova et al., 2016). This suggests that the micro-architecture of scaffolds, particularly fiber orientation, plays a significant role in directing cell morphology and growth, which is crucial for bone tissue engineering applications. Another biomimetic strategy consists of reproducing the resorption pits generated by osteoclasts during bone remodeling. These

microcavities, typically ranging from 20 to 50 μm in diameter, represent natural microtopographical features of bone surfaces and may act as biological signals indicating that a remodeling process is occurring. Wilkinson et al. identified that micropits harboring large diameter favored bone neoformation (Wilkinson et al., 2011), supporting the work of Kilian and co-authors (Kilian et al., 2010).^{[LG29][LG30][BD31]}

This body of evidence regarding the pico-, nano- and micro-scale structures of biomaterials dedicated to bone regeneration and implant osseointegration applications demonstrates the significance of the very local structural environment on cell behavior such as adhesion, migration, proliferation and differentiation. Overall, studies showed that mimicking the natural bone topographical features tends to improve the biomaterial performances. However, beyond these pico-, nano- and microscale structural cues, successful bone regeneration also requires an appropriate three-dimensional scaffold architecture that allows vascular invasion and tissue maturation while ensuring mechanical stability, highlighting the importance of structural design at the macroscale level.

2.4. FROM A MACROSCALE PERSPECTIVE

In addition to pico-, nano- and microscale surface features, the macroscale architecture of scaffolds also plays a critical role in determining the regenerative performance of bone biomaterials (Bobbert and Zadpoor, 2017, Sadeghian Dehkord et al., 2024). The internal macro-architecture of a scaffold refers to its porosity and spatial organization, which are primarily defined by parameters such as pore size, pore geometry, interconnectivity, and pore orientation.

Pore size is a critical parameter, as it determines the available space for tissue ingrowth and vascularization. Early studies suggested that pores of approximately 100 μm represent the minimum size required to support bone formation (Hulbert et al., 1970). Later, it was established that larger pore sizes up to 800 μm promoted significant bone formation due to an appropriate neovascularization into the scaffold ^[FL34] (Cheng et al., 2016, De Wild et al., 2019, Roosa et al., 2010, Sadeghian Dehkord et al., 2023). However, Ghayor and Weber identified an optimal pore diameter of 1.2 mm with 0.7 mm interconnecting bottlenecks for osteoconduction by testing a library of 3D-printed β -TCP scaffolds in a rabbit calvarial defect model (Ghayor et al., 2021). Importantly, conclusions drawn from such studies should be interpreted with caution, since fabrication strategies that vary pore size often alter pico-, nano- and microscale features in parallel.

Additionally, the gradient porosity that exists between cortical and cancellous bone has inspired

the development of scaffolds with cortical to cancellous porosity gradients, with several studies demonstrating their positive impact on bone regenerative performance, particularly in trabecular and osteochondral reconstructions (Abbasi et al., 2020b, Di Luca et al., 2016a, Di Luca et al., 2016b, Diloksumpan et al., 2020, Oh et al., 2007). Beyond pore size, surface curvature within scaffold pores can also significantly influence cell behavior. Concave surfaces have been shown to promote greater cell adhesion, proliferation, and tissue formation compared with flat or convex surfaces (Callens et al., 2020, Rumpler et al., 2008). This effect has been attributed to differences in local surface energy and to the geometric and mechanical cues experienced by cells spanning curved features. More recently, several authors have demonstrated that substrate curvature can be deliberately incorporated into 3D-printed scaffolds, with biomimetic curvature design supporting and actively guiding bone tissue regeneration (Callens et al., 2020, Zadpoor, 2015).

Beyond size and curvature, pore geometry and interconnectivity further shape the regenerative response (Barba et al., 2018, Bianchi et al., 2014, Boccaccio et al., 2016, Knychala et al., 2013). Van Bael et al. (2012) compared selective laser-melted Ti6Al4V scaffolds with triangular, hexagonal, and rectangular pores (500 μm and 1000 μm , defined as the diameter of the inscribed circle) seeded with human periosteum-derived cells (Van Bael et al., 2012). Cell growth was driven by pore size rather than pore shape, while shape influenced differentiation and pore occlusion: hexagonal pores closed fastest because cells bridged their corners more readily, an outcome consistent with the curvature-driven bridging mechanism described by Rumpler et al. (2008). Importantly, because pore size in this study was defined by the inscribed-circle diameter, the triangular and rectangular pores had substantially larger cross-sectional areas than the hexagonal ones at any nominal pore size; the observed shape-dependent differences therefore reflect a combined effect of local curvature and effective pore size rather than geometry alone. Pore interconnectivity is equally critical, as it allows vascular networks to penetrate and distribute throughout the scaffold; high interconnectivity has been associated with improved neovascularization and tissue colonization (Mastrogiacomo et al., 2006). In contrast, closed pores remain inaccessible to cells and vasculature and therefore do not contribute to the regenerative volume of the scaffold, although they can affect its mechanical properties (Entezari et al., 2019). Lastly, besides the impact on scaffold mechanical performances, pore orientation may also influence bone regeneration (Jones et al., 2009, Liu et al., 2013, Serrano-Bello et al., 2020). For example, Dewey et al. (2020) compared anisotropic and isotropic mineralized collagen scaffolds *in vitro* and showed that, while short-term MSC migration and activity were not affected by pore orientation, anisotropic pore alignment enhanced bone mineral synthesis over the culture period (Dewey et al., 2020).

While submillimeter structural features (pico-, nano- and microscale) can effectively enhance bone regeneration by mimicking natural bone surface characteristics, direct replication of native bone macro-architecture does not always lead to optimal regenerative outcomes (Willie et al., 2010). Several *in vivo* studies evaluating the clinical, histological, and histomorphometric outcomes of bone blocks derived from natural sources (autologous, allogenic, or xenogenic), each with distinct processing histories that affect their cellular content and biological activity (Monje and Wang, 2017), have produced mixed results. In particular, allogenic fresh-frozen bone blocks display large fractions of non-vital bone (empty osteocyte lacunae) and impaired remodeling and incorporation compared with autogenous grafts, with block architecture itself influencing the extent of resorption (Acocella et al., 2012, Spin-Neto et al., 2015). Xenogeneic blocks, despite their excellent ability to maintain space, often demonstrate limited new bone formation within the biomaterial and poor neovascularization capacity (Araújo et al., 2002). These observations suggest that effective scaffold design requires not only biomimicry but also careful optimization of vascularization pathways, material properties, and the incorporation of biologically active substances (Krishnan et al., 2014).

2.5. MULTISCALE HYBRID TOPOGRAPHIES

Although structural features are often investigated independently according to discrete dimensional scales, biological systems interact simultaneously with physicochemical and topographical cues spanning multiple hierarchical levels. Consequently, increasing attention has been directed toward multiscale hybrid architectures integrating nano-, micro-, and macroscale features within a single biomaterial system. Rather than acting independently, these structural levels may exert synergistic effects on protein adsorption, mechanotransduction, cellular adhesion, immune regulation, vascularization, and tissue organization, collectively shaping regenerative outcomes. A growing body of recent literature has converged on the view that hierarchical, cross-scale integration represents a paradigm shift in biomaterial design, moving beyond the optimization of individual length scales toward the deliberate engineering of multiscale interfaces (Wang et al., 2025a, Wang et al., 2025b, Wei et al., 2023). Several overarching principles are emerging. The biological functions traditionally associated with each scale do not act in isolation but interact, and their combinations can produce responses not predictable from any single scale alone (Kim et al., 2025). Multiscale design is therefore not merely additive, since the architecture that is achievable at one scale frequently constrains the architecture achievable at adjacent scales, with molecular-level control often coming at the cost of macroscopic geometric flexibility, and vice versa (Tseng et al., 2017). Specific

physiological functions, including immune polarization, mechanical compatibility with native tissue, and stimuli-responsive behavior are increasingly understood as cross-scale phenomena requiring coordinated design at multiple hierarchical levels rather than tuning of any single feature (Kim et al., 2025, Wang et al., 2025a).

Several unresolved challenges remain. Reproducing nanoscale features uniformly across macroscopic scaffold volumes is technically difficult, and standardized protocols for characterizing multiscale architectures across length scales are lacking (Wei et al., 2023). Demonstrating synergistic, rather than additive biological responses also requires study designs that systematically control for features at the other scales, which is a methodological standard rarely met in the current literature. Finally, clinical translation is constrained by manufacturing scalability, long-term biocompatibility, and regulatory frameworks that are not yet adapted to architecturally complex constructs (Wang et al., 2025a, Wang et al., 2025b). Computational and AI-driven materials design has been proposed as a means to navigate this multidimensional design space connecting the multiscale-hybrid framework to the broader vision of personalized, computer-assisted scaffold design developed in Section [Future Directions].

2.6. FROM A CLINICAL HANDLING AND DESIGN PERSPECTIVE

From a clinical perspective, biomaterial performance is not only determined by intrinsic material properties, but also how the material behaves during surgical manipulation and adaptation to the defect environment. Particulate bone substitutes, such as bovine-derived hydroxyapatite particles (e.g., Bio-Oss®) are currently among the most widely used biomaterials in clinical practice for alveolar bone regeneration and have demonstrated long-term clinical effectiveness (Botilde et al., 2020, Wang and Lang, 2012). Although these particulate bone substitutes present many advantages, such as easy handling, which makes them perfectly appropriate to treat self-contained bone defects, they lack 3D stability and are not suitable for more complex cases such as large or non-contained bone defects. In such situations, bone blocks may be used to provide structural support and maintain the required regenerative space. However, currently available bone blocks, whether harvested as autologous grafts of variable dimensions, or supplied in standardized commercial allogenic or xenogenic forms, typically require manual intraoperative shaping to adapt them to the defect morphology. This step can be time-consuming and technically demanding, potentially affecting the clinical workflow and surgical precision (Carrel et al., 2016, Giuliani et al., 2016, Sawada et al., 2018). In addition, many of these materials exhibit intrinsic brittleness, which may increase the risk of fracture during shaping or fixation and further complicates their clinical handling. To overcome these limitations, increasing attention has been directed toward the development of patient-specific

three-dimensional scaffolds designed to match the external geometry of the bone defect. Such custom-made scaffolds can be produced using advanced fabrication technologies, particularly additive manufacturing (3D printing), which is especially well-suited to producing the complex internal porous geometries required for bone scaffolds [LG35]. These technologies allow precise control over both the external shape of the scaffold and its internal architecture, offering promising perspectives for improving surgical handling and regenerative outcomes (Bartnikowski et al., 2020, Le Guéhennec et al., 2020, Lee et al., 2018, Mangano et al., 2015, Vaquette et al., 2018). However, scaffold design for alveolar bone regeneration inherently involves biological–mechanical trade-offs, where optimization of one parameter frequently compromises another. Highly porous and interconnected architectures favor vascular ingrowth, cell migration, nutrient diffusion, and osteogenic activity (Abbasi et al., 2020a, Mangano et al., 2025) but often at the expense of mechanical resistance, dimensional stability, and long-term space maintenance, particularly in non-contained or vertical defects where collapse resistance is critical (Valamvanos et al., 2024). Conversely, denser or less porous structures may better preserve the regenerative compartment and withstand physiological loading, yet can restrict tissue infiltration, vascularization, and remodeling dynamics (Huang et al., 2025, Abbasi et al., 2020a). Similar compromises exist regarding degradation kinetics where rapidly resorbing scaffolds may facilitate tissue substitution and remodeling but risk premature loss of structural support, whereas slowly degrading constructs may ensure prolonged mechanical stability while interfering with physiological bone turnover and tissue maturation (Mangano et al., 2025). These trade-offs cannot be resolved by biological optimization alone. Clinical translation also depends on how the material integrates with surgical workflows and on operator-dependent constraints, since the regenerative outcome is partly shaped intraoperatively by the clinician's handling decisions. Such considerations motivate the design of biomimetic multiscale scaffolds capable of balancing biological and mechanical demands not only spatially but also dynamically over time.

2.7. DYNAMIC MULTISCALE BIOMATERIALS

Dynamic multiscale biomaterials represent an emerging generation of regenerative scaffolds designed not only to reproduce the hierarchical structural organization of native bone across pico-, nano-, micro-, and macroscale levels, but also to actively adapt to the evolving biological environment during healing. In contrast to conventional static biomaterials, these systems integrate structural, mechanical, and biochemical responsiveness over time, enabling continuous interactions with surrounding tissues throughout the regenerative process.

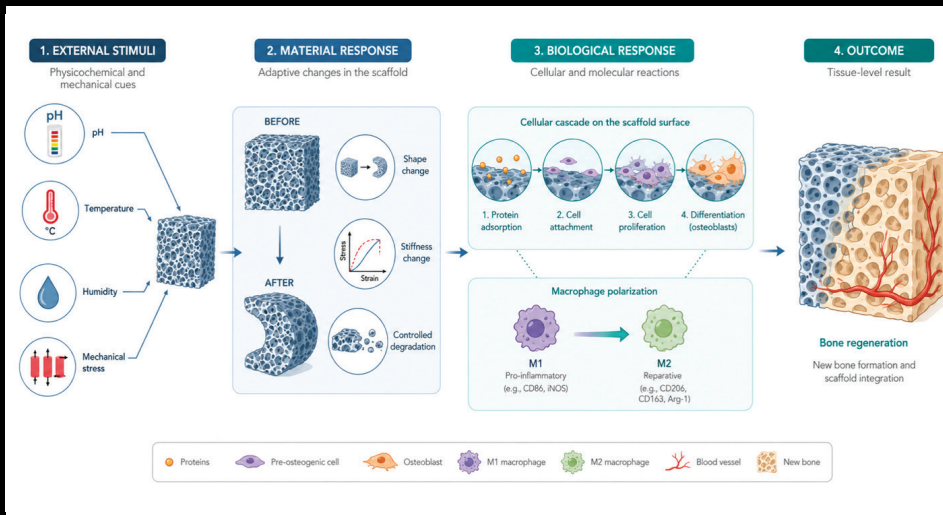
Such biomaterials may exhibit active properties including bioresponsiveness, bioadaptiveness, and shape-memory behavior (Sailaja et al., 2016, Zhang et al., 2018). Bioresponsive

biomaterials possess the ability to sense and react to internal stimuli such as pH changes, proteins, antigens, ionic fluctuations, or inflammatory mediators, as well as external stimuli including magnetic fields, electric fields, temperature, light, or mechanical stress. These interactions can trigger the release of bioactive molecules or induce structural modifications capable of modulating cellular activity and tissue regeneration (Montoya et al., 2021, Morris et al., 2016, Sankaran et al., 2018, Ju et al., 2009). Beyond biochemical responsiveness, bioadaptive biomaterials are designed to dynamically adjust their mechanical and structural properties according to the progression of tissue healing. The concept envisions scaffolds capable of providing temporary mechanical support while degrading in synchrony with new tissue formation, thereby maintaining structural integrity throughout the regenerative process (Mao et al., 2024, Kumar et al., 2025). As a recent experimental example, Mao et al. (2024) developed a silk fibroin/magnesium oxide composite scaffold with water-responsive shape memory that maintained mechanical stability and structural integrity throughout the degradative process while supporting bone regeneration in irregular cranial defects in vivo. The adaptation of the biomaterials to inflammation can have a great impact in avoiding foreign body reaction after implantation. Ma et al. described how surface chemistry and topography influence macrophage polarization from pro- to anti-inflammatory phenotypes (M1 to M2), reducing chronic inflammation and foreign body reaction (Ma et al., 2016, Sailaja et al., 2016). Conventional manufacturing processes, however, often provide limited control over the preservation or reproduction of the hierarchical structures across pico-, nano-, micro-, and macroscale levels in natural or synthetic bone substitutes.

Advances in additive manufacturing technologies have enabled the fabrication of biomimetic scaffolds with highly controlled internal architectures and microenvironments. These technologies allow the reproduction of hierarchical structures inspired by native bone tissue, improving the adhesion, proliferation, and differentiation of osteogenic cells (Le Guéhennec et al., 2020, Limmahakhun et al., 2017). However, most conventional 3D-printed scaffolds still possess static architectures, which do not adapt to the evolving biological environment of the defect site. This limitation may restrict their long-term integration and functional performance, particularly in dynamic environments such as the oral and craniofacial region. To overcome this, leading research groups have introduced the concept of 4D bioprinting, which integrates time as a fourth dimension into scaffold design. This technology uses stimuli-responsive smart materials that can change shape, stiffness, or functionality in response to environmental cues such as temperature, pH, moisture, or mechanical stress (Morouço et al., 2017, Wan et al., 2020). These dynamic scaffolds better mimic the natural remodeling of bone and surrounding tissues, allowing self-adjustment to complex oral environments and promoting more effective, biologically synchronized regeneration (Song et al., 2024). (Figure 4)

Figure 4

Schematic representation of stimuli-responsive (4D) smart scaffolds for bone regeneration. External stimuli (pH, temperature, humidity, and mechanical stress) trigger adaptive changes in scaffold properties, including shape, stiffness, and controlled degradation. These material adaptations regulate protein adsorption, cell attachment, proliferation, osteogenic differentiation, and macrophage polarization, ultimately promoting vascularization, scaffold integration and bone regeneration.



Nevertheless, dynamic multiscale biomaterials and 4D-printed constructs remain at an early stage of development. Major challenges persist regarding mechanical robustness, predictability of transformation behavior, long-term biocompatibility, and the integration of bioactive and structural functionalities within clinically relevant constructs, particularly for load-bearing applications such as alveolar bone regeneration (Ionov, 2018, Wan et al., 2020). Continued interdisciplinary research will be required to integrate shape-memory materials, bioactive components, and advanced manufacturing strategies to develop next-generation scaffolds capable of intelligent and patient-specific bone repair.

2.8. Future directions for multi-scaled designed scaffold

Understanding the interactions between regenerative biomaterials and cellular and tissue actors at the bone defect site across multiple scales provides valuable insights into the mechanisms for successful bone regeneration. As discussed throughout this review, the performance of biomaterials is influenced by various structural and functional parameters, including atomic and

nanoscale surface chemistry, microscale topography, macroscale architecture, and scaffold design. However, there is a wide diversity of biomaterials marketed for bone regeneration that have sub-optimal properties at one or more scales, leaving much room for further research in this area. Future biomaterials should aim to integrate multiscale structural design with dynamic biological responsiveness, allowing the simultaneous control of pico-scale surface chemistry, nano- and micro-topography, macro-scale architecture, and mechanical stability. Such approach could improve the regulation of key biological processes including cell adhesion, immune modulation, vascularization, and tissue remodeling. Advances in surface biofunctionalization already enable the incorporation of bioactive molecules, peptides, or ions at the nanoscale, while additive manufacturing technologies allow the precise control of scaffold geometry and internal architecture at the microscale and macroscale. In the future, the design of bone regenerative biomaterials may increasingly rely on personalized approaches, where scaffold properties are tailored to the biological characteristics of individual patients, also as consequence of chronic diseases that patients may manifest. Identifying patient-specific regenerative profiles, including biochemical, immunological, and metabolic parameters, could allow the selection or design of biomaterials adapted to each clinical situation. Bioresponsive and bioadaptive materials are well-suited to this paradigm. Their surface biofunctionalization parameters could in principle be adapted to patient-specific needs through algorithm-guided design. In this context, artificial intelligence and computational modeling may play a central role in the next generation of biomaterial design. Machine learning algorithms could integrate large datasets describing biomaterial properties, manufacturing parameters, and clinical outcomes to predict the optimal multiscale scaffold configuration for a given defect and patient profile (Ibrahimi et al., 2024, Basu et al., 2022, Cranford and Buehler, 2010). Such approaches could guide the rational design of biomaterials capable of dynamically interacting with their biological environment. Ultimately, combining multiscale scaffold engineering, smart biomaterials, and computer-assisted design strategies may enable the development of adaptive and patient-specific biomaterials capable of promoting predictable and efficient bone regeneration (Foroughi et al., 2024, Kolomenskaya et al., 2023, Suwardi et al., 2022).



3. CONCLUSION

This review examined how the structural properties of biomaterials at the pico-, nano-, micro-, and macroscale influence bone regeneration, with the aim of identifying design principles for the next generation of regenerative biomaterials. The advances in bioengineering, including biomimetic surface design, additive manufacturing, and stimulus-responsive materials, appear to support the development of more efficient biomaterials that combine biomimetic features with the capacity to adapt to and respond to their biological environment. This will allow the development of personalized biomaterials tailored not only in shape but also at the molecular and physicochemical level, in line with each patient's specific clinical needs. Producing such personalized biomaterials reliably and at clinical scale remains a major challenge in terms of cost, manufacturing consistency, and regulatory approval. Achieving this will require integrating multiscale structural principles with dynamic biomaterial behavior, combining hierarchical architectural control across different scales with stimuli-responsive functionality and patient-specific design, supported by computational tools that navigate the resulting multidimensional design space.

REFERENCES

- 1** ABBASI, N., HAMLET, S., LOVE, R. M. & NGUYEN, N.-T. 2020a. **Porous scaffolds for bone regeneration.** *Journal of Science: Advanced Materials and Devices*, 5, 1–9.
- 2** ABBASI, N., IVANOVSKI, S., GULATI, K., LOVE, R. M. & HAMLET, S. 2020b. **Role of offset and gradient architectures of 3-D melt electrowritten scaffold on differentiation and mineralization of osteoblasts.** *Biomater Res*, 24, 2.
- 3** ACOCELLA, A., BERTOLAI, R., ELLIS, E., NISSAN, J. & SACCO, R. 2012. **Maxillary alveolar ridge reconstruction with monocortical fresh-frozen bone blocks: A clinical, histological and histomorphometric study.** *Journal of Cranio-Maxillo-Facial Surgery: Official Publication of the European Association for Cranio-Maxillo-Facial Surgery*, 40, 525–533.
- 4** ALPASLAN, E. & WEBSTER, T. J. 2014. **Nanotechnology and picotechnology to increase tissue growth: A summary of in vivo studies.** *International Journal of Nanomedicine*, 9, 7–12.
- 5** AMIN YAVARI, S., VAN DER STOK, J., CHAI, Y. C., WAUTHLE, R., TAHMASEBI BIRGANI, Z., HABIBOVIC, P., MULIER, M., SCHROOTEN, J., WEINANS, H. & ZADPOOR, A. A. 2014. **Bone regeneration performance of surface-treated porous titanium.** *Biomaterials*, 35, 6172–81.
- 6** AMINI, A. R., LAURENCIN, C. T. & NUKAVARAPU, S. P. 2012. **Bone tissue engineering: Recent advances and challenges.** *Critical Reviews in Biomedical Engineering*, 40, 363–408.
- 7** ANDRÉS, N. C., D'ELÍA, N. L., RUSSO, J. M., CAMPELO, A. E., MASSHEIMER, V. L. & MESSINA, P. V. 2017. **Manipulation of Mg(2+)-Ca(2+) Switch on the Development of Bone Mimetic Hydroxyapatite.** *ACS Appl Mater Interfaces*, 9, 15698–15710.
- 8** ANDRUKHOV, O., HUBER, R., SHI, B., BERNER, S., RAUSCH-FAN, X., MORITZ, A., SPENCER, N. D. & SCHEDLE, A. 2016. **Proliferation, behavior, and differentiation of osteoblasts on surfaces of different microroughness.** *Dental Materials*, 32, 1374–1384.

- 9 ARAÚJO, M. G., SONOHARA, M., HAYACIBARA, R., CARDAROPOLI, G. & LINDHE, J. 2002. **Lateral ridge augmentation by the use of grafts comprised of autologous bone or a biomaterial. An experiment in the dog.** Journal of Clinical Periodontology, 29, 1122–1131.
- 10 ASHFAQ, R., KOVÁCS, A., BERKÓ, S. & BUDAI-SZŰCS, M. 2024. **Developments in Alloplastic Bone Grafts and Barrier Membrane Biomaterials for Periodontal Guided Tissue and Bone Regeneration Therapy.** Int J Mol Sci, 25.
- 11 BAIER, R., MEYER, A., NATIELLA, J., NATIELLA, R. & CARTER, J. 1984. **Surface properties determine bioadhesive outcomes: Methods and results.** Journal of Biomedical Materials Research; J Biomed Mater Res, 18, 337–55.
- 12 BARBA, A., MAAZOUZ, Y., DIEZ-ESCUADERO, A., RAPPE, K., ESPANOL, M., MONTUFAR, E. B., ÖHMAN-MÄGI, C., PERSSON, C., FONTECHA, P., MANZANARES, M. C., FRANCH, J. & GINEBRA, M. P. 2018. **Osteogenesis by foamed and 3D-printed nanostructured calcium phosphate scaffolds: Effect of pore architecture.** Acta Biomaterialia, 79, 135–147.
- 13 BARTNIKOWSKI, M., VAQUETTE, C. & IVANOVSKI, S. 2020. **Workflow for highly porous resorbable custom 3D printed scaffolds using medical grade polymer for large volume alveolar bone regeneration.** Clin Oral Implants Res, 31, 431–441.
- 14 BASU, B., GOWTHAM, N. H., XIAO, Y., KALIDINDI, S. R. & LEONG, K. W. 2022. **Biomaterialomics: Data science-driven pathways to develop fourth-generation biomaterials.** Acta Biomater, 143, 1–25.
- 15 BERNHARDT, A., DITTRICH, R., LODE, A., DESPANG, F. & GELINSKY, M. 2012. **Nanocrystalline spherical hydroxyapatite granules for bone repair - in vitro evaluation with osteoblasts and osteoclasts.** J Tissue Eng Regen Med, 6, 37.
- 16 BIANCHI, M., URQUIA EDREIRA, E. R., WOLKE, J. G. C., BIRGANI, Z. T., HABIBOVIC, P., JANSEN, J. A., TAMPIERI, A., MARCACCI, M., LEEUWENBURGH, S. C. G. & BEUCKEN, J. J. J. P. 2014. **Substrate geometry directs the in vitro mineralization of calcium phosphate ceramics.** Acta Biomaterialia, 10, 661–669.

- 17 BOBBERT, F. S. L. & ZADPOOR, A. A. 2017. **Effects of bone substitute architecture and surface properties on cell response, angiogenesis, and structure of new bone.** *Journal of Materials Chemistry, B*, 5, 6175–6192.
- 18 BOCCACCIO, A., UVA, A. E., FIORENTINO, M., MORI, G. & MONNO, G. 2016. **Geometry Design Optimization of Functionally Graded Scaffolds for Bone Tissue Engineering: A Mechanobiological Approach.** *PLoS One*, 11, 146935.
- 19 BOSCH-RUÉ, E., DIEZ-TERCERO, L., GIORDANO-KELHOFFER, B., DELGADO, L. M., BOSCH, B. M., HOYOS-NOGUÉS, M., MATEOS-TIMONEDA, M. A., TRAN, P. A., GIL, F. J. & PEREZ, R. A. 2020. **Biological Roles and Delivery Strategies for Ions to Promote Osteogenic Induction.** *Front Cell Dev Biol*, 8, 614545.
- 20 BOTILDE, G., COLIN, P. E., GONZÁLEZ-MARTÍN, O., LECLOUX, G., ROMPEN, E. & LAMBERT, F. 2020. **Hard and soft tissue analysis of alveolar ridge preservation in esthetic zone using deproteinized bovine bone mineral and a saddle connective tissue graft: A long-term prospective case series.** *Clinical Implant Dentistry and Related Research*, 22, 387–396.
- 21 CALLENS, S. J. P., UYTENDAELE, R. J. C., FRATILA-APACHITEI, L. E. & ZADPOOR, A. A. 2020. **Substrate curvature as a cue to guide spatiotemporal cell and tissue organization.** *Biomaterials*, 232, 119739.
- 22 CARREL, J. P., WISKOTT, A., SCHERRER, S. & DURUAL, S. 2016. **Large Bone Vertical Augmentation Using a Three-Dimensional Printed TCP/HA Bone Graft: A Pilot Study in Dog Mandible.** *Clinical Implant Dentistry and Related Research*, 18, 1183–1192.
- 23 CHEN, X., FAN, H., DENG, X., WU, L., YI, T., GU, L., ZHOU, C., FAN, Y. & ZHANG, X. 2018. **Scaffold Structural Microenvironmental Cues to Guide Tissue Regeneration in Bone Tissue Applications.**
- 24 CHENG, M., WAHAFU, T., JIANG, G., LIU, W., QIAO, Y., PENG, X., CHENG, T., ZHANG, X., HE, G. & LIU, X. 2016. **A novel open-porous magnesium scaffold with controllable microstructures and properties for bone regeneration.** *Scientific Reports*, 6, 24134.

- 25 CRAMER, M. C. & BADYLAK, S. F. 2020. **Extracellular Matrix-Based Biomaterials and Their Influence Upon Cell Behavior.** *Annals of Biomedical Engineering*, 48, 2132–2153.
- 26 CRANFORD, S. & BUEHLER, M. J. 2010. **Materiomics: biological protein materials, from nano to macro.** *Nanotechnol Sci Appl*, 3, 127–48.
- 27 DAVISON, M. J., MCMURRAY, R. J., SMITH, C. A., DALBY, M. J. & MEEK, R. D. 2016. **Nanopit-induced osteoprogenitor cell differentiation: The effect of nanopit depth.** *Journal of Tissue Engineering*, 7, 2041731416652778.
- 28 DE CARVALHO, B., ROMPEN, E., LECLoux, G., SCHUPBACH, P., DORY, E., ART, J. F. & LAMBERT, F. 2019. **Effect of Sintering on In Vivo Biological Performance of Chemically Deproteinized Bovine Hydroxyapatite.** *Materials (Basel)*, 12.
- 29 DE WILD, M., GHAYOR, C., ZIMMERMANN, S., RÜEGG, J., NICHOLLS, F., SCHULER, F., CHEN, T.-H. & WEBER, F. E. 2019. **Osteoconductive Lattice Microarchitecture for Optimized Bone Regeneration.** *3D Printing and Additive Manufacturing*, 6, 40–49.
- 30 DELIGIANNI, D. D., KATSALA, N., LADAS, S., SOTIROPOULOU, D., AMEDEE, J. & MISSIRLIS, Y. F. 2001a. **Effect of surface roughness of the titanium alloy Ti-6Al-4V on human bone marrow cell response and on protein adsorption.** *Biomaterials*, 22, 1241–51.
- 31 DELIGIANNI, D. D., KATSALA, N. D., KOUTSOUKOS, P. G. & MISSIRLIS, Y. F. 2001b. **Effect of surface roughness of hydroxyapatite on human bone marrow cell adhesion, proliferation, differentiation and detachment strength.** *Biomaterials*, 22, 87–96.
- 32 DEWEY, M. J., NOSATOV, A. V., SUBEDI, K. & HARLEY, B. 2020. **Anisotropic mineralized collagen scaffolds accelerate osteogenic response in a glycosaminoglycan-dependent fashion.** *RSC Adv*, 10, 15629–15641.
- 33 DI LUCA, A., LONGONI, A., CRISCENTI, G., MOTA, C., VAN BLITTERSWIJK, C. & MORONI, L. 2016a. **Toward mimicking the bone structure: design of novel hierarchical scaffolds with a tailored radial porosity gradient.** *Biofabrication*, 8, 045007.

- 34 DI LUCA, A., OSTROWSKA, B., LORENZO-MOLDERO, I., LEPEDDA, A., SWIESZKOWSKI, W., VAN BLITTERSWIJK, C. & MORONI, L. 2016b. **Gradients in pore size enhance the osteogenic differentiation of human mesenchymal stromal cells in three-dimensional scaffolds.** *Sci Rep*, 6, 22898.
- 35 DILOKSUMPAN, P., BOLANOS, R. V., COKELAERE, S., POURAN, B., DE GRAUW, J., VAN RIJEN, M., VAN WEEREN, R., LEVATO, R. & MALDA, J. 2020. **Orthotopic Bone Regeneration within 3D Printed Bioceramic Scaffolds with Region-Dependent Porosity Gradients in an Equine Model.** *Adv Healthc Mater*, 9, e1901807.
- 36 DU, Y., GUO, J. L., WANG, J., MIKOS, A. G. & ZHANG, S. 2019. **Hierarchically designed bone scaffolds: From internal cues to external stimuli.** *Biomaterials*, 218, 119334.
- 37 DULAK, J., SZADE, K., SZADE, A., NOWAK, W. & JOZKOWICZ, A. 2015. **Adult stem cells: hopes and hypes of regenerative medicine.** *Acta Biochim Pol*, 62, 329–37.
- 38 ELBOURNE, A., CRAWFORD, R. J. & IVANOVA, E. P. 2017. **Nano-structured antimicrobial surfaces: From nature to synthetic analogues.** *Journal of Colloid and Interface Science*, 508, 603–616.
- 39 ENTEZARI, A., ROOHANI, I., LI, G., DUNSTAN, C. R., ROGNON, P., LI, Q., JIANG, X. & ZREIQAT, H. 2019. **Architectural Design of 3D Printed Scaffolds Controls the Volume and Functionality of Newly Formed Bone.** *Advanced Healthcare Materials*, 8, 1801353.
- 40 FOROUGH, A. H., VALERI, C. & RAZAVI, M. J. 2024. **A review of computational optimization of bone scaffold architecture: methods, challenges, and perspectives.** *Prog Biomed Eng (Bristol)*, 7.
- 41 GENTLEMAN, M. M. & GENTLEMAN, E. 2014. **The role of surface free energy in osteoblast–biomaterial interactions.** *International Materials Reviews*, 59, 417–429.
- 42 GENTLEMAN, M. M. & RUUD, J. A. 2010. **Role of Hydroxyls in Oxide Wettability.** *Langmuir*, 26, 1408–1411.

- 43 GHAYOR, C., BHATTACHARYA, I. & WEBER, F. E. 2021. **The optimal microarchitecture of 3D-printed β -TCP bone substitutes for vertical bone augmentation differs from that for osteoconduction.** *Materials & Design*, 204, 109650.
- 44 GIANNOUDIS, P. V., CHRIS ARTS, J. J., SCHMIDMAIER, G. & LARSSON, S. 2011. **What should be the characteristics of the ideal bone graft substitute?** *Injury*, 42 Suppl 2, 1–2.
- 45 GIULIANI, A., MANESCU, A., MOHAMMADI, S., MAZZONI, S., PIATTELLI, A., MANGANO, F., IEZZI, G. & MANGANO, C. 2016. **Quantitative Kinetics Evaluation of Blocks Versus Granules of Biphasic Calcium Phosphate Scaffolds (HA/ β -TCP 30/70) by Synchrotron Radiation X-ray Microtomography: A Human Study.** *Implant Dentistry*, 25, 6–15.
- 46 GRADO, G., KELLER, L., IDOUX-GILLET, Y., WAGNER, Q., MUSSET, A. M., BENKIRANE-JESSEL, N., BORNERT, F. & OFFNER, D. 2018. **Bone substitutes: A review of their characteristics, clinical use, and perspectives for large bone defects management.** *Journal of Tissue Engineering*, 9, 2041731418776819.
- 47 GUADARRAMA BELLO, D., FOUILLEN, A., BADIA, A. & NANJI, A. 2017. **A nanoporous titanium surface promotes the maturation of focal adhesions and formation of filopodia with distinctive nanoscale protrusions by osteogenic cells.** *Acta Biomater*, 60, 339–349.
- 48 GUI, N., XU, W., MYERS, D. E., SHUKLA, R., TANG, H. P. & QIAN, M. 2018. **The effect of ordered and partially ordered surface topography on bone cell responses: A review.** *Biomaterials Science*, 6, 250–264.
- 49 HAUGEN, H. J., LYGSTADAAS, S. P., ROSSI, F. & PERALE, G. 2019. **Bone grafts: Which is the ideal biomaterial?** *Journal of Clinical Periodontology*, 46 Suppl 2, 92–102.
- 50 HESARAKI, S., NAZARIAN, H., POURBAGHI-MASOULEH, M. & BORHAN, S. 2014. **Comparative study of mesenchymal stem cells osteogenic differentiation on low-temperature biomineralized nanocrystalline carbonated hydroxyapatite and sintered hydroxyapatite.** *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, 102, 108–118.

- 51 HOANG, V. T., NGUYEN, Q. T., PHAN, T. T. K., PHAM, T. H., DINH, N. T. H., ANH, L. P. H., DAO, L. T. M., BUI, V. D., DAO, H. N., LE, D. S., NGO, A. T. L., LE, Q. D. & NGUYEN THANH, L. 2025. **Tissue Engineering and Regenerative Medicine: Perspectives and Challenges.** *MedComm* (2020), 6, e70192.
- 52 HU, X., WANG, T., LI, F. & MAO, X. 2023. **Surface modifications of biomaterials in different applied fields.** *RSC Adv*, 13, 20495–20511.
- 53 HUANG, D., LI, Z., LI, G., ZHOU, F., WANG, G., REN, X. & SU, J. 2025. **Biomimetic structural design in 3D-printed scaffolds for bone tissue engineering.** *Mater Today Bio*, 32, 101664.
- 54 HULBERT, S. F., YOUNG, F. A., MATHEWS, R. S., KLAUITTER, J. J., TALBERT, C. D. & STELLING, F. H. 1970. **Potential of ceramic materials as permanently implantable skeletal prostheses.** *Journal of Biomedical Materials Research*, 4, 433–456.
- 55 HUMES, H. D. 2005. **Stem cells: The next therapeutic frontier.** *Transactions of the American Clinical and Climatological Association*, 116, 167–183 183–184.
- 56 IBRAHIMI, S., D'ANDREA, L., GASTALDI, D., RIVOLTA, M. W. & VENA, P. 2024. **Machine Learning approaches for the design of biomechanically compatible bone tissue engineering scaffolds.** *Computer Methods in Applied Mechanics and Engineering*, 423, 116842.
- 57 IONOV, L. 2018. **4D Biofabrication: Materials, Methods, and Applications.** *Advanced Healthcare Materials*, 7, 1800412.
- 58 JONES, A. C., ARNS, C. H., HUTMACHER, D. W., MILTHORPE, B. K., SHEPPARD, A. P. & KNACKSTEDT, M. A. 2009. **The correlation of pore morphology, interconnectivity and physical properties of 3D ceramic scaffolds with bone in growth.** *Biomaterials*, 30, 1440–1451.
- 59 JU, X.-J., XIE, R., YANG, L. & CHU, L.-Y. 2009. **Biodegradable 'intelligent' materials in response to chemical stimuli for biomedical applications.** *Expert Opinion on Therapeutic Patents*, 19, 683–696.

- 60 KHAJURIA, D. K., KUMAR, V. B., GIGI, D., GEDANKEN, A. & KARASIK, D. 2018. **Accelerated Bone Regeneration by Nitrogen-Doped Carbon Dots Functionalized with Hydroxyapatite Nanoparticles.** *ACS applied materials & interfaces*, 10, 19373–19385.
- 61 KILIAN, K. A., BUGARIJA, B., LAHN, B. T. & MRKSICH, M. 2010. **Geometric cues for directing the differentiation of mesenchymal stem cells.** *Proceedings of the National Academy of Sciences of the United States of America*, 107, 4872–4877.
- 62 KIM, K., MIN, S., THANGAM, R., TAG, K. R., LEE, H. J., HEO, J., JUNG, H., SWE, T. T., ZARE, I., SONG, G., NAJAFABADI, A. H., LEE, J., JUNG, H. D., KIM, J. S., HUR, S., SONG, H. C., PARK, S. G., ZHANG, K., ZHAO, P., BIAN, L., KIM, S. H., YOON, J., AHN, J. P., KIM, H. K. & KANG, H. 2025. **Dynamic hierarchical ligand anisotropy for competing macrophage regulation in vivo.** *Bioact Mater*, 47, 121–135.
- 63 KNYCHALA, J., BOUROPOULOS, N., CATT, C. J., KATSAMENIS, O. L., PLEASE, C. P. & SENGERS, B. G. 2013. **Pore geometry regulates early stage human bone marrow cell tissue formation and organisation.** *Annals of Biomedical Engineering*, 41, 917–930.
- 64 KOŁBUK, D., HELJAK, M., CHOLIŃSKA, E. & URBANEK, O. 2020. **Novel 3D Hybrid Nanofiber Scaffolds for Bone Regeneration.** *Polymers*, 12.
- 65 KOLOMENSKAYA, E., BUTOVA, V., POLTAVSKIY, A., SOLDATOV, A. & BUTAKOVA, M. 2023. **Application of Artificial Intelligence at All Stages of Bone Tissue Engineering.** *Biomedicines*, 12.
- 66 KRISHNAN, L., WILLETT, N. J. & GULDBERG, R. E. 2014. **Vascularization strategies for bone regeneration.** *Ann Biomed Eng*, 42, 432–44.
- 67 KUMAR, R., SPORN, K., PRABHAKAR, P., PALADUGU, P., KHANNA, A., NGO, A., GOWDA, C., WAISBERG, E., JAGADEESAN, R., ZAMAN, N. & TAVAKKOLI, A. 2025. **Next-Generation Biomaterials for Load-Bearing Tissue Interfaces: Sensor-Integrated Scaffolds and Mechanoadaptive Constructs for Skeletal Regeneration.** *J Funct Biomater*, 16.

- 68** LAMBERT, F., BACEVIC, M., LAYROLLE, P., SCHÜPBACH, P., DRION, P., ROMPEN, E. 2017. **Impact of biomaterial microtopography on bone regeneration: comparison of three hydroxyapatites.** *Clinical Oral Implants Research*, 28, e201–e207.
- 69** LE GUÉHENNEC, L., VAN HEDE, D., PLOUGONVEN, E., NOLENS, G., VERLÉE, B., DE PAUW, M. C. & LAMBERT, F. 2020. **In vitro and in vivo biocompatibility of calcium-phosphate scaffolds three-dimensional printed by stereolithography for bone regeneration.** *Journal of Biomedical Materials Research Part A*, 108, 412–425.
- 70** LEE, K. G., LEE, K. S., KANG, Y. J., HWANG, J. H., LEE, S. H., PARK, S. H., PARK, Y., CHO, Y. S. & LEE, B. K. 2018. **Rabbit Calvarial Defect Model for Customized 3D-Printed Bone Grafts.** *Tissue Eng Part C Methods*, 24, 255–262.
- 71** LEVINGSTONE, T. J., HERBAJ, S. & DUNNE, N. J. 2019. **Calcium-Phosphate Nanoparticles for Therapeutic Applications in Bone Regeneration.** *Nanomaterials*, 9.
- 72** LIMMAHAKHUN, S., OLOYEDE, A., SITTHISERIPRATIP, K., XIAO, Y. & YAN, C. 2017. **3D-printed cellular structures for bone biomimetic implants.** *Additive Manufacturing*, 15, 93–101.
- 73** LIU, X., RAHAMAN, M. N. & FU, Q. 2013. **Bone regeneration in strong porous bioactive glass (13-93) scaffolds with an oriented microstructure implanted in rat calvarial defects.** *Acta Biomaterialia*, 9, 4889–4898.
- 74** MA, C. Y., ZHAI, Y., LI, C. T., LIU, J., XU, X., CHEN, H., TSE, H. F. & LIAN, Q. 2024. **Translating mesenchymal stem cell and their exosome research into GMP compliant advanced therapy products: Promises, problems and prospects.** *Med Res Rev*, 44, 919–938.
- 75** MA, J., ZHOU, Z., GAO, M., YU, B., XIAO, D., ZOU, X. & BÜNGER, C. 2016. **Biosynthesis of Bioadaptive Materials: A Review on Developing Materials Available for Tissue Adaptation.** *Journal of Materials Science & Technology*, 32, 810–814.

- 76** MANGANO, C., RIBERTI, N., ORILISI, G., TECCO, S., FURLANI, M., GIOMMI, C., MENGUCCI, P., GIORGINI, E. & GIULIANI, A. 2025. **Morphometric, Biomechanical and Macromolecular Performances of β -TCP Macro/Micro-Porous Lattice Scaffolds Fabricated via Lithography-Based Ceramic Manufacturing for Jawbone Engineering.** *J Funct Biomater*, 16.
- 77** MANGANO, F. G., ZECCA, P. A., NOORT, R., APRESYAN, S., IEZZI, G., PIATTELLI, A., MACCHI, A. & MANGANO, C. 2015. **Custom-Made Computer-Aided-Design/Computer-Aided-Manufacturing Biphasic Calcium-Phosphate Scaffold for Augmentation of an Atrophic Mandibular Anterior Ridge.** *Case Reports in Dentistry*, 2015, 941265.
- 78** MAO, Z., BI, X., YU, C., CHEN, L., SHEN, J., HUANG, Y., WU, Z., QI, H., GUAN, J., SHU, X., YU, B. & ZHENG, Y. 2024. **Mechanically robust and personalized silk fibroin-magnesium composite scaffolds with water-responsive shape-memory for irregular bone regeneration.** *Nat Commun*, 15, 4160.
- 79** MARINER, P. D., WUDEL, J. M., MILLER, D. E., GENOVA, E. E., STREUBEL, S. O. & ANSETH, K. S. 2013. **Synthetic hydrogel scaffold is an effective vehicle for delivery of INFUSE (rhBMP2) to critical-sized calvaria bone defects in rats.** *Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society*, 31, 401–406.
- 80** MASTROGIACOMO, M., SCAGLIONE, S., MARTINETTI, R., DOLCINI, L., BELTRAME, F., CANCEDDA, R. & QUARTO, R. 2006. **Role of scaffold internal structure on in vivo bone formation in macroporous calcium phosphate bioceramics.** *Biomaterials*, 27, 3230–3237.
- 81** MCBEATH, R., PIRONE, D. M., NELSON, C. M., BHADRIRAJU, K. & CHEN, C. S. 2004. **Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment.** *Developmental Cell*, 6, 483–495.
- 82** MONJE, A. & WANG, H. L. 2017. **Biological and physical properties of bone block grafting biomaterials for alveolar ridge augmentation.**

- 83 MONTOYA, C., DU, Y., GIANFORCARO, A. L., ORREGO, S., YANG, M. & LELKES, P. I. 2021. **On the road to smart biomaterials for bone research: definitions, concepts, advances, and outlook.** *Bone Research*, 9.
- 84 MOROUCO, P., LATTANZI, W. & ALVES, N. 2017. **Four-Dimensional Bioprinting As a New Era for Tissue Engineering and Regenerative Medicine.** *Frontiers in Bioengineering and Biotechnology*, 5, 61.
- 85 MORRIS, E., CHAVEZ, M. & TAN, C. 2016. **Dynamic biomaterials: toward engineering autonomous feedback.** *Current Opinion in Biotechnology*, 39, 97–104.
- 86 MOSTAFAVI, E., SOLTANTABAR, P. & WEBSTER, T. J. 2018. **Nanotechnology and picotechnology: A new arena for translational medicine.** *Biomaterials in Translational Medicine: A Biomaterials Approach*, 191–212.
- 87 NADEEM, D., SMITH, C.-A., DALBY, M. J., MEEK, R. M. D., LIN, S., LI, G. & SU, B. 2015. **Three-dimensional CaP/gelatin lattice scaffolds with integrated osteoinductive surface topographies for bone tissue engineering.** *Biofabrication*, 7, 15005.
- 88 NECULA, M. G., MAZARE, A., ION, R. N., OZKAN, S., PARK, J., SCHMUKI, P. & CIMPEAN, A. 2019. **Lateral Spacing of TiO₂ Nanotubes Modulates Osteoblast Behavior.** *Materials (Basel, Switzerland)*, 12.
- 89 NGUYEN, D. H. K., BAZAKA, O., BAZAKA, K., CRAWFORD, R. J. & IVANOVA, E. P. 2020. **Three-Dimensional Hierarchical Wrinkles on Polymer Films: From Chaotic to Ordered Antimicrobial Topographies.** *Trends in Biotechnology*, 38, 558–571.
- 90 OH, S. H., PARK, I. K., KIM, J. M. & LEE, J. H. 2007. **In vitro and in vivo characteristics of PCL scaffolds with pore size gradient fabricated by a centrifugation method.** *Biomaterials*, 28, 1664–1671.
- 91 PASHAZADEH-PANAHI, P. & HASANZADEH, M. 2019. **Revolution in biomedicine using emerging of picomaterials: A breakthrough on the future of medical diagnosis and therapy.** *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, 120, 109484.

- 92 PATEL, K. D., KIM, T. H., MANDAKHBAYAR, N., SINGH, R. K., JANG, J. H., LEE, J. H. & KIM, H. W. 2020. **Coating biopolymer nanofibers with carbon nanotubes accelerates tissue healing and bone regeneration through orchestrated cell- and tissue-regulatory responses.** *Acta Biomaterialia*, 108, 97–110.
- 93 PRATHYUSHA, K., REDDY, J. G. M., VENKATASWAMY, M. & RAMESH, A. 2018. **Pico technology: Instruments used and Applications in pharmaceutical field.** *Research Journal of Pharmaceutical Dosage Forms and Technology*, 10, 34.
- 94 QASIM, M. Z., HAMMAD, H. M., ABBAS, F., SAEED, S., BAKHAT, H. F., NASIM, W., FARHAD, W., RABBANI, F. & FAHAD, S. 2020. **The potential applications of picotechnology in biomedical and environmental sciences.** *Environmental Science and Pollution Research International*, 27, 133–142.
- 95 RAHMATI, M., SILVA, E. A., RESELAND, J. E., HEYWARD, C. A. & HAUGEN, H. J. 2020. **Biological responses to physicochemical properties of biomaterial surface.** *Chemical Society Reviews*, 49, 5178–5224.
- 96 RAMLY, E. P., ALFONSO, A. R., KANTAR, R. S., WANG, M. M., SISO, J. R. D., IBRAHIM, A., COELHO, P. G. & FLORES, R. L. 2019. **Safety and Efficacy of Recombinant Human Bone Morphogenetic Protein-2 (rhBMP-2) in Craniofacial Surgery.** *Plast Reconstr Surg Glob Open*, 7, e2347.
- 97 ROOSA, S. M. M., KEMPPAINEN, J. M., MOFFITT, E. N., KREBSBACH, P. H. & HOLLISTER, S. J. 2010. **The pore size of polycaprolactone scaffolds has limited influence on bone regeneration in an in vivo model.** *Journal of Biomedical Materials Research. Part A*, 92, 359–368.
- 98 RUMPLER, M., WOESZ, A., DUNLOP, J. W. C., VAN DONGEN, J. T. & FRATZL, P. 2008. **The effect of geometry on three-dimensional tissue growth.** *Journal of The Royal Society Interface*, 5, 1173–1180.
- 99 SADEGHIAN DEHKORD, E., DE CARVALHO, B., ERNST, M., ALBERT, A., LAMBERT, F. & GERIS, L. 2024. **Influence of physicochemical characteristics of calcium phosphate-based biomaterials in cranio-maxillofacial bone regeneration. A systematic literature review and meta-analysis of preclinical models.** *Mater Today Bio*, 26, 101100.

- 100** SADEGHIAN DEHKORD, E., KERCKHOFS, G., COMPÈRE, P., LAMBERT, F. & GERIS, L. 2023. **An Empirical Model Linking Physico-Chemical Biomaterial Characteristics to Intra-Oral Bone Formation.** *J Funct Biomater*, 14.
- 101** SAILAJA, G. S., RAMESH, P., VELLAPPALLY, S., ANIL, S. & VARMA, H. K. 2016. **Biomimetic approaches with smart interfaces for bone regeneration.** *Journal of Biomedical Science*, 23, 1–13.
- 102** SAMSONRAJ, R. M., RAGHUNATH, M., NURCOMBE, V., HUI, J. H., WIJNEN, A. J. & COOL, S. M. 2017. **Concise Review: Multifaceted Characterization of Human Mesenchymal Stem Cells for Use in Regenerative Medicine.** *Stem Cells Translational Medicine*, 6, 2173–2185.
- 103** SANKARAN, S., ZHAO, S., MUTH, C., PAEZ, J. & DEL CAMPO, A. 2018. **Toward Light-Regulated Living Biomaterials.** *Advanced Science*, 5, 1800383.
- 104** SANZ, M., DAHLIN, C., APATZIDOU, D., ARTZI, Z., BOZIC, D., CALCIOLARI, E., DE BRUYN, H., DOMMISCH, H., DONOS, N., EICKHOLZ, P., ELLINGSEN, J. E., HAUGEN, H. J., HERRERA, D., LAMBERT, F., LAYROLLE, P., MONTERO, E., MUSTAFA, K., OMAR, O. & SCHLIEPHAKE, H. 2019. **Biomaterials and regenerative technologies used in bone regeneration in the craniomaxillofacial region: Consensus report of group 2 of the 15th European Workshop on Periodontology on Bone Regeneration.** *J Clin Periodontol*, 46 Suppl 21, 82–91.
- 105** SATO, M., SAMBITO, M. A., ASLANI, A., KALKHORAN, N. M., SLAMOVICH, E. B. & WEBSTER, T. J. 2006. **Increased osteoblast functions on undoped and yttrium-doped nanocrystalline hydroxyapatite coatings on titanium.** *Biomaterials*, 27, 2358–2369.
- 106** SAWADA, K., NAKAHARA, K., HAGA-TSUJIMURA, M., IIZUKA, T., FUJIOKA-KOBAYASHI, M., IGARASHI, K. & SAULACIC, N. 2018. **Comparison of three block bone substitutes for bone regeneration: Long-term observation in the beagle dog.** *Odontology*, 106, 398–407.

- 107** SCHAKENRAAD, J. M., BUSSCHER, H. J., WILDEVUUR, C. R. & ARENDS, J. 1986. **The influence of substratum surface free energy on growth and spreading of human fibroblasts in the presence and absence of serum proteins.** *Journal of Biomedical Materials Research*, 20, 773–784.
- 108** SEO, S. & NA, K. 2011. **Mesenchymal Stem Cell-Based Tissue Engineering for Chondrogenesis.** *Journal of Biomedicine and Biotechnology*, 2011, 806891.
- 109** SERRANO-BELLO, J., CRUZ-MAYA, I., SUASTE-OLMOS, F., GONZÁLEZ-ALVA, P., ALTOBELLI, R., AMBROSIO, L., MEDINA, L. A., GUARINO, V. & ALVAREZ-PEREZ, M. A. 2020. **In vivo Regeneration of Mineralized Bone Tissue in Anisotropic Biomimetic Sponges.** *Frontiers in Bioengineering and Biotechnology*, 8, 587.
- 110** SIAWASCH, S. A. M., YU, J., CASTRO, A. B., DHONDT, R., TEUGHEL, W., TEMMERMAN, A. & QUIRYNEN, M. 2025. **Autologous platelet concentrates in alveolar ridge preservation: A systematic review with meta-analyses.** *Periodontol* 2000, 97, 104–130.
- 111** SIMMONS, P., MCELROY, T. & ALLEN, A. R. 2020. **A Bibliometric Review of Artificial Extracellular Matrices Based on Tissue Engineering Technology Literature: 1990 through 2019.** *Materials* (Basel, Switzerland), 13.
- 112** SONG, W., HUANG, W., QU, J., XIAO, C., YIN, H., LIU, X. & XU, W. 2024. **The application prospects of 4D printing tissue engineering materials in oral bone regeneration.** *IJB*, 11.
- 113** SPIN-NETO, R., STAVROPOULOS, A., COLETTI, F. L., PEREIRA, L. A. V. D., MARCANTONIO, E. & WENZEL, A. 2015. **Remodeling of cortical and corticocancellous fresh-frozen allogeneic block bone grafts—A radiographic and histomorphometric comparison to autologous bone grafts.** *Clinical Oral Implants Research*, 26, 747–752.
- 114** SUN, Z. L., WATAHA, J. C. & HANKS, C. T. 1997. **Effects of metal ions on osteoblast-like cell metabolism and differentiation.** *J Biomed Mater Res*, 34, 29–37.

- 115 SUWARDI, A., WANG, F., XUE, K., HAN, M. Y., TEO, P., WANG, P., WANG, S., LIU, Y., YE, E., LI, Z. & LOH, X. J. 2022. **Machine Learning-Driven Biomaterials Evolution.** *Adv Mater*, 34, e2102703.
- 116 SZTERNER, P., ANTOSIK, A., PAGACZ, J. & TYMOWICZ-GRZYB, P. 2023. **Morphology Control of Hydroxyapatite as a Potential Reinforcement for Orthopedic Biomaterials: The Hydrothermal Process.** *Crystals*, 13, 793.
- 117 TERRANOVA, L., MALLET, R., PERROT, R. & CHAPPARD, D. 2016. **Polystyrene scaffolds based on microfibers as a bone substitute; development and in vitro study.** *Acta Biomaterialia*, 29, 380–388.
- 118 THIAN, E. S., HUANG, J., BEST, S. M., BARBER, Z. H., BROOKS, R. A., RUSHTON, N. & BONFIELD, W. 2006. **The response of osteoblasts to nanocrystalline silicon-substituted hydroxyapatite thin films.** *Biomaterials*, 27, 2692–2698.
- 119 TSENG, P., NAPIER, B., ZHAO, S., MITROPOULOS, A. N., APPEGATE, M. B., MARELLI, B., KAPLAN, D. L. & OMENETTO, F. G. 2017. **Directed assembly of bio-inspired hierarchical materials with controlled nanofibrillar architectures.** *Nat Nanotechnol*, 12, 474–480.
- 120 UNADKAT, H. V., HULSMAN, M., CORNELISSEN, K., PAPENBURG, B. J., TRUCKENMÜLLER, R. K., CARPENTER, A. E., WESSLING, M., POST, G. F., UETZ, M., REINDERS, M. J. T., STAMATIALIS, D., BLITTERSWIJK, C. A. & BOER, J. 2011. **An algorithm-based topographical biomaterials library to instruct cell fate.** *Proceedings of the National Academy of Sciences of the United States of America*, 108, 16565–16570.
- 121 VALAMVANOS, T. F., DEREKA, X., KATIFELIS, H., GAZOULI, M. & LAGOPATI, N. 2024. **Recent Advances in Scaffolds for Guided Bone Regeneration.** *Biomimetics (Basel)*, 9.
- 122 VAN BAELE, S., CHAI, Y. C., TRUSCELLO, S., MOESEN, M., KERCKHOFS, G., VAN OOSTERWYCK, H., KRUTH, J. P. & SCHROOTEN, J. 2012. **The effect of pore geometry on the in vitro biological behavior of human periosteum-derived cells seeded on selective laser-melted Ti6Al4V bone scaffolds.** *Acta Biomaterialia*, 8, 2824–2834.

- 123** VAQUETTE, C., PILIPCHUK, S. P., BARTOLD, P. M., HUTMACHER, D. W., GIANNOBILE, W. V. & IVANOVSKI, S. 2018. **Tissue Engineered Constructs for Periodontal Regeneration: Current Status and Future Perspectives.** *Advanced Healthcare Materials*, 7, 1800457.
- 124** WÄHNERT, D., GREINER, J., BRIANZA, S., KALTSCHMIDT, C., VORDEMVENNE, T. & KALTSCHMIDT, B. 2021. **Strategies to Improve Bone Healing: Innovative Surgical Implants Meet Nano-/Micro-Topography of Bone Scaffolds.** *Biomedicines*, 9.
- 125** WAN, Z., ZHANG, P., LIU, Y., LV, L. & ZHOU, Y. 2020. **Four-dimensional bioprinting: Current developments and applications in bone tissue engineering.** *Acta Biomaterialia*, 101, 26–42.
- 126** WANG, F., LI, C., ZHANG, S. & LIU, H. 2020. **Role of TiO₂ Nanotubes on the Surface of Implants in Osseointegration in Animal Models: A Systematic Review and Meta-Analysis.** *Journal of Prosthodontics: Official Journal of the American College of Prosthodontists*, 29, 501–510.
- 127** WANG, Q., HUANG, Y. & QIAN, Z. 2018. **Nanostructured Surface Modification to Bone Implants for Bone Regeneration.** *Journal of Biomedical Nanotechnology*, 14, 628–648.
- 128** WANG, R. E. & LANG, N. P. 2012. **Ridge preservation after tooth extraction.** *Clinical Oral Implants Research*, 23 Suppl 6, 147–156.
- 129** WANG, W. & YEUNG, K. W. K. 2017. **Bone grafts and biomaterials substitutes for bone defect repair: A review.** *Bioact Mater*, 2, 224–247.
- 130** WANG, Y., JAN, H., ZHONG, Z., ZHOU, L., TENG, K., CHEN, Y., XU, J., XIE, D., CHEN, D., XU, J., QIN, L., TUAN, R. S. & LI, Z. A. 2025a. **Multiscale metal-based nanocomposites for bone and joint disease therapies.** *Mater Today Bio*, 32, 101773.
- 131** WANG, Y., WANG, T., TANG, R., WANG, D., ZHANG, X., NAGAUMI, H., YANG, B., REN, X., HUANG, J., ZHANG, Y., HAO, J. & MA, Q. 2025b. **Multiscale interface engineering in biohybrid composites for biomedical applications.** *Mater Today Bio*, 35, 102382.

- 132** WEI, J., PAN, F., PING, H., YANG, K., WANG, Y., WANG, Q. & FU, Z. 2023. **Bioinspired Additive Manufacturing of Hierarchical Materials: From Biostructures to Functions.** *Research (Wash D C)*, 6, 0164.
- 133** WILKINSON, A., HEWITT, R. N., MCNAMARA, L. E., MCCLOY, D., DOMINIC MEEK, R. M. & DALBY, M. J. 2011. **Biomimetic microtopography to enhance osteogenesis in vitro.** *Acta Biomaterialia*, 7, 2919–2925.
- 134** WILLIE, B. M., PETERSEN, A., SCHMIDT-BLEEK, K., CIPITRIA, A., MEHTA, M., STRUBE, P., LIENAU, J., WILDEMANN, B., FRATZL, P. & DUDA, G. 2010. **Designing biomimetic scaffolds for bone regeneration: why aim for a copy of mature tissue properties if nature uses a different approach?** *Soft Matter*, 6, 4976–4987.
- 135** YANG, Y., WANG, X., HUANG, T. C., HU, X., KAWAZOE, N., TSAI, W. B., YANG, Y. & CHEN, G. 2018. **Regulation of mesenchymal stem cell functions by micro-nano hybrid patterned surfaces.** *Journal of Materials Chemistry. B*, 6, 5424–5434.
- 136** ZADPOOR, A. A. 2015. **Bone tissue regeneration: the role of scaffold geometry.** *Biomaterials Science*, 3, 231–245.
- 137** ZHANG, J., LUO, X., BARBIERI, D., BARRADAS, A. M. C., BRUIJN, J. D., BLITTERSWIJK, C. A. & YUAN, H. 2014. **The size of surface microstructures as an osteogenic factor in calcium phosphate ceramics.** *Acta Biomaterialia*, 10, 3254–3263.
- 138** ZHANG, K., WANG, S., ZHOU, C., CHENG, L., GAO, X., XIE, X., SUN, J., WANG, H., WEIR, M. D., REYNOLDS, M. A., ZHANG, N., BAI, Y. & XU, H. H. K. 2018. **Advanced smart biomaterials and constructs for hard tissue engineering and regeneration.** *Bone Research*, 6.
- 139** ZHANG, Q., MA, L., ZHENG, S., WANG, Y., FENG, M., SHUAI, Y., DUAN, B., FAN, X., YANG, M. & MAO, C. 2019. **Air-plasma treatment promotes bone-like nano-hydroxylapatite formation on protein films for enhanced in vivo osteogenesis.** *Biomaterials Science*, 7, 2326–2334.

- 140** ZHAO, C., XIA, L., ZHAI, D., ZHANG, N., LIU, J., FANG, B., CHANG, J. & LIN, K. 2015. **Designing ordered micropatterned hydroxyapatite bioceramics to promote the growth and osteogenic differentiation of bone marrow stromal cells.** *Journal of Materials Chemistry. B*, 3, 968–976.
- 141** ZHAO, Q., NI, Y., WEI, H., DUAN, Y., CHEN, J., XIAO, Q., GAO, J., YU, Y., CUI, Y., OUYANG, S., MIRON, R. J., ZHANG, Y. & WU, C. 2024. **Ion incorporation into bone grafting materials.** *Periodontol 2000*, 94, 213–230.
- 142** ZHOU, P., MAO, F., HE, F., HAN, Y., LI, H., CHEN, J. & WEI, S. 2019. **Screening the optimal hierarchical micro/nano pattern design for the neck and body surface of titanium implants.** *Colloids and Surfaces. B, Biointerfaces*, 178, 515–524.
- 143** ZHUJIANG, A. & KIM, S. G. 2016. **Regenerative Endodontic Treatment of an Immature Necrotic Molar with Arrested Root Development by Using Recombinant Human Platelet-derived Growth Factor : A Case Report.** *Journal of Endodontics*, 42, 72–75.

An aerial photograph of a river delta, showing a complex network of water channels and sandbars. The water is a light, silty color, contrasting with the darker, textured sandbars. The channels meander and branch out across the landscape, creating a web-like pattern. The overall tone is somewhat desaturated, with a focus on the geometric and organic shapes of the river system.

“What we do in life echoes in eternity.”

Gladiator (2000)



GENERAL DISCUSSION

DISCUSSION

Physicochemical Determinants of Regenerative Behavior

The work compiled in this thesis supports a central concept: calcium phosphate biomaterials should not be evaluated solely according to their class, origin, or commercial brand, but according to how their physicochemical and structural characteristics influence biological behavior across regenerative outcomes.

The systematic synthesis of preclinical evidence (Chapter 1) demonstrated that distinct physicochemical parameters influence distinct aspects of regeneration. Macropore size, Ca/P ratio, and compressive strength were associated with newly formed bone, whereas particle size, Ca/P ratio, and surface area influenced bone-to-material contact. Residual graft persistence was primarily affected by macropore size, particle size, and mechanical properties. Importantly, no single parameter explained all regenerative outcomes simultaneously, and no biomaterial category consistently outperformed all others across every endpoint. These findings suggest that biomaterial performance cannot be reduced to a single axis of evaluation. A material favorable for early bone formation may exhibit less favorable behavior regarding biodegradation or volumetric stability (Jiao et al., 2024, Yue et al., 2023), and the relevance of each parameter ultimately depends on the clinical indication. However, this interpretation must be considered taking into account the substantial methodological heterogeneity identified in the literature. Mechanical properties and surface roughness were inconsistently reported, while bone-to-material contact was evaluated in only a limited number of studies. Consequently, the structure–function relationships identified in this thesis should be interpreted as a robust conceptual framework rather than an exhaustive characterization of all determinants governing regeneration.

The importance of within-class variability was further substantiated experimentally in two complementary models. In a clinically relevant guided bone regeneration model (Chapter 3) bovine hydroxyapatite produced under sub-critical conditions and a continuous flow of a basic solution, following the patented extraction method (Wishbone Hydroxyapatite, WHA), achieved significantly higher newly formed bone at four weeks than the chemically-deproteinized commercial reference (Bio-Oss, BO), while both variants converged on equivalent regeneration by twelve and twenty-six weeks, with comparable bone-to-material contact and biocompatibility throughout the observation period. The earlier study isolating sintering temperature alone within a single chemically-pre-processed chemistry (Chapter 2) had already established that within-class variability is real. The highly-sintered (1200 °C) variant showed significantly lower bone-to-material contact than both the unsintered and 820 °C variants, and significantly lower

newly formed bone than the unsintered variant, despite identical chemistry and origin. Together, these results establish that two biomaterials sharing class, chemistry, origin, and indication can differ in their regenerative behavior in ways that matter clinically (Garcia et al., 2024), and these differences are related to manufacturing and physicochemical signatures rather than to confounding compositional or biological variables. The mechanistic basis underlying these observations was further explored in subsequent *in vitro* investigations (Chapter 4). WHA also demonstrated greater hydrophilicity, increased protein and albumin adsorption, enhanced cell attachment and proliferation, and a more controlled macrophage response. Together, these findings suggest that WHA established a more favorable early biological interface. One central aspect of this interface concerns calcium exchange at the material–fluid boundary. Specific surface area is a major determinant of the surface reactivity and dissolution behavior of calcium-phosphate biomaterials (Diez-Escudero et al., 2017). In the present work, BO exhibited a specific surface area approximately five times greater than WHA. In the calcium-containing serum medium, this larger reactive surface was associated with a significantly greater depletion of calcium from the surrounding medium, suggesting increased sequestration of extracellular calcium by BO. Beyond its physicochemical role, extracellular calcium also acts as a biological signaling mediator. Elevated extracellular calcium levels have been shown to stimulate the expression of bone morphogenetic proteins and enhance downstream osteogenic signaling pathways involving transcription factors such as Runx2 and Osterix (Aquino-Martínez et al., 2017, Nakade et al., 2001, Barradas et al., 2012, Yanai et al., 2019). The greater extracellular calcium availability maintained by WHA may therefore provide a plausible mechanistic explanation for its faster bone formation during the early stages of regeneration. These findings also help reinterpret the role of specific surface area across the broader body of work developed in this thesis. An earlier study demonstrated that surface area alone did not predict biological performance. Non-sintered and moderately sintered variants of bovine hydroxyapatite achieved comparable osteoconductivity despite an almost tenfold difference in specific surface area, while surface morphology emerged as the more influential parameter (Chapter 2). The present *in vitro* investigation extends this observation. Despite its lower specific surface area, WHA adsorbed greater amounts of serum protein and albumin, likely as a consequence of its superior hydrophilicity. Specific surface area therefore cannot be interpreted as a universally beneficial parameter. Its biological effects depend on the broader physicochemical environment with which it is associated, including surface morphology, wettability, porosity, and calcium-exchange behavior. A higher specific surface area does not inherently predict superior regenerative performance. Instead, the structure–function relationship identified throughout this thesis reflects a biological continuum linking manufacturing parameters to physicochemical properties, early cellular interactions, and ultimately regenerative outcomes. Calcium availability

unlikely fully explain the observed biological differences. WHA material also maintained a higher magnesium release throughout the observation period. Magnesium is known to contribute to alkaline phosphatase activity (He et al., 2016, Makris et al., 2023) and to modulate macrophage polarization toward a more reparative phenotype (Costantino et al., 2020). In parallel, WHA recruited fewer macrophages, suggesting a more controlled early inflammatory response. The early regenerative advantage observed in vivo therefore appears to be multifactorial rather than driven by a single mechanism. Greater extracellular calcium availability, sustained magnesium release, and a more balanced inflammatory environment may have acted synergistically during the early phases of healing to support faster bone formation.

Collectively, these findings support a shift in how calcium phosphate biomaterials are evaluated. Comparative approaches centered primarily on biomaterial class overlook the substantial variability that may exist within the same category of materials. From a clinical perspective, this distinction is not merely conceptual but may directly influence how biomaterials are selected and interpreted in regenerative practice (Lambert et al., 2017, Tadic and Eppe, 2004, Yue et al., 2023).

Improving the Reliability of Preclinical Bone Regeneration Assessment

The structure–function relationships described above rely on quantitative measurements obtained from preclinical histomorphometric analyses. The reliability of those inferences is therefore bounded by the rigor of those workflows and the methodological heterogeneity founded by the systematic synthesis (WP1) is not limited to inconsistent reporting of physicochemical features, it extends to the methods used in the literature to define the analytic ROI within which regeneration is measured, which varied widely across studies and were often poorly described. Quantitative integration of evidence across studies is therefore constrained as much by methodological heterogeneity as by biological complexity (Gerstenfeld et al., 2005, Rogova et al., 2024).

A particular vulnerability lies in the spatial reference against which regeneration is measured. In conventional preclinical workflows, the region of interest is generally defined retrospectively on histologic sections after healing, when the original defect geometry can no longer be directly visualized (Ghiacci et al., 2016, Shaheen et al., 2021, Toledano et al., 2022). The boundary of the analysis is therefore reconstructed by the observer, on tissue that the regeneration process itself has reshaped. The reference and the object being measured evolves during the healing period and the outcomes depend not only on what regenerated but also on the rules used to redraw the original contour. When the post-healing envelope contracts or expands relative to

the original defect, the apparent proportion of regenerated tissue shifts accordingly, and differences between treatment and control conditions can be artificially compressed.

The digital-twin workflow developed in this thesis (WP4) addresses this vulnerability directly. Intraoperative IOS, applied immediately after extraction, produces a high-resolution surface dataset of the original defect geometry, which is then aligned with the post-defect scan to reconstruct an animal-specific three-dimensional digital twin of the surgical defect. Cross-sectional projections of this digital twin, registered to histologic sectioning planes, provide a prospective spatial reference for ROI definition. Validation across the dog mandibular cohort demonstrated submillimetric agreement between IOS-derived and histologically measured wall thickness, strong correspondence between section-level ROI projections and reconstructed defect volume, and consistent positional alignment between planned and actual section planes. The methodology preserves the original defect envelope as a stable spatial reference, independent of the healing process, and enables outcome measurements to be normalized to a geometry that is no longer being reshaped by the experiment itself.

The contribution of this workflow extends beyond the present body of work. The digital-twin approach is not specific to bovine hydroxyapatite, to a particular defect geometry, or to a single animal model. It is a measurement framework applicable to any preclinical bone regeneration study in which the original defect surface can be acquired intraoperatively. By making the baseline geometry explicit and stable, it removes one of the principal sources of cross-study heterogeneity surfaced by the systematic synthesis. In this way, future preclinical studies could adopt prospective rather than retrospective ROI definition, the comparability of structure–function evidence across studies should improve, and with it the reliability of clinical inferences drawn from preclinical work.

Two limitations bound this contribution. The workflow was validated in one species and one defect geometry, and its accuracy under different anatomical configurations remains to be confirmed. Intraoral optical scanning captures surface geometry only. It does not replace micro-computed tomography for assessment of internal mineralized architecture, and the two modalities are complementary rather than substitutive. The scope of the contribution should therefore be understood as a methodology for prospective spatial normalization, not as a comprehensive solution to the broader heterogeneity of preclinical bone regeneration reporting an area in which surface roughness measurement, mechanical characterization, and standardized bone-to-material contact assessment remain underdeveloped across the field.

For the field, the implication is that the strength of clinical inferences drawn from preclinical evidence is conditional on the rigor of the underlying histomorphometry measurements, and that closing methodological gaps can produce gains in inferential reliability that are independent of any new biological finding. For the clinician, the practical implication is more indirect but no

less consequential since the confidence that can be placed in any reported biomaterial difference depends on whether the regions in which differences were measured were accurate or not.

From descriptive to instructive design

The structure–function evidence consolidated in the preceding sections positions current calcium-phosphate biomaterials within a descriptive paradigm: their physicochemical features are characterized, their biological responses are quantified, and their performance is interpreted retrospectively against the regenerative environment in which they are placed. This paradigm has carried the field a substantial distance, and the work compiled in this thesis sits squarely within it. It also exposes the paradigm's limits. Even when the structure–function profile is fully mapped, the resulting biomaterial remains a passive substrate whose properties are fixed at the moment of implantation, while bone regeneration develops across multiple structural scales and multiple biological phases over weeks to months. The mismatch between a static, single scale and a dynamic, multiscale process places an upper bound on what a passive scaffold can accomplish, regardless of how well its descriptive profile has been characterized. The narrative review included in this thesis, describes the key elements that a next-generation biomaterial design paradigm should integrate structural design across the pico-, nano-, micro-, and macroscales involved in cellular response; bioresponsive and bioadaptive behavior over time; computationally optimized architectures; and patient-specific customization through algorithm-guided design. While these concepts are individually present in the literature, their integration into a single, coherent framework specifically dedicated to alveolar and cranio-maxillofacial bone regeneration had not previously been clearly consolidated (Guo et al., 2026).

Two limitations should nevertheless be acknowledged. First, the next-generation paradigm described here remains largely conceptual. It is based on design principles emerging from different experimental fields, but these principles have not yet been validated together as a unified system for alveolar or cranio-maxillofacial bone regeneration. The transition from descriptive biomaterials toward truly instructive biomaterials should therefore be considered a future direction rather than an established clinical reality. Second, some of the most ambitious aspects of next-generation biomaterials, including patient-specific biomaterial design, dynamic bioresponsive behavior, and AI-guided structural optimization, still depend on technological and translational infrastructures that are currently at an early stage of development.

Within this perspective, the implications for clinicians remain mainly long-term rather than immediate. The biomaterials currently available in clinical practice are, and will likely remain for

some time, materials whose performance can be refined within the existing descriptive paradigm but not fundamentally transformed by it. Rather than a sudden shift, the concepts described here will probably enter clinical practice progressively through the development of specific next-generation biomaterials designed for targeted clinical indications over the coming decade.

For the scientific community, however, the implications are more immediate. The current descriptive evidence base has now reached a level of maturity that justifies systematic investigation of multiscale, dynamic, and personalized biomaterials in alveolar and cranio-maxillofacial regeneration models. In this context, the empirical findings and methodological approaches developed throughout this thesis provide a rigorous foundation for future research in this direction.

Clinical translation: scope, generalization, and implications

The experimental work developed in this thesis focused primarily on bovine hydroxyapatite.. Whether the same manufacturing–biology relationships apply identically to synthetic hydroxyapatite, β -tricalcium phosphate, or biphasic calcium phosphate scaffolds was not directly investigated. Nevertheless, indirect evidence supports broader generalization. The systematic synthesis identified comparable structure–function relationships across xenografts, alloplasts, and biphasic materials, while manufacturing processes such as sintering are widely used across calcium phosphate biomaterials. (Champion, 2013, Odusote et al., 2019, Trzaskowska et al., 2023). The principle that manufacturing influences biological behavior through measurable physicochemical modifications therefore likely extends beyond bovine hydroxyapatite, even though the dominant mechanisms and their magnitude may differ between material classes.

From a clinical perspective, three implications emerge. First, biomaterial selection should not rely solely on biomaterial class, chemistry, or commercial brand. Two biomaterials sharing the same nominal composition may still exhibit distinct regenerative behaviors if their manufacturing pathways differ (Joyce et al., 2021). Biomaterial selection should therefore consider the specific physicochemical and biological profile of the individual product rather than assuming equivalence within the same category. Second, manufacturing-driven differences appear to influence primarily the early regenerative phase. Across the experimental studies included in this thesis, the largest differences between biomaterials were observed during the first weeks of healing, whereas later healing periods demonstrated progressive convergence. Since accelerated implant protocols rely heavily on early healing dynamics and rapid tissue integration, manufacturing-related differences influencing early regenerative behavior may be especially

relevant in these clinical situations (Morton et al., 2023, Sanz et al., 2012). Third, biomaterial selection should remain indication-specific and outcome-oriented. No single physicochemical characteristic optimized all regenerative endpoints simultaneously. Biomaterials favorable for early bone formation were not necessarily optimal for volumetric stability or long-term graft persistence. The relevance of each characteristic therefore depends on the biological and clinical objective of the regenerative procedure.

For the scientific community, these findings also carry methodological implications. Comparative biomaterial studies should move beyond broad material categories and more systematically characterize manufacturing conditions and physicochemical signatures. Similarly, improving methodological standardization and geometrically accurate region-of-interest definition may help reduce the heterogeneity that currently limits cross-study comparison and quantitative evidence synthesis.

Finally, this work does not challenge the established clinical principles governing regenerative therapy, including defect morphology, flap management, soft-tissue stability, surgical technique, and patient-related factors. Rather, it refines one specific component within this framework: the evaluation and selection of biomaterials. In this context, the main contribution of this thesis is not the identification of a universally superior biomaterial, but the proposal of a more biologically grounded framework for understanding and interpreting biomaterial behavior in oral bone regeneration.

Limitations of the thesis

Beyond the limitations specific to each study, several considerations bound the body of work as a whole. The empirical investigations were centered on bovine hydroxyapatite, while the systematic synthesis encompassed the broader range of calcium-phosphate biomaterials, the manufacturing–biology relationships demonstrated experimentally were not directly tested for synthetic hydroxyapatite, β -tricalcium phosphate, or biphasic calcium phosphate scaffolds, and their generalization to these classes rests on indirect evidence. The body of work is, moreover, preclinical and in vitro in nature: the clinical implications discussed throughout this thesis are inferred from animal and laboratory models and have not been demonstrated in human studies. The in vitro mechanistic investigation relied on a defined panel of cell lines and on short-to-medium-term assays, which capture the early biological response but not the full trajectory of bone remodeling.

Finally, part of the experimental work was conducted in collaboration with, and funded by, an industrial partner, and several co-authors hold founding or shareholding interests in that partner. These relationships are disclosed in the constituent publications, and the data analysis and interpretation were conducted with measures to preserve scientific independence.

REFERENCES

- 1 AQUINO-MARTÍNEZ, R., ARTIGAS, N., GÁMEZ, B., ROSA, J. L. & VENTURA, F. 2017. **Extracellular calcium promotes bone formation from bone marrow mesenchymal stem cells by amplifying the effects of BMP-2 on SMAD signalling.** *PLoS One*, 12, e0178158.
- 2 BARRADAS, A. M., FERNANDES, H. A., GROEN, N., CHAI, Y. C., SCHROOTEN, J., VAN DE PEPPEL, J., VAN LEEUWEN, J. P., VAN BLITTERSWIJK, C. A. & DE BOER, J. 2012. **A calcium-induced signaling cascade leading to osteogenic differentiation of human bone marrow-derived mesenchymal stromal cells.** *Biomaterials*, 33, 3205–15.
- 3 CHAMPION, E. 2013. **Sintering of calcium phosphate bioceramics.** *Acta Biomater*, 9, 5855–75.
- 4 COSTANTINO, M. D., SCHUSTER, A., HELMHOLZ, H., MEYER-RACHNER, A., WILLUMEIT-RÖMER, R. & LUTHRINGER-FEYERABEND, B. J. C. 2020. **Inflammatory response to magnesium-based biodegradable implant materials.** *Acta Biomater*, 101, 598–608.
- 5 DIEZ-ESCUADERO, A., ESPANOL, M., BEATS, S. & GINEBRA, M. P. 2017. **In vitro degradation of calcium phosphates: Effect of multiscale porosity, textural properties and composition.** *Acta Biomater*, 60, 81–92.
- 6 GARCIA, V. G., DALL AGNOL, G. S., CAMPISTA, C. C. C., BURY, L. L., ERVOLINO, E., LONGO, M., MULINARI-SANTOS, G., LEVIN, L. & THEODORO, L. H. 2024. **Evaluation of Two Anorganic Bovine Xenogenous Grafts in Bone Healing of Critical Defect in Rats Calvaria.** *Braz Dent J*, 35, e246119.
- 7 GERSTENFELD, L. C., WRONSKI, T. J., HOLLINGER, J. O. & EINHORN, T. A. 2005. **Application of histomorphometric methods to the study of bone repair.** *J Bone Miner Res*, 20, 1715–22.
- 8 GHIACCI, G., GRAIANI, G., RAVANETTI, F., LUMETTI, S., MANFREDI, E., GALLI, C., CACCHIOLI, A., MACALUSO, G. M. & SALA, R. 2016. **«Over-inlay» block graft and differential morphometry: a novel block graft model to study bone regeneration and host-to-graft interfaces in rats.** *J Periodontal Implant Sci*, 46, 220–33.

- 9 GUO, H., LI, Q., ZREIQAT, H. & ENTEZARI, A. 2026. **Emerging technologies for personalised oro-maxillofacial bone regeneration: Design strategies and fabrication techniques.** *Biomaterials*, 326, 123709.
- 10 HE, L. Y., ZHANG, X. M., LIU, B., TIAN, Y. & MA, W. H. 2016. **Effect of magnesium ion on human osteoblast activity.** *Braz J Med Biol Res*, 49.
- 11 JIAO, X., WU, F., YUE, X., YANG, J., ZHANG, Y., QIU, J., KE, X., SUN, X., ZHAO, L., XU, C., LI, Y., YANG, X., YANG, G., GOU, Z. & ZHANG, L. 2024. **New insight into biodegradable macropore filler on tuning mechanical properties and bone tissue ingrowth in sparingly dissolvable bioceramic scaffolds.** *Mater Today Bio*, 24, 100936.
- 12 JOYCE, K., FABRA, G. T., BOZKURT, Y. & PANDIT, A. 2021. **Bioactive potential of natural biomaterials: identification, retention and assessment of biological properties.** *Signal Transduct Target Ther*, 6, 122.
- 13 LAMBERT, F., BACEVIC, M., LAYROLLE, P., SCHÜPBACH, P., DRION, P., ROMPEN, E., SCHUPBACH, P., DRION, P. & ROMPEN, E. 2017. **Impact of biomaterial microtopography on bone regeneration: comparison of three hydroxyapatites.** *Clinical Oral Implants Research*, 28, e201–e207.
- 14 MAKRIS, K., MOUSA, C. & CAVALIER, E. 2023. **Alkaline Phosphatases: Biochemistry, Functions, and Measurement.** *Calcif Tissue Int*, 112, 233–242.
- 15 MORTON, D., WISMEIJER, D., CHEN, S., HAMILTON, A., WITTNEBEN, J., CASENTINI, P., GONZAGA, L., LAZARIN, R., MARTIN, W., MOLINERO-MOURELLE, P., OBERMAILER, B., POLIDO, W. D., TAHMASEB, A., THOMA, D. & ZEMBIC, A. 2023. **Group 5 ITI Consensus Report: Implant placement and loading protocols.** *Clin Oral Implants Res*, 34 Suppl 26, 349–356.
- 16 NAKADE, O., TAKAHASHI, K., TAKUMA, T., AOKI, T. & KAKU, T. 2001. **Effect of extracellular calcium on the gene expression of bone morphogenetic protein-2 and -4 of normal human bone cells.** *J Bone Miner Metab*, 19, 13–9.
- 17 ODUSOTE, J. K., DANYUO, Y., BARUWA, A. D. & AZEEZ, A. A. 2019. **Synthesis and characterization of hydroxyapatite from bovine bone for production of dental implants.** *J Appl Biomater Funct Mater*, 17, 2280800019836829.

- 18 ROGOVA, V. V., PEEV, S., YOTSOVA, R., GEROVA-VATSOVA, T. & PARUSHEV, I. 2024. **Histomorphometric Assessment of Non-Decalcified Plastic-Embedded Specimens for Evaluation of Bone Regeneration Using Bone Substitute Materials-A Systematic Review.** *Materials (Basel)*, 18.
- 19 SANZ, I., GARCIA-GARGALLO, M., HERRERA, D., MARTIN, C., FIGUERO, E. & SANZ, M. 2012. **Surgical protocols for early implant placement in post-extraction sockets: a systematic review.** *Clin Oral Implants Res*, 23 Suppl 5, 67–79.
- 20 SHAHEEN, M. Y., BASUDAN, A. M., NIAZY, A. A., VAN DEN BEUCKEN, J., JANSEN, J. A. & ALGHAMDI, H. S. 2021. **Histological and Histomorphometric Analyses of Bone Regeneration in Osteoporotic Rats Using a Xenograft Material.** *Materials (Basel)*, 14.
- 21 TADIC, D. & EPPL, M. 2004. **A thorough physicochemical characterisation of 14 calcium phosphate-based bone substitution materials in comparison to natural bone.** *Biomaterials*, 25, 987–94.
- 22 TOLEDANO, M., VALLECILLO, C., GUTIERREZ-CORRALES, A., TORRES-LAGARES, D., TOLEDANO-OSORIO, M. & SERRERA-FIGALLO, M. A. 2022. **Histomorphometric Analysis of Differential Regional Bone Regeneration Induced by Distinct Doped Membranes.** *Polymers (Basel)*, 14.
- 23 TRZASKOWSKA, M., VIVCHARENKO, V. & PRZEKORA, A. 2023. **The Impact of Hydroxyapatite Sintering Temperature on Its Microstructural, Mechanical, and Biological Properties.** *Int J Mol Sci*, 24.
- 24 YANAI, R., TETSUO, F., ITO, S., ITSUMI, M., YOSHIZUMI, J., MAKI, T., MORI, Y., KUBOTA, Y. & KAJIOKA, S. 2019. **Extracellular calcium stimulates osteogenic differentiation of human adipose-derived stem cells by enhancing bone morphogenetic protein-2 expression.** *Cell Calcium*, 83, 102058.
- 25 YUE, X., ZHAO, L., YANG, J., JIAO, X., WU, F., ZHANG, Y., LI, Y., QIU, J., KE, X., SUN, X., YANG, X., GOU, Z., ZHANG, L. & YANG, G. 2023. **Comparison of osteogenic capability of 3D-printed bioceramic scaffolds and granules with different porosities for clinical translation.** *Front Bioeng Biotechnol*, 11, 1260639.



CONCLUSION

GENERAL CONCLUSION

The work compiled in this thesis addressed six interconnected research questions spanning biomaterial characterization, biological performance, methodological development, and future biomaterial design.

1. How do physicochemical and structural properties influence bone regeneration outcomes?

The systematic synthesis of preclinical evidence demonstrated that different physicochemical parameters influence distinct regenerative outcomes (Chapter 1). Macropore size, Ca/P ratio, and compressive strength were associated with newly formed bone, whereas particle size, surface area, and Ca/P ratio influenced bone-to-material contact. Residual graft persistence was mainly affected by macropore size, particle size, and mechanical properties. Importantly, no single parameter optimized all regenerative outcomes simultaneously, highlighting that biomaterial evaluation must remain indication-specific rather than based on a single performance criterion.

2. Does sintering temperature influence the biological performance of bovine hydroxyapatite?

The sintering study demonstrated that modifying a single manufacturing parameter significantly altered the physicochemical profile and in vivo biological behavior of bovine hydroxyapatite despite identical chemistry and origin (Chapter 2). High-temperature sintering reduced surface area, increased crystallinity, altered surface morphology, and resulted in lower bone-to-material contact and newly formed bone. These findings established that manufacturing decisions directly influence regenerative performance.

3. Can two manufacturing variants of the same bovine hydroxyapatite behave differently in guided bone regeneration?

In the guided bone regeneration model, WHA achieved significantly greater early bone formation than the chemically deproteinized commercial reference while maintaining comparable biocompatibility and later regenerative outcomes (Chapter 3). These findings confirmed that clinically relevant variability may exist even between biomaterials sharing the same origin, chemistry, and indication, particularly during early healing.

4. Which mechanisms explain the distinct biological behavior of manufacturing-variant bovine hydroxyapatites?

WHA demonstrated greater hydrophilicity, more favorable ion-release dynamics, increased protein adsorption, enhanced cell attachment and proliferation, and a more controlled

macrophage response. Together, these findings support a measurable continuum linking manufacturing conditions to physicochemical properties, early cellular interactions, and regenerative outcomes (Chapter 4).

5. Can intraoral optical scanning improve region-of-interest determination for histomorphometric analysis?

The digital workflow developed in this thesis demonstrated that intraoperative intraoral optical scanning can reconstruct original defect geometry with high spatial accuracy (Chapter 5). This digital-twin approach provides a reproducible framework for anatomically guided region-of-interest definition and addresses an important source of methodological heterogeneity in preclinical bone regeneration research.

6. Which principles may guide the design of next-generation biomaterials?

The narrative review integrated emerging concepts related to multiscale architecture, bioadaptive behavior, computational design, and patient-specific biomaterial engineering within a unified framework for cranio-maxillofacial regeneration. By consolidating these concepts into a coherent design paradigm, this work provides a conceptual roadmap for the future development of structurally instructive and personalized regenerative biomaterials (Chapter 6).

Overall synthesis

Taken together, the six contributions of this thesis support a different way of evaluating calcium-phosphate biomaterials for alveolar and cranio-maxillofacial bone regeneration. Rather than focusing only on biomaterial class, origin, or commercial brand, the findings highlight the importance of the relationship between manufacturing, physicochemical characteristics, and biological behavior. Within this framework, manufacturing emerges as a key determinant of regenerative performance, while rigorous and anatomically guided methodologies become essential for reliable preclinical evaluation. The work also supports the transition toward a new generation of biomaterials based on multiscale design, dynamic biological interactions, and patient-specific approaches. Overall, this thesis contributes to the field at multiple levels: by improving the understanding of structure–function relationships in bone substitute biomaterials, by providing methodological advances for preclinical assessment, and by proposing a conceptual framework to guide the future design of regenerative biomaterials.



List of publications

De Carvalho B., Rompen E., Lecloux G., Schupbach P., Dory E., Art J.-F., Lambert F. (2019). Effect of Sintering on In Vivo Biological Performance of Chemically Deproteinized Bovine Hydroxyapatite. *Materials*.; 12(23):3946.

Sadeghian Dehkord, E., **De Carvalho B.**, Ernst M., Albert A., Lambert F., & Geris L. (2024). Influence of physicochemical characteristics of calcium phosphate-based biomaterials in cranio-maxillofacial bone regeneration. A systematic literature review and meta-analysis of preclinical models. *Materials today. Bio*, 26, 101100. <https://doi.org/10.1016/j.mtbio.2024.101100>

De Carvalho B., Dory E., Trus C., Pirson J., Germain L., Lecloux G., Lambert F., & Rompen E. (2023). Biological performance of a novel bovine hydroxyapatite in a guided bone regeneration model: A preclinical study in a mandibular defect in dogs. *Clinical implant dentistry and related research*, 10.1111/cid.13260.

De Carvalho B., Anania S., Van Hede D., Dory E., Rompen E., Lambert F. (2025). Influence of in vitro physicochemical and biological characteristics on hydroxyapatite xenografts performance. *J Periodontal Res*. 2026 Feb 11

De Carvalho B., Lecloux G., Beaudry D., Knight B., Rompen E. (ready for submission). Digital twin–guided optical impression analysis for anatomically accurate ROI determination and histologic alignment. A methodology validation study in preclinical bone regeneration models.

De Carvalho B., Ivanets M., Lambert F., Geris L., Van Hede D. (ready for submission). Next-generation biomaterials for alveolar bone regeneration: a structurally instructive, multiscale design paradigm.

Van Hede D., **De Carvalho B.**, Rompen E., Lecloux G., Pirson J., Cobraiville E. and Lambert F. Clinical-Scale Application of Bioactive 3D-Printed Hydroxyapatite Scaffolds for Alveolar Bone Regeneration: A Preclinical Study in a Canine Model. *Clinical Oral Implants Research* (Submitted)

Determe S., **De Carvalho B.**, Lecloux G., Lambert F. and Van Hede D. (in preparation). Effectiveness of Dental Implant Placement in Optimized 3D-Printed Hydroxyapatite Scaffolds: A Preclinical Pilot Study

De Carvalho B., Hurtgen A., Finelle G., Lecloux G., Lambert F. (in preparation). Immediate implant placement in the posterior region combining sealing socket abutment and peri-implant socket filling. A Randomized Controlled Clinical Trial

Lambert F., **De Carvalho B.** (2018). Impact de la santé buccodentaire sur les performances sportives, *Journal de Traumatologie du Sport*, Volume 35, Issue 3

Le Roch S., Rouche F., Valet F., Bouchard P.; on behalf of the ESCAPE group. European survey on criteria of aesthetics for periodontal evaluation: The ESCAPE study. *J Clin Periodontol.* 2019; 46: 1116– 1123.

Salhi L., **De Carvalho B.**, Reners M.. Update on the Roles of Oral Hygiene and Plaque Control on Periodontal Disease. *Adv Exp Med Biol.* 2022;1373:329-339. doi: 10.1007/978-3-030-96881-6_17. PMID: 35612806.

Lectures and presentations

19 May 2019 – TERMIS Congress, Rhodes, Greece

Oral communication: Effect of Sintering on Biological Performance of Bovine Hydroxyapatite

19 November 2021 – Belgian Society of Periodontology – Past, Present and Future of Periodontology, Belgium

Lecture: A New Approach for Peri-Implantitis Treatment with Electrolytic Cleaning

23 January 2025 – VBT Congress

Lecture: Optimization of Results in Immediate Implant Placement: Patient, Biological and Surgical Considerations

16 May 2025 – EuroPerio11, Vienna, Austria

Oral communication: Influence of In Vitro Physicochemical and Biological Characteristics on Hydroxyapatite Xenografts Performance

16 September 2025 – ITI Master Class and Hands-on

Lecture and hands-on course: Enhancing Peri-Implant Soft Tissues with Mucogingival Surgeries

8 November 2025 – OMD Portugal

Lecture: Peri-Implant Biocompatibility: The Role of Mucointegration

25 November 2025 – ADF Congress, Paris, France

Invited lecture: Repousser les limites de la régénération parodontale : l'interaction entre le design du lambeau, les biomatériaux et le minimalisme chirurgical

11 December 2025 – ITI Study Club Lisbon, Portugal

Lecture: Optimization of Results in Immediate Implant Placement: Patient, Biological and Surgical Considerations

25 March 2026 – Cercle Franco-Libanais d'Implantologie et de Parodontologie

Lecture: Biomateriaux 360° : de la régénération osseuse à la stabilité transmucosale





BRUNO DE CARVALHO

Born the 3rd of July 1989

Periodontist and researcher originally from Portugal, working in the fields of periodontology, implant dentistry, and regenerative biomaterials. He obtained his Integrated Master's Degree in Dentistry in Porto back in 2013, following an Erasmus exchange at the University of Barcelona, Spain. He subsequently completed the full-time EFP-accredited postgraduate program in Periodontology and Implant Dentistry at the University of Liège (Belgium) between 2014 and 2017. Since 2019, he has served as Head of Clinic in the Department of Periodontology, Oro-Dental and Implant Surgery at the University Hospital of Liège (CHU de Liège), where he is also co-coordinator of the EFP-accredited postgraduate program in Periodontology and Implant Dentistry. His academic activities include teaching theoretical and practical courses in periodontology, implant dentistry, microsurgery, suturing techniques, minimally invasive dental extractions, and digital workflows. He is also an invited lecturer in the master's Program in Periodontology and Implantology at the University of Porto and University of Lisbon.

His research activities are centered on biomaterials for oral bone regeneration, tissue engineering, peri-implant tissue integration, and implant rehabilitation protocols. As part of the research group led by Prof. France Lambert, his work focuses particularly on the relationship between physicochemical characteristics of calcium-phosphate biomaterials and their biological performance in alveolar and cranio-maxillofacial regeneration.

Throughout his academic career, Bruno De Carvalho has been actively involved in international scientific and professional organizations. He currently serves as President of the Belgian Society of Periodontology and is an ITI Fellow and member of the ITI Future Leaders Committee. He also participated in the Communication Committee of the European Federation of Periodontology and previously hosted the EFP PerioTalks Podcast. His research has been recognized with several distinctions, including the 2nd EFP Prize for Preclinical & Basic Research in 2020 and selection as a PED Talks speaker at EuroPerio 9.

Alongside his academic and clinical work, he maintains a strong interest in visual communication, photography, graphic design, music, digital media and AI, fields that continue to shape his approach to education and scientific dissemination.





ACKNOWLEDGEMENTS

This thesis may represent the completion of a PhD, but its story began long before the first experiment. It is inseparable from the twelve years I have spent in Belgium, from the people who trained me, challenged me, supported me and ultimately helped shape the clinician, researcher, teacher, and person I became.

PROMOTOR AND CO-PROMOTOR

To my promotor, **Professor France Lambert**.

Thank you for shaping me not only as a clinician and researcher, but also as a teacher, speaker, and, most importantly, as a person. Looking back over the past decade, it has been both an adventure and a privilege to work alongside you. I could not have wished for better conditions in which to pursue this PhD, and you always made sure I had the support, freedom, and opportunities to grow. Thank you for believing in my ideas, for our countless discussions, and for teaching me to question, to think critically, and to always look beyond the obvious. The opportunities I have had under your guidance are impossible to summarize in a single paragraph. Above all, thank you for investing in me, for becoming my mentor, my older sister, and my friend.

Professor Eric Rompen.

Thank you for planting the seed of this PhD long before it became a reality. During a dinner in Monaco in 2016, you simply told me: «You're going to finish your Periodontology specialization, and then you're going to do a PhD.» Well... I have never really argued with your orders, so here we are, closing another chapter. Thank you for continuously motivating me, for allowing me to learn by your side, and for transmitting your passion for biology during surgery, which has always fascinated me. More importantly, thank you for building the foundations of this Periodontology program, allowing so many of us to become periodontists, and for trusting us to continue raising its standards year after year.

MEMBERS OF THE JURY

Dr. Dorien Van Hede.

Thank you for your constant support throughout this thesis. Your ability to explain complex concepts with such simplicity, your availability to answer my endless questions, and your kindness have made this journey much easier. Over these years, we shared not only research but also countless conversations and memorable moments that I will always carry with me.

Professor Amélie Mainjot.

Thank you for your trust and encouragement throughout these years. Your enthusiasm is truly contagious, and I have learned a great deal from you, both professionally and personally.

Professor Chantal Humblet.

Thank you for your valuable comments during every thesis committee meeting. Your ability to analyze my work from a different perspective challenged me to think more critically and undoubtedly improved the quality of this thesis.

Professor Andy Temmerman.

Andy, it has been a real pleasure collaborating with you on so many projects beyond this thesis. Some people simply connect effortlessly, sharing the same sense of humor and enthusiasm, and you are certainly one of them. I admire you as a clinician, researcher, and exceptionally gifted surgeon. Thank you for your valuable contribution to this work and, above all, for your friendship.

Professor Kasia Gurzawska-Comis.

Thank you for your structured and thoughtful approach throughout the evaluation of this thesis and during my defense. It was a pleasure to have you as part of my jury, challenging me and helping me look at my work from different perspectives.

COLLABORATORS**Geoffrey Lecloux.**

Where do I even begin? You have been family to me in Belgium. Thank you for shaping me as a surgeon, for constantly pushing me to become better, and for sharing your unique way of approaching research. Your comments and ideas have influenced this thesis in countless ways. Above all, thank you for always being there, through the successes, the doubts, and, of course, for your legendary dark humor.

Sandy.

Thank you for your invaluable help with the in vitro analyses. Your expertise was essential to making sense of the data, and you were always available with a smile and genuine enthusiasm. It was truly a pleasure to collaborate with you.

Ehsan.

I must admit that, at one point, I thought it would be impossible to publish our systematic review. Your perseverance was remarkable, and your coordination throughout the project taught me a great deal. Thank you for your dedication and for being part of this thesis.

Margarita and Liesbet.

Thank you both for your insightful comments and invaluable suggestions that significantly improved Chapter 6. I learned a great deal from working with you.

Peter Schüpbach.

Thank you for welcoming me into your lab in Zurich and for introducing me to the fascinating world of histology. Your generosity and expertise left a lasting impression on me.

To the Wishbone team — Emilie, Justine, Caroline, Jean-François and Loïc.

Thank you for your continuous support, your availability, and your patience in answering my many questions throughout these years.

Leila Salhi.

Thank you for your guidance in helping me organize this thesis, but even more for your remarkable personality and unwavering optimism. Your resilience, even when life was not always kind, has been a true source of inspiration.

FRIENDS

Adrien Bolette & Claire.

Adrien, thank you for being the big brother I never had. During the moments when I felt lost, you were always only one message away, ready to meet, listen, and remind me of who I was. The adventures we shared will stay with me forever. Claire, thank you for the countless conversations, unforgettable moments, thoughtful advice, and for simply being the wonderful person you are.

Guillaume Roig.

Thank you for being there from the very beginning. Your support meant more than you probably realize and made adapting to a completely new country so much easier. I always knew you had my back. Through all these years, we have shared challenges, achievements, and unforgettable memories. You truly became a brother to me.

Lou Li Manni.

My Perio sister. My Perio love. We connected instantly. Your personality is one of the rarest I have ever met. Watching you become such an outstanding and confident clinician has been inspiring. Our Miami trip and all the watermelooooon moments, will always have a special place in my heart. Love you.

Gaëlle Boltide.

My Perio sister. Your energy, strength, and determination have always amazed me. No matter what life put in front of you, you always found the strength to get back up and keep moving forward. That resilience is something I will carry with me for the rest of my life. And, of course, thank you for proving that you are also the greatest party animal I know.

Brieuc and Manon.

You are both very special to me. I first met you as students, then as residents in our Periodontology program, and later had the privilege of briefly mentoring you. Watching your growth has been one of the greatest rewards of my academic career.

To all the past and current Periodontology residents.

This thesis is also for you. As a mentor, I always believed that my role was to teach and help shape you as future periodontists. What I never expected was how much I would learn from each of you in return. Every personality, every discussion, every challenge helped me grow as well. Thank you, Romane, Marianzela, Lemmy, Yaman, Samuel, Gilles, Alexandre, Clémentine, Sébastien, Sina, Haitam, Francesca, Zoï, Elisa, Faissal, Alexandra, and everyone who has been part of this journey.

Michèle Reners.

Thank you for your contagious smile, your trust, and your inspiring mindset. I have wonderful memories of the moments we shared over these years. Thank you for always believing in me.

Nathalie Robert & Julie Oudkerk.

Thank you for sharing the path of this PhD at the same time with your owns, for the mutual support and for so many patients we treat together.

Kim Vincent & Elise Gaudin.

Thank you for being my teachers, being my friends and for caring so much about me. With you I feel I'm surrounded with big sisters that really want me to achieve each day.

Isabelle Bugli.

My safe place. My Belgian mom. Thank you for always taking care of me, for making me feel at home, and for being there whenever I needed you.

Madame Paule.

Thank you for helping me find the path when everything seemed dark. Thank you for helping me reconnect with myself and discover the Bruno I wanted to become. Our sessions have had a profound impact on my life, far beyond this thesis.

Wendy.

My Flemish queen. Thank you for your amazing mind, for being a true friend and for all the amazing moments we pass together.

Jean-Noël, Anne France, Corrine.

Thank you for always make sure I was “still breathing” with all the running and daily tasks in the department. You are unforgettable for me during all this times.

To all the clinicians.

Thank you so much for your trust, for all the teaching throughout this years and to always make me feel accepted since day 1. A big thank you to Jean Phi, Amandine, Alice, Charlotte, Deborah, Marcia, Alexandre, Martin, Vero Varlet, Vero Urréa, Pascal, Florence, Vinciane, Audrey, Marc and Alain.

To all my colleagues from the **Belgian Society of Periodontology**, also a big thank you for believing in me, to allow me to learn so much from you and for your continuous collaboration and amazing love from Perio.

Sart-Tilman Family.

You were my anchor when I arrived. Over the years we shared so many moments, so much growth together. I was able to learn a lot from every single person. A big thank you to Cecile, Maryam, Emilie, Elodie, Jen Marechal, Jeny, Thierry, Nisa, Claire, Nathalie.

Brull Family.

To my wonder trio, Christelle, Isabelle et Cedric. You make the Brull a living ecosystem and without you over there would not be the same energy. Thank you for your continuous support, motivation, to always listen to me and to be amazing friends.

To finish with Belgium, I would like to thank Dr. Tommie Van de Velde for introducing and to send me to Liège in 2013 for a quick visit. Thanks to you I got to know all these amazing people that shaped my career.

Portugal.

To all my dear friends, that couldn't wait for me to finish this chapter, a big thank you for ALWAYS being there for me. For accepting me exactly as I am in my crazy days and in my boring days. To David, Maga, Rafa, Paulo, Maria Inês, Rita, Andreia and Rute, a big thank you. Also to my sporty friends Filipa and Marta, for so many sweaty and dancing moments in the last years.

Para terminar:

Para ti Mãe, que sempre estas aí para me ouvir e que sempre me inspiras todos os dias a querer ser mais e melhor e a alcançar novas metas. Esta tese é sem dúvida a melhor dedicatória que te posso dar por todos os esforços e sacrifícios que fizeste e fazes em prol da minha carreira. Amo-te...

“ I’m coming to the cottage ”

Heated Rivalry (2025)

Understanding biomaterials.
Inspiring regeneration.



INFLUENCE OF MULTILEVEL BIOMATERIAL
CHARACTERISTICS ON ORAL BONE REGENERATION

BRUNO DE CARVALHO

