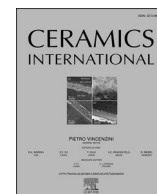




Contents lists available at ScienceDirect

Ceramics International

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

Thermally stable strontium- and magnesium-enriched bioactive glass scaffolds promote bone regeneration and angiogenesis: An *in vivo* comparative study with 45S5

Marzia Ferretti ^{a,1} , Francesco Cavani ^{a,1} , Alexandre Anesi ^b, Francesca Chiarini ^a, Roberta Salvatori ^c , Marta Checchi ^a, Ilaria Zanoni ^d , Francesca Paganelli ^a , Devis Bellucci ^{e,*} , Valeria Cannillo ^{e,**,2} , Carla Palumbo ^{a,2}

^a Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, 41124, Italy

^b Department of Medical and Surgical Sciences for Children & Adults, Cranio-Maxillofacial Surgery, University of Modena and Reggio Emilia, Modena, 41124, Italy

^c Department of Medical and Surgical Sciences for Children & Adults, University of Modena and Reggio Emilia, Modena, 41124, Italy

^d Institute of Science, Technology and Sustainability for Ceramics (CNR-ISSMC), National Research Council of Italy, Faenza, 48018, Italy

^e Department of Engineering "Enzo Ferrari", University of Modena and Reggio Emilia, Modena, 41125, Italy

ARTICLE INFO

Keywords:

Bioactive glasses

Strontium

Magnesium

CAM

In vivo evaluation

Angiogenesis

Bone regeneration

ABSTRACT

The development of biomaterials to actively support both bone formation and vascularization represents a key challenge in regenerative medicine. Two novel bioactive glasses, Bio_MS and BGMS10, enriched with therapeutic ions such as strontium and magnesium and characterized by high thermal stability, were fabricated into porous scaffolds using the foam replica method and compared to the conventional 45S5 Bioglass®. The materials were evaluated for their ionic release, angiogenic potential (using the chorioallantoic membrane-CAM assay) and bone regenerative capacity *in vivo* (using rabbit models with femur/tibia defects). Ion release analyses revealed controlled and sustained release of biologically active ions (Ca, Mg, Sr, Si) from Bio_MS and BGMS10 scaffolds. By CAM assay, both the novel scaffolds enhanced vessel density and branching, indicating a good pro-angiogenic effect. After 60 days of implantation, SEM/X-EDS and histomorphometric analyses demonstrated extensive new bone formation and effective osteo-scaffold coupling for all three BGs, even if Bio_MS scaffold showed the highest affinity index. Histological observations confirmed abundant osteoblast laminae and limited osteoclastic activity around Bio_MS, whereas BGMS10 samples showed higher osteoclast presence, possibly due to their elevated Mg release. In summary, these results showed that all bioactive glasses show no significant differences in terms of osteoconductive potential, although Bio_MS, having faster ionic dissolution and higher affinity index than the others, seems in a more advanced stage of dissolution, inducing early osteogenesis, as evidenced by the presence of abundant osteoblastic laminae close to its surface; these features make it a strong candidate for bone-defect repair and broader regenerative medicine applications.

1. Introduction

The aging population and the consequent increase in age-related diseases, including osteoporosis, osteoarthritis, and other degenerative conditions, together with the rising incidence of severe trauma and bone tumors, represent a major challenge in clinical management. The human body's natural ability to heal and restore bone defects through

complex regeneration processes, involving both anatomical and physiological repair, is not always sufficient to fully solve the problem. In many cases, when bone defects occur, the intrinsic healing ability of the body is incomplete or inadequate. The defect size is crucial among the different factors influencing bone regeneration, beyond the patient's overall health status. The so-called "Critical-Size Defect" (CSD), typically defined as 2.5 cm or larger defects, cannot be spontaneously

* Corresponding author.

** Corresponding author.

E-mail addresses: devis.bellucci@unimore.it (D. Bellucci), valeria.cannillo@unimore.it (V. Cannillo).

¹ Authors equally contributed as first author.

² Authors equally contributed as last author.

<https://doi.org/10.1016/j.ceramint.2026.08.174>

Received 26 May 2026; Accepted 12 August 2026

Available online 12 August 2026

0272-8842/© 2026 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Table 1

Composition in mol% of the BGs used.

Sample	SiO ₂	P ₂ O ₅	Na ₂ O	K ₂ O	CaO	MgO	SrO
45S5	46,1	2,6	24,4	-	26,9	-	-
Bio_MS	46,1	2,6	5	-	31,3	5	10
BGMS10	47,2	2,6	2,3	2,3	25,6	10	10

Table 2

BG scaffold (45S5, Bio_MS and BGMS10) implantation scheme in rabbits' left and right femoral distal epiphysis (F) and tibial proximal diaphysis (T).

Rabbit 1	Rabbit 2	Rabbit 3
right F 45S5	right F Bio_MS	right F BGMS10
right T 45S5	right T Bio_MS	right T BGMS10
left F BGMS10	left F 45S5	left F Bio_MS
left T BGMS10	left T 45S5	left T Bio_MS
Rabbit 4	Rabbit 5	Rabbit 6
right F 45S5	right F Bio_MS	right F BGMS10
right T BGMS10	right T Bio_MS	right T BGMS10
left F BGMS10	left F 45S5	left F Bio_MS
left T 45S5	left T 45S5	left T Bio_MS
Rabbit 7	Rabbit 8	Rabbit 9
right F 45S5	right F Bio_MS	right F BGMS10
right T 45S5	right T Bio_MS	right T BGMS10
left F BGMS10	left F 45S5	left F Bio_MS
left T BGMS10	left T 45S5	left T Bio_MS

repaired [1,2]. In such cases, surgical interventions involving autologous grafts, heterologous grafts, or synthetic biomaterials are necessary to restore the defect [3].

Autologous grafts are considered the gold standard, as they are obtained by harvesting bone tissue from the same patient and offer many advantages like compatibility with the host tissue and the best chance of proliferation and differentiation into osteoblasts to promote the healing process [4]. The presence of growth factors, such as Bone Morphogenetic Proteins (BMPs), further enhances osteogenesis [5]; however, the health status of the patient significantly influences the feasibility of harvesting autologous grafts and individuals with comorbidities such as osteoporosis, diabetes, or vascular diseases may not be suitable candidates, due to their compromised healing potential [6].

In contrast, heterologous grafts, derived from human or animal donors, offer the enormous advantage of eliminating donor site morbidity, a significant concern associated with autologous grafts. Patients avoid

the additional surgical procedures required for autologous grafts, thereby reducing complications such as pain, infection risk, and prolonged recovery. Moreover, heterologous grafts provide a more abundant supply of bone tissue, making them particularly beneficial in cases that require extensive bone reconstruction [7,8]. For bone allografts and xenografts, the principal concerns include the risk of disease transmission, potential immunogenicity and host rejection, and variability in graft incorporation and remodeling. Despite rigorous donor screening and processing protocols that substantially reduce infectious risk, the possibility of pathogen transmission cannot be entirely eliminated. In addition, residual antigenicity may elicit immune responses that impair graft integration. Furthermore, these grafts often exhibit inconsistent biological behavior, with variability in revascularization, osteointegration, and long-term remodeling, which can ultimately affect clinical outcomes [9].

Alternatively, synthetic biomaterials – defined as materials designed for use in the human body – can be utilized. They are available in various forms, ranging from nano-to macro-scales, and have been extensively studied and applied in different skeletal regions to promote bone regeneration and/or replace bone defects [10]. Similar to heterologous grafts, biomaterials have certain disadvantages, such as the potential for residual immunogenicity, foreign body reactions, delayed or incomplete osteointegration, slow or inconsistent resorption. However, they also offer significant advantages, particularly the ability to create customizable designs [11]. This ability to adapt biomaterials to specific clinical needs allows bone regeneration to be optimized by mimicking the structural and functional properties of natural bone.

Among the biomaterials used as bone substitutes, bioactive glasses (BGs) hold a prominent position due to their ability to enhance osteointegration and induce the formation of hydroxyapatite, which is essential for bonding to bone tissue [12]. Bioactive glasses are also used in tissue engineering to construct three-dimensional scaffolds, porous structures designed to replace bone defects, while providing not only temporary mechanical support but also the release of bioactive substances that promote *in situ* tissue regeneration [13]. These scaffolds facilitate colonization by osteoprogenitor cells, actively supporting new bone growth.

The first bioactive glass, 45S5 Bioglass (composition: 46.1 mol% SiO₂, 26.9 mol% CaO, 24.4 mol% Na₂O, 2.6 mol% P₂O₅), created by Prof. Larry Hench in the late 1960s, is the most well-established of the BGs and is widely used in reconstructive and regenerative medicine [14], [15], both in granular and scaffold forms; further, 45S5 releases

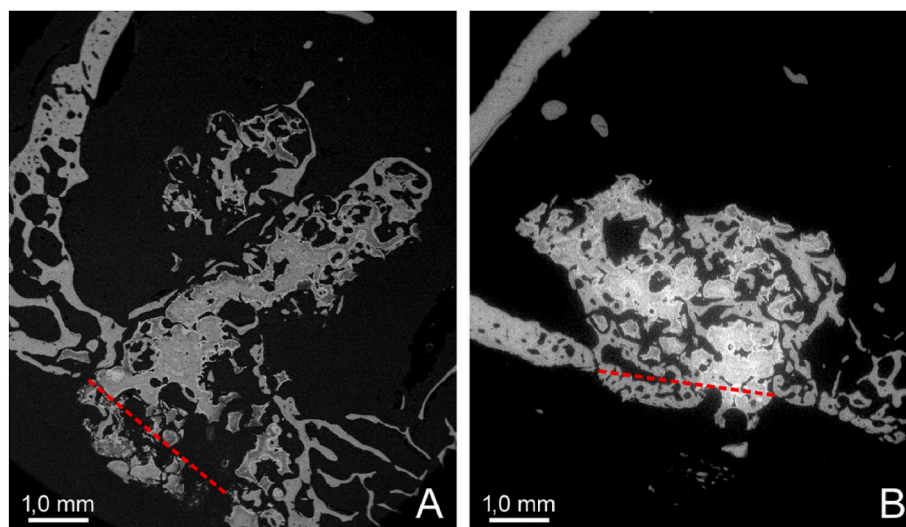


Fig. 1. Representative SEM images of femoral distal epiphysis (A) and tibial proximal diaphysis (B) sections corresponding to the center of the drilled hole (indicated by red dotted line) containing the BG scaffold. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

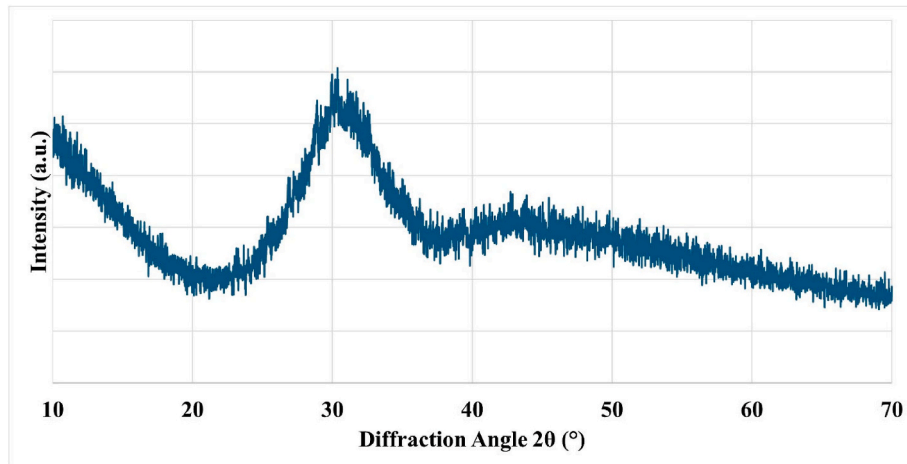


Fig. 2. XRD spectrum obtained on the Bio_MS scaffold.

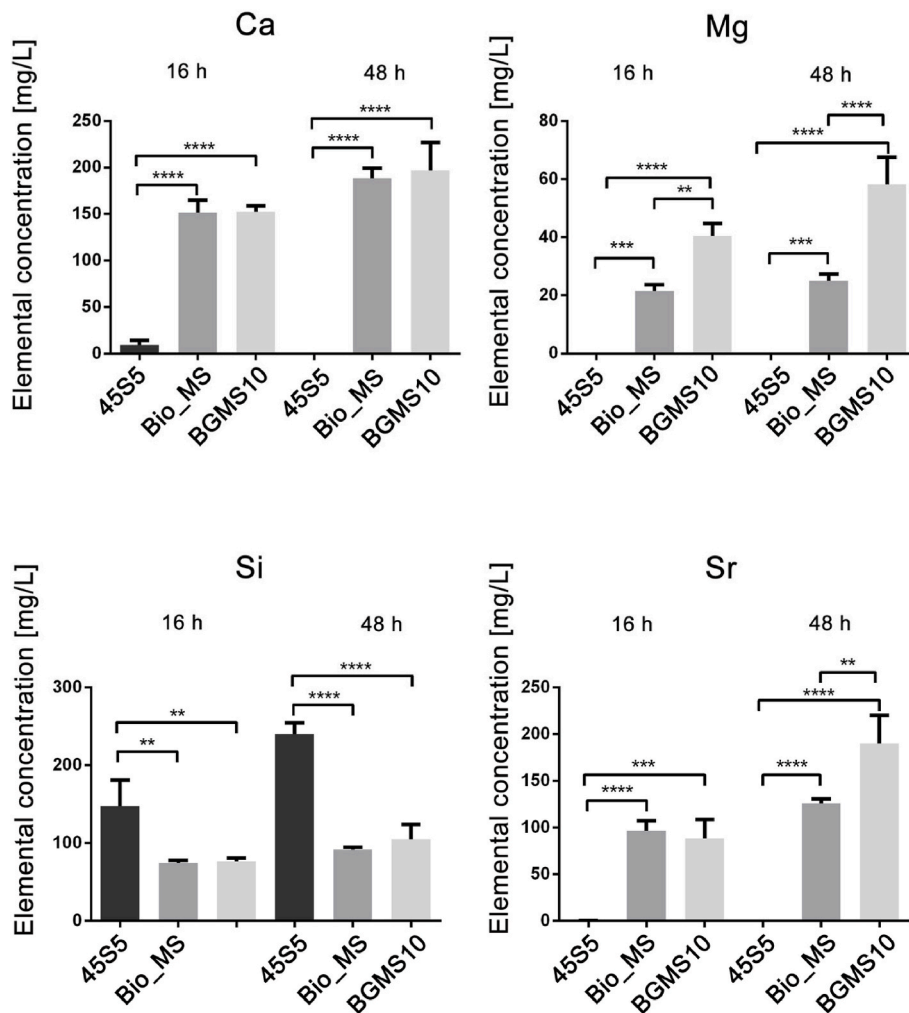


Fig. 3. Assessment of Ca, Mg, Si, and Sr elemental concentrations (mg/L) of inductively coupled plasma (ICP) optical emission spectrometry of the three BGs scaffolds after 16h and 48h.

ions that stimulate cell proliferation and bone matrix deposition [16–19].

Scaffolds made from 45S5 powder can be fabricated using various methods, including foam replication, and additive manufacturing; these methods are selected based on the desired scaffold porosity, post-

production treatments, and cost considerations [20]. Among the most commonly used low-cost protocols, foam replication involves impregnating foam with a mixture of bioactive glass powder, binder, and solvent, then burning it in a furnace at approximately 400–600°C. Higher temperatures are subsequently applied to consolidate the glass particles

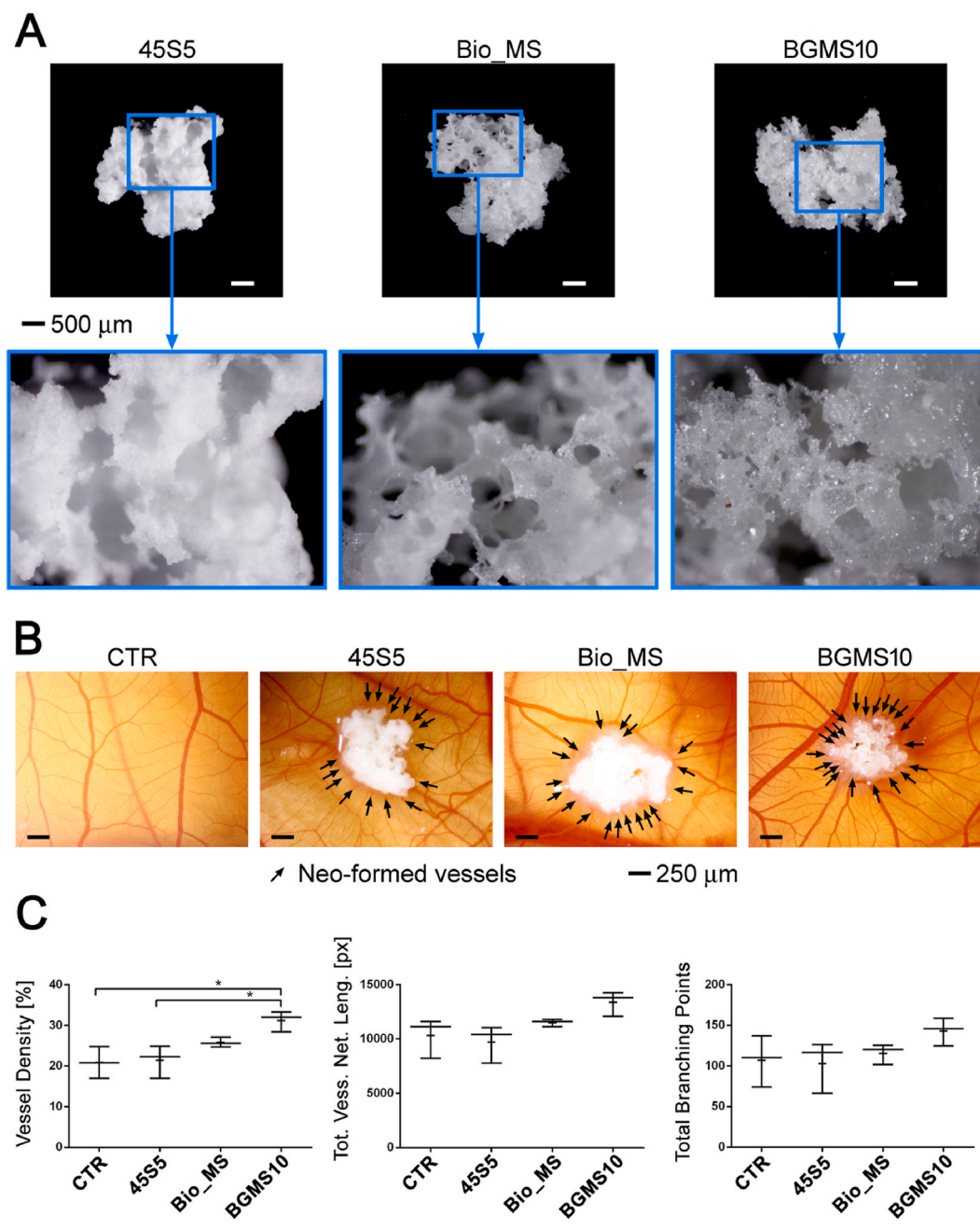


Fig. 4. In (A) the photographs of the three BG scaffolds before grafting (enlarged squares x4). In (B) images taken at ED13, showing the vascular reaction triggered by the biomaterials after 4 days of engraftment with BGs; the black arrows indicate the vessels of neoformation sprouting radially from the BGs. In (C) CAM assay results from WimCAM software to test angiogenesis after the engraftment, *i.e.*, vessel density, total vessel network length and total branching point. Statistical analyses were performed employing one-way ANOVA with Tukey's correction for multiple comparisons (significance: * $p < 0.05$).

through sintering while preserving porosity [13,21].

However, heat treatment during scaffold production, aimed at consolidating and imparting mechanical strength to porous scaffolds, can induce crystallization of bioactive glasses, thereby reducing their bioactivity and, consequently, their clinical efficacy [22–24].

This is particularly problematic for bioactive glasses such as 45S5, which have a high content of alkaline ions like Na_2O , promoting crystallization if cooling is not rapid enough [25]; moreover, prolonged exposure of the bioactive glass to elevated temperatures significantly increases the likelihood of nucleation and crystal growth [16,26,27]. In this context, adjusting the SiO_2 (network former) ratio relative to modifiers such as Na_2O and CaO , or substituting some of these ions with others, such as replacing Na_2O with K_2O and CaO with MgO or SrO , can

refine the glass structure, enhancing its resistance to crystallization during sintering [28–32]. These modifications not only increase thermal stability but also may enhance biological responses; for example, magnesium plays a crucial role in bone metabolism and can stimulate both the differentiation and proliferation of osteoblasts, thus promoting new bone formation [33]. On the other hand, strontium is an important element in bone turnover; in fact, it is known to stimulate bone formation by osteoblasts while simultaneously inhibiting bone resorption by osteoclasts. For this reason, strontium is used as the active ingredient in medications for the treatment of osteoporosis [34,35].

In recent years, two new bioactive glasses containing strontium, magnesium, and a low content of alkaline ions, Bio_MS and BGMS10, have been developed [36–38]. These materials were specifically

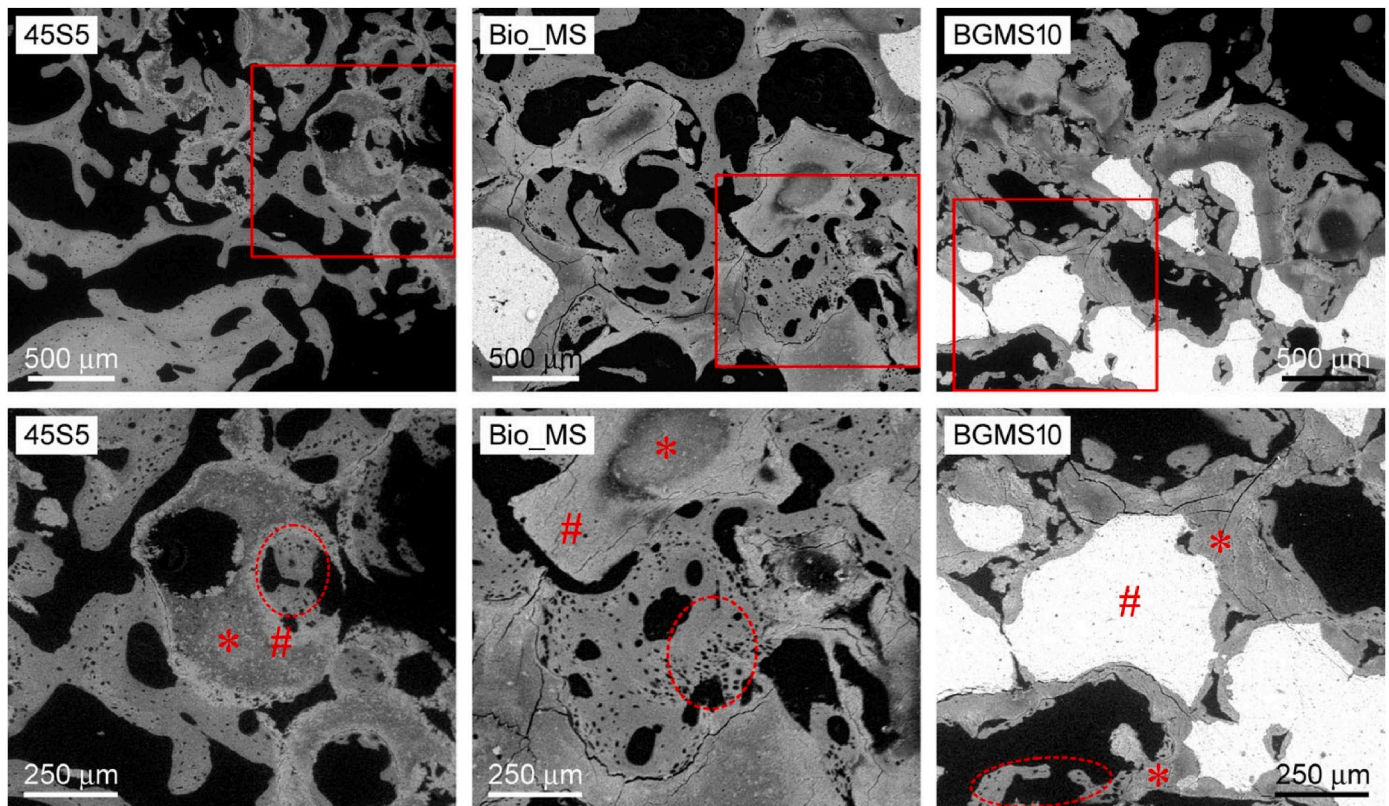


Fig. 5. SEM representative images of the femoral distal epiphyses implanted with the 45S5, Bio_MS, BGMS10 scaffolds. The images below are the enlargements of the squared area above. Note, in each of the three scaffolds, the different morphological aspect with darker (*) and brighter (#) areas. Note also the black osteocyte lacunae (circled) well visible inside the new-formed bone adjacent to the BGs.

designed to combine high thermal stability (allowing for sintering with minimal or no crystallization) with excellent bioactivity and a favorable biological response. Such properties make them ideal candidates for the production of scaffolds for bone tissue engineering. These two glasses in granule form have already been tested for *in vitro* and *in vivo* properties. In particular, the new Sr- and Mg-enriched bioglasses BGMS10 and Bio_MS exhibited high biocompatibility and strong pro-angiogenic activity, effectively promoting cell adhesion, proliferation, which are essential for orchestrating the complex cascade of tissue healing [39]. Moreover, their regenerative potential was assessed by implanting the granules into rabbit femurs, using 45S5 as the gold-standard reference under identical conditions. The novel BG formulations exhibited excellent biocompatibility and osteoconductivity, promoting the formation of uniformly distributed new bony trabeculae, whereas 45S5 resulted in less uniform coarse trabeculae separated by larger soft-tissue areas [40].

It is important to underline that, in addition to biocompatibility and osteoconductivity, a fundamental property for bone tissue engineering applications of biomaterials is their angiogenic potential. In fact, it is currently strongly argued that the effectiveness of a biomaterial depends largely on its ability to induce the formation of new blood vessels, particularly in the case of osteogenesis [41,42].

The purpose of this study is to evaluate the regenerative potential of Bio_MS and BGMS10 scaffolds produced by the foam replica method, compared with 45S5 scaffolds, by investigating, with different techniques, the release of specific ions, the angiogenic response and the osteoconductive potential, inducing the formation of different bone architectures in different skeletal regions with different metabolic and mechanical meaning/implications.

2. Materials and methods

2.1. Production of bioactive glasses and scaffold preparation and characterization

The composition of the glasses used in the present study is reported in Table 1. The glasses were produced by melt-quenching, following a procedure previously described [38]. Briefly, the chemical powders (SiO_2 , $\text{Ca}_3(\text{PO}_4)_2$, Na_2CO_3 , CaCO_3 , K_2CO_3 , SrCO_3 , $\text{Mg}(\text{OH})25\text{H}_2\text{O}$; Carlo Erba Reagenti, Rodano-Milano, Italy) were carefully weighed, mixed for 6 h in a plastic vessel rolling on a shaker and melted in a platinum crucible at 1450°C for 1 h. The molten glass was rapidly cast into water at room temperature to obtain a frit, which was dried for 12 h at 110°C , then ground in a porcelain jar for 40 min and sieved to produce a powder (grain size $< 63 \mu\text{m}$).

To produce scaffolds using the foam replication method, the glass powders were mixed with water and polyvinyl binder to form a slurry, which was stirred for 30 min in a beaker under magnetic agitation. Polyurethane sponges were used as templates to create samples with the desired shape and porosity. The sponges were immersed in the slurry, thoroughly impregnated, removed, and squeezed. This process was repeated five times, after which the sponges were rapidly dried in a stream of hot air.

Finally, the samples underwent thermal treatment to gradually remove the sponge and simultaneously sinter the glass powders. The thermal treatment involved a heating rate of $2^\circ\text{C}/\text{min}$ up to 500°C , followed by $5^\circ\text{C}/\text{min}$ up to the final temperature, which was selected based on prior studies and varied depending on the type of glass (1050°C for 45S5, 750°C for Bio_MS and BGMS10). The samples were held at the final temperature for 3 h, then removed from the oven and allowed to cool to room temperature.

X-ray diffraction (XRD) analysis was performed to evaluate the

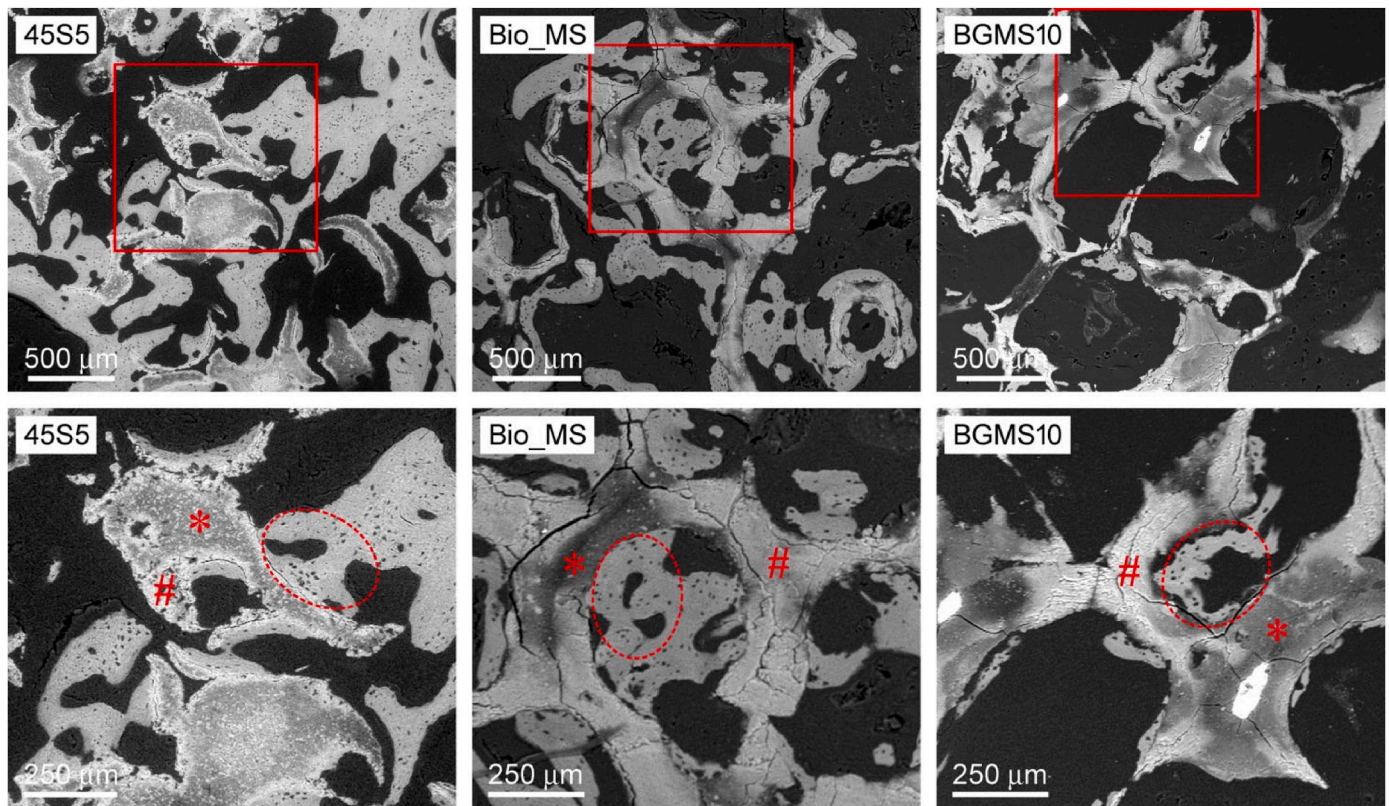


Fig. 6. SEM representative images of the tibial proximal diaphyses implanted with the 45S5, Bio_MS, BGMS10 scaffolds. The images below are the enlargements of the squared area above. Note, in each of the three scaffolds, the different morphological aspect with darker (*) and brighter (#) areas. Note also the black osteocyte lacunae (circled) well visible inside the new-formed bone adjacent to the BGs.

potential formation of crystalline phases within the fabricated scaffolds as a result of the thermal treatment. To this end, the scaffolds were finely powdered and placed onto a sample holder in order to obtain a flat and uniform surface. The measurements were carried out using an X-ray diffractometer (X'Pert PRO, PANalytical, Almelo, The Netherlands) equipped with a Cu K α radiation source ($\lambda = 1.5406 \text{ \AA}$), operating at 40 kV and 40 mA. Diffraction patterns were recorded over a 2θ range from 10° to 70° , with a step size of 0.017° . Data acquisition was conducted in continuous scanning mode using a PIXcel3D detector.

2.2. Ionic release

The ionic release from the three scaffolds immersed in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% Fetal Bovine Serum (FBS) (Sigma Aldrich, Darmstadt, Germany), was evaluated at 16 and 48 h using inductively coupled plasma optical emission spectrometry (ICP-OES). The analysis was performed with an ICP-OES 5100 – vertical dual view instrument (Agilent Technologies, Santa Clara, CA, USA), equipped with a OneNeb nebulizer and an autosampler. Argon was used as the internal standard, with a reference wavelength of 420 nm. Calibration of the measurements was carried out using calibration curves with a correlation coefficient exceeding 0.999. A linear fit was applied to the calibration, including a blank sample. The precision of the measurements, expressed as the relative standard deviation (RSD) percentage, was always below 5%. The detection limit (LOD) at the operating wavelength was 0.01 mg L^{-1} for Sr, 0.1 mg L^{-1} for Si, and 1 mg L^{-1} for Ca and Mg. In selecting the analytical wavelength for each element, both signal intensity and potential interferences with the medium were considered. Prior to analysis, the samples underwent acid digestion by adding 10% v/v phosphoric acid (H_3PO_4 96%) and 10% v/v hydrogen peroxide (H_2O_2 30%). Calibration curves were generated within the

range of $0.01 - 100.00 \text{ } \mu\text{g/mL}$, using standards prepared in DMEM + 10% FBS and applying the same digestion procedure as used for the samples.

2.3. CAM assay

Fertilized Isa Brown chicken eggs (sourced from Società Agricola Vinifera S.S., Forlì, Italy) were gathered post-fertilization and placed in an incubator (FIEM Mercurius MG244, Como, Italy) under controlled conditions of 48% humidity and a temperature of 37.8°C . Following our established protocol, after three days of incubation and embryonic development (ED3), classified according to Hamburger and Hamilton's system, 3 mL of albumen were extracted from one end of the eggshell to facilitate the detachment of the developing CAM from the shell membrane [43]. Subsequently a small opening was created in the eggshell to assess embryo viability before being sealed with $18 \times 18 \text{ mm}^2$ coverslips and paraffin. At ED9, bioactive glass scaffolds (45S5, Bio_MS and BGMS10), which had been sterilized under UV light, were implanted onto the CAMs. Eggs that were either opened and left intact without grafted materials served as control groups. For each experimental condition, five eggs were used, with a minimum of three independent replicates performed. On the fourth day of incubation (ED13), the eggs were opened, and images of the CAMs were captured *in ovo* using a Nikon SMZ745T stereomicroscope (Nikon Instruments Europe BV, Amstelveen, The Netherlands) equipped with a digital camera and X-Entry software (both developed by Alexasoft, Florence, Italy). Photographs taken at ED13 were analyzed to observe vessel formation around the BG scaffolds, with angiogenic responses assessed by comparing grafted CAMs to control samples at 1X magnification. A consistent circular region surrounding the implanted materials was selected for analysis. To further assess angiogenesis, WimCAM, a dedicated WIMASIS Image Software for CAM assays, was utilized. This

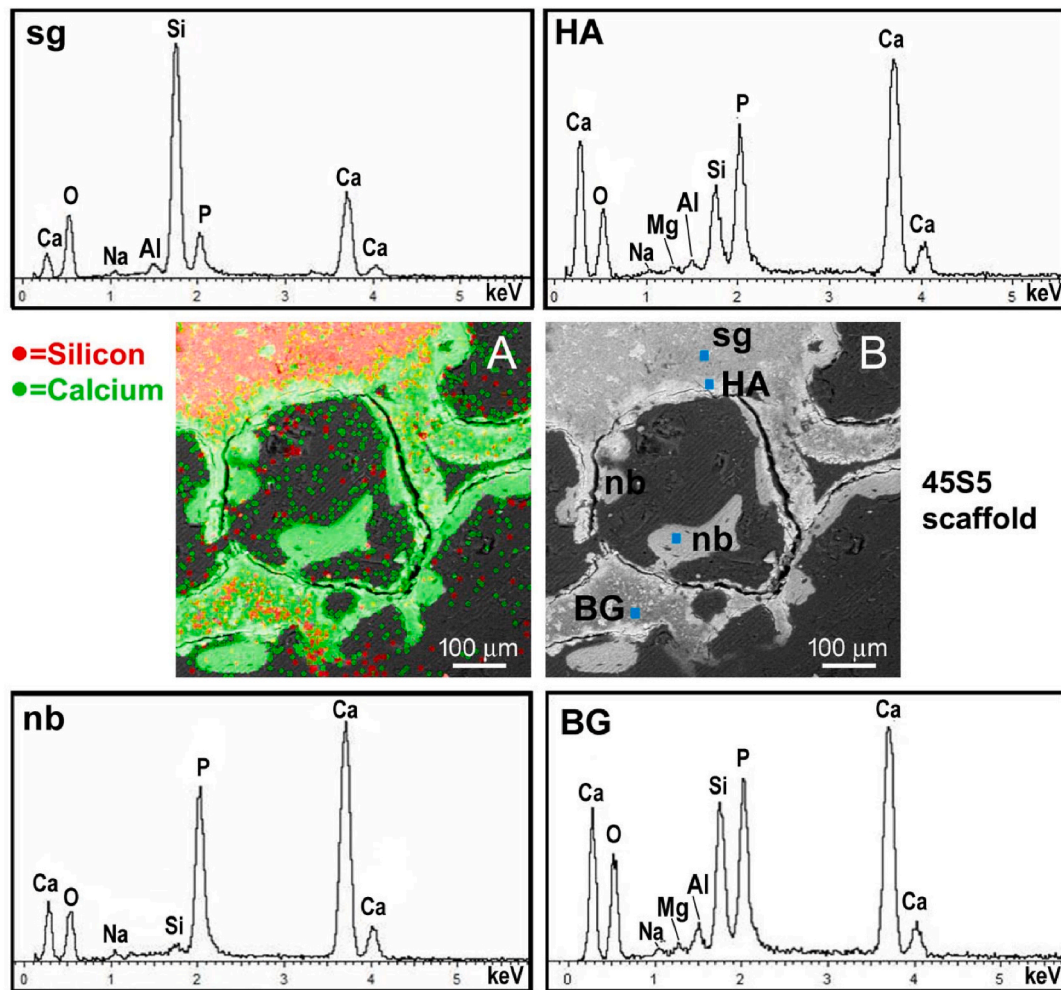


Fig. 7. 45S5 scaffold: representative SEM micrographs and corresponding X-ray microanalysis results. Specifically, the X-EDS map (A) shows the distribution of Silicon, labeling both glass and silica gel (sg), and Calcium labeling hydroxyapatite (HA) (or calcium phosphate-rich phase) and newly-formed bone (nb). The SEM micrograph (B) shows the morphology of the implanted scaffold with the labelled regions: BG, bioactive glass; sg, silica gel; HA, hydroxyapatite; nb, newly-formed bone. Panels show the X-EDS spectra acquired at selected sites indicated by the label (nb, HA, sg, BG) which confirm the elemental composition of these regions. The blue squares denote the EDS analysis locations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

software examines the newly formed vessels radiating from the implanted bioactive glasses by measuring key parameters such as vessel density, total network length, and the number of branching points.

2.4. Animal model and surgery

Two implantation sites were used in each animal leg. According to Kanazawa et al., both the epiphysis of the distal femur and the metaphysis of the proximal tibia were selected because they provide sufficient bone volume [44]. Nine healthy New Zealand White rabbits (Harlan Laboratories S.r.l., Correzzana, Monza, and Brianza, Italy), each weighing between 3.5 and 4.5 kg, were used in this study. All procedures were approved by the Bioethical Committee of the Italian National Institute of Health and authorized by the Italian Ministry of Health (Protocol No. 210/2013-B). Prior to surgery, animals were acclimatized and had free access to food and water. On the day of surgery, animals were weighed and anesthetized using an intramuscular injection of xylazine (4 mg/kg; Sedaxylan®, Dechra Veterinary Products S.r.l., Turin, Italy) and ketamine (30 mg/kg; Imalgene 1000®, Merial Italia S.p.A., Milan, Italy). After induction, the surgical sites (hind limbs) were shaved and disinfected. Cylindrical bone defects, 4 mm in diameter, were created using a Piezosurgery® device equipped with the IM4A

insert tip (Mectron, Carasco, Genova, Italy). Each animal received four defects: two in the distal femoral epiphysis (one per femur, 6 mm deep) and two in the proximal tibial diaphysis (one per tibia, 4 mm deep).

Scaffolds were implanted into the defects according to the experimental design outlined in Table 2. Following implantation, surgical incisions were closed in layers using 4-0 glycolide/L-lactide copolymer sutures (Vicryl®, Ethicon, Johnson & Johnson S.p.A., Pomezia, Rome, Italy) for deep tissues, and 3-0 silk sutures (Perma-hand® Silk Suture, Ethicon, Johnson & Johnson S.p.A., Pomezia, Rome, Italy) for skin closure. Iodine solution was applied to the surgical sites postoperatively to prevent infection. Postoperative care included a three-day course of intramuscular antibiotics (enrofloxacin, 10 mg/kg; Baytril® 5%, Bayer S.p.A., Milan, Italy) and analgesics (buprenorphine, 0.05 mg/kg; Temgesic®, Indivior Italia S.r.l., Milan, Italy) for pain management. Animals were housed individually, received veterinary care, and were provided with nutritional support throughout the 60-day follow-up period.

At the end of the study, animals were euthanized under general anesthesia (xylazine, 4 mg/kg, and ketamine, 30 mg/kg) using an intravenous injection of embutramide/mebezonium iodide (0.3 mL/kg; Tanax®, MSD Animal Health S.r.l., Segrate, Milan, Italy). The femurs and tibias from both hind limbs were harvested and fixed in 10% neutral buffered formalin (Bio Optica Milano S.p.A., Milan, Italy) for five days.

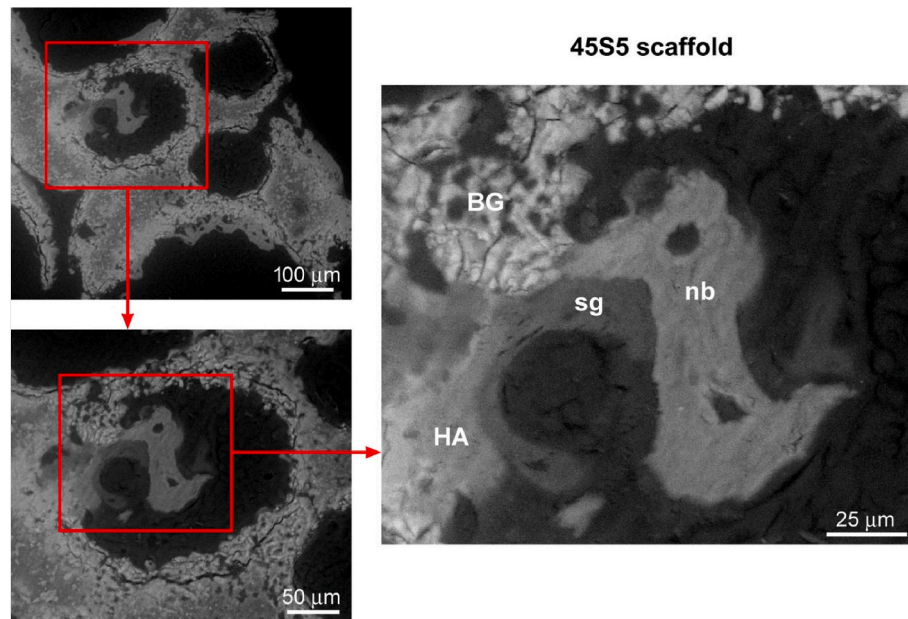


Fig. 8. 45S5 scaffold: SEM micrographs showing the morphology of the scaffold with pores within which newly-formed bone can be observed. Labelled regions: BG, bioactive glass; sg, silica gel; HA, hydroxyapatite; nb, newly-formed bone. The arrows indicate the enlargements of red squared areas. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

It should be noted from the outset that not all samples have been suitable for the histomorphometric and histological analyses described below, as in some cases the scaffold detached and/or the embedding procedure encountered complications.

On *ex vivo* samples, SEM observations, X-EDS analysis, histomorphometric and histological evaluations were performed.

2.5. SEM, X-EDS and histomorphometric analyses

After fixation, the specimens were dehydrated in an ascending series of ethanol and embedded in polymethylmethacrylate. Serial sections (300 µm thick) were obtained perpendicularly to the longitudinal axis of both femoral distal epiphyses and tibial proximal diaphyses, with a Leica SP 1600 cutting system (Leica SpA, Milan, Italy). For each specimen, the section corresponding to the center of the drilled hole containing the BG scaffold was selected under optical microscope (Eclipse Ni-E, Nikon) and sputter-coated with an 8–10 nm gold–palladium layer (Emitech K550, Emitech Ltd, Ashford, Kent, UK) at 25 mA for 60 s for Scanning Electron Microscope (SEM) analysis. The selected sections were observed with a SEM-QUANTA 200 equipped with an X-EDS detector (SEM-QUANTA 200, FEI Company, the Netherlands) under low vacuum, using the backscatter mode and saved (Fig. 1). Local compositional analysis was performed with energy dispersion X-ray spectroscopy (X-EDS, Inca, Oxford Instruments, UK).

A collage image using the 100X magnification was obtained by means of the ImageJ software to perform histomorphometry. To calculate the affinity index - AI (defined as the perimeter of bone in contact with the scaffold over the scaffold total perimeter), the scaffold perimeter was manually outlined with the freehand line function of ImageJ as well as the bone perimeter in contact with the scaffold.

2.6. Light microscopy histological analysis

Histological analysis was performed under Light Microscope—LM (Eclipse Ni-E, Nikon): the same section or one adjacent to that used for SEM analysis (in case this was damaged during stab removal) was glued to a microscope slide, thinned with fine grain emery papers, and polished with alumina. A superficial staining was performed with an alcoholic solution of toluidine blue (2 min at 60°C), then rinsed with tap

water; a few drops of HCl 0.1 N solution were added on top of the section for 30 s and rinsed; finally, a few drops of an alcoholic solution of basic fuchsin were added for 30 s and rinsed.

2.7. Statistical analysis

A power analysis was conducted to determine the sample size, using Cohen's *f* as the effect size measure. A *post hoc* estimation of statistical power based on the total number of analyzed defects ($N = 36$) suggests moderate to high power (approximately 0.75–0.95), assuming independent observations. The number of animals was determined in accordance with the 3R principles (Reduction, Replacement, Refinement), which advocate minimizing animal use while preserving scientific validity. As emphasized by Moran et al. [45], preclinical *in vivo* study designs should be optimized to reduce animal numbers without compromising data quality. In this context, the use of multiple defects per animal represents a well-established and widely accepted strategy in bone regeneration models.

Implementing multiple defects within the same animal enables intra-individual comparisons, thereby reducing inter-subject variability and enhancing experimental efficiency. Previous studies have demonstrated that such approaches can substantially decrease the number of animals required while maintaining statistical robustness. This adopted experimental design is consistent with commonly used preclinical approaches in bone regeneration research and allows an appropriate balance between ethical considerations and methodological rigor [45,46].

Data obtained from the analysis of angiogenic responses are presented as mean values $\pm$ standard deviation (SD). To determine statistical significance among the different BG scaffolds, a one-way ANOVA was performed, followed by Tukey's post-hoc test for multiple comparisons. These statistical analyses and graphical representations were generated using GraphPad Prism 5 (version 5.00, San Diego, USA).

Histomorphometric data were compared among groups using the ANOVA test, followed by Bonferroni post hoc analysis. The Student's *t*-test was used to compare the AI results for each BG scaffold type between femur and tibia. All values were expressed as mean $\pm$ standard deviation. Statistical analyses were performed using Stata 11 software (StataCorp LLC, College Station, TX).

The specific *p*-values indicating statistical significance (**p* < 0.05,

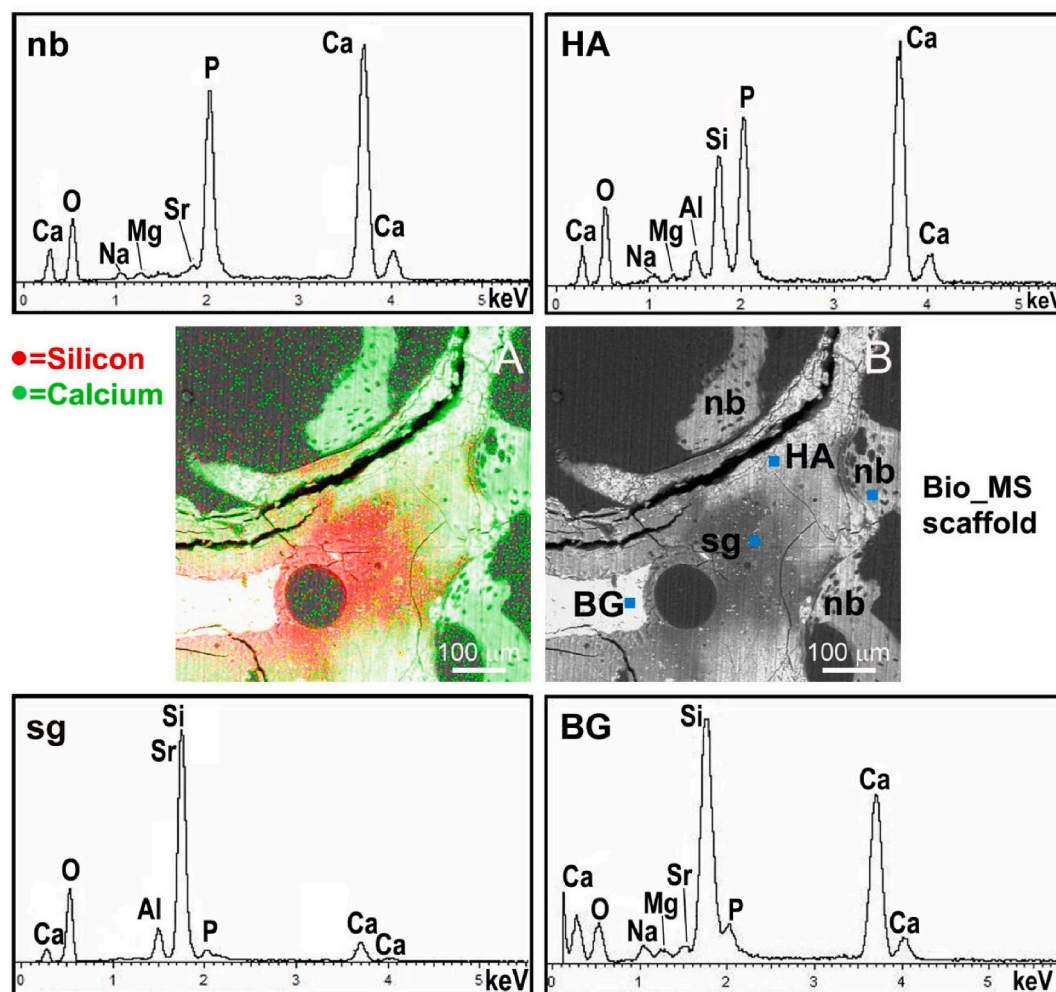


Fig. 9. Bio_MS scaffold: representative SEM micrographs and corresponding X-ray microanalysis results. In particular, the X-EDS map (A) shows the distribution of Silicon labelling both glass and silica gel (sg), and Calcium labelling of hydroxyapatite (HA) (or a calcium phosphate-rich phase) and newly-formed bone (nb). The SEM micrograph (B) shows the morphology of the implanted scaffold with the labelled regions: BG, bioactive glass; sg, silica gel; HA, hydroxyapatite; nb, newly-formed bone. Panels show the X-EDS spectra acquired at selected sites indicated by the label (nb, HA, sg, BG) which confirm the elemental composition of these regions. The blue squares denote the EDS analysis locations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

p < 0.01, *p < 0.001) are provided in the corresponding figure legends.

3. Results

3.1. XRD analysis of scaffolds

The fabricated scaffolds were ground, and the potential formation of crystalline phases induced by the thermal treatment was investigated by XRD. The degree of crystallinity of the glass is known to influence its ionic release and, more broadly, the biological response of the material.

In the case of the 45S5 scaffolds, the presence of the crystalline phase $\text{Na}_4\text{Ca}_4\text{Si}_6\text{O}_{18}$ was detected, in agreement with previous reports in the literature [30]. By contrast, the BGMS10 and BioMS scaffolds were found to be predominantly amorphous. For illustrative purposes, the XRD pattern of the BioMS scaffold is presented in Fig. 2.

3.2. Ionic release

The ion release patterns of 45S5, Bio_MS and BGMS10 scaffolds were analyzed following incubation of the glasses in DMEM supplemented with 10% FBS for 16 and 48 h to compare the differences among the

three scaffolds. When evaluating their release profiles in direct culture conditions, the calcium ion release exhibited a similar trend in both Bio_MS and BGMS10 scaffolds, showing a time-dependent increase and a significant release compared to the 45S5 scaffold (Fig. 3). The release of Mg, Si, and Sr ions from Bio_MS and BGMS10 scaffolds was also evaluated, revealing a significantly higher and time-dependent magnesium ion release (which is also expected, given the higher content) in BGMS10 compared to Bio_MS. In contrast, the silicon ion release levels were comparable between Bio_MS and BGMS10 scaffolds, but significantly lower than 45S5 scaffold release (Fig. 3). Finally, the release of strontium ions was comparable in Bio_MS and BGMS10 scaffolds at 16 h, while after 48 h, BGMS10 scaffold released significantly more ions than Bio_MS scaffold (Fig. 3).

3.3. CAM assay

To evaluate the angiogenic ability of BG scaffolds in *in vivo*-like settings, we utilized the Chorioallantoic Membrane (CAM) assay, an ethical model that mimics physiological conditions, indicated as “*in ovo*” model. At embryonic day 9 (ED9), the BG scaffolds (Fig. 4A) were implanted onto the CAMs. As controls, we included eggs opened but did not receive any grafted material. After four days (ED13), the vascular

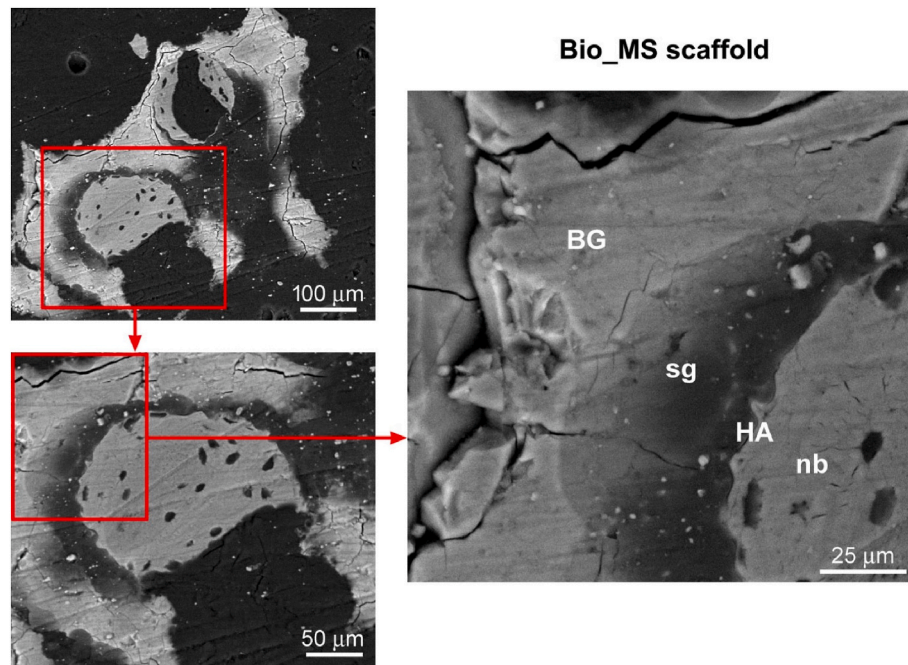


Fig. 10. Bio_MS scaffold: SEM micrographs showing a scaffold region containing a rounded islet of newly-formed bone. Labelled regions: BG, bioactive glass; sg, silica gel; HA, hydroxyapatite; nb, newly-formed bone. The arrows indicate the enlargements of red squared areas. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

reaction induced by the BG scaffolds displayed newly formed capillaries spreading radially from the BGs in an irregular pattern (Fig. 4B). Additionally, a significant number of these vessels exhibited dichotomous branching, a hallmark of a pronounced angiogenic response, across all analyzed BG scaffolds. Quantitative analysis of angiogenesis demonstrated that all BG scaffolds exhibited a significantly greater pro-angiogenic effect than the control CAMs, as evidenced by increased vessel density and total vessel network length (Fig. 4C), where the vessel density quantifies the proportion of tubular formations within the entire image, expressed as a percentage, and the total vessel network length represents the cumulative length of the tubular structures measured in pixels. Moreover, among the three scaffolds, BGMS10 showed a significantly higher vessel density and total vessel network length compared to 45S5; no significant differences between BGMS10 and Bio_MS scaffolds were observed.

3.4. SEM, X-EDS and histomorphometric analyses

Figs. 5 and 6 show SEM representative images of the femoral distal epiphyses and tibial proximal diaphyses for each of the three BG scaffolds (45S5, Bio_MS, BGMS10) tested in this study. At low magnification, it is possible to observe the various morphological aspects of the three BGs, with darker and brighter areas corresponding to differences in chemical composition and phases, as reported in the following X-EDS results (related to Figs. 7–12).

To evaluate scaffold/bone interactions, SEM analyses (including X-EDS spectra and elemental maps) were performed. The results are shown in Figs. 7–12: SEM observations of variations in grayscale brightness allowed the identification of the implantation area and bioactive glass residues (BG), hydrated silica gel (sg), hydroxyapatite (HA), and new bone (nb) [47].

After 60 days, the cross-section of the 45S5 scaffold implant (Fig. 7) revealed the presence of residual BG, which appeared bright and exhibited a porous structure. In accordance with the known bioactivity mechanism of BGs [48], a dissolution process was observed: hydrated silica (sg), together with calcium- and phosphorus-rich precipitates (from which hydroxyapatite would eventually form, according to the

model proposed in the literature to explain the *in vitro* bioactivity of BGs), appeared as a pale gray shade, while hydroxyapatite exhibited a lighter color adjacent to sg. However, accurately identifying the different regions remains challenging. Fig. 7 also shows that new bone (nb) has grown close to the scaffold; this bone is characterized by numerous osteocyte lacunae. To confirm these observations, an elemental map (Fig. 7A) was obtained to visualize the chemical elements present in the bone/implant area. The maps showed calcium (green) concentrated in the regions of new bone formation and hydroxyapatite, while silica (red) was present in the hydrated silica (sg) and residual BG scaffold areas. Furthermore, X-EDS analyses were performed to determine the composition of each observed structure (Fig. 7).

To investigate scaffold integration within the treated bone, SEM images at different magnifications were observed (Fig. 8). Specifically, the three fields of Fig. 8 clearly show the newly-formed bone (nb) in contact with the residual BG scaffold that adapts to the morphology of the pores and, at higher magnifications, the irregular surface and margins of the residual BG structure, as well as the presence of newly-formed bone as a result of the bioactivity and osteoconductive properties of the 45S5 bioglass. In broad terms, the various phases of the BG/bone integration can be identified by the presence of hydroxyapatite precipitates on the bioactive glass surface, which initially appear as white aggregates as observed by SEM. These aggregates gradually increase in size until they form an almost continuous crystalline layer similar to biological hydroxyapatite, which is essential for scaffold attachment to the bone. Subsequently, the scaffold supports the growth of newly-formed bone.

Figs. 9 and 10 present the results of the Bio_MS scaffold. Like the 45S5 scaffold, Bio_MS exhibits pores with similar circular morphology. The bone/scaffold interface is highlighted in Fig. 9. Figs. 9B and 10 show in detail the different phases of the Bio_MS bioactivity process, characterized by the formation of silica gel (sg), which locally appears cracked in accordance with several studies reported in the literature [38], and hydroxyapatite subsequently precipitates on the sg layer. In principle, the chemical reactions leading to the formation of HA on Bio_MS, as well as those occurring for BGMS10, are analogous to those of 45S5; the main difference lies in timing [30,38].

As shown in Fig. 10, new bone grows within the pores, starting from

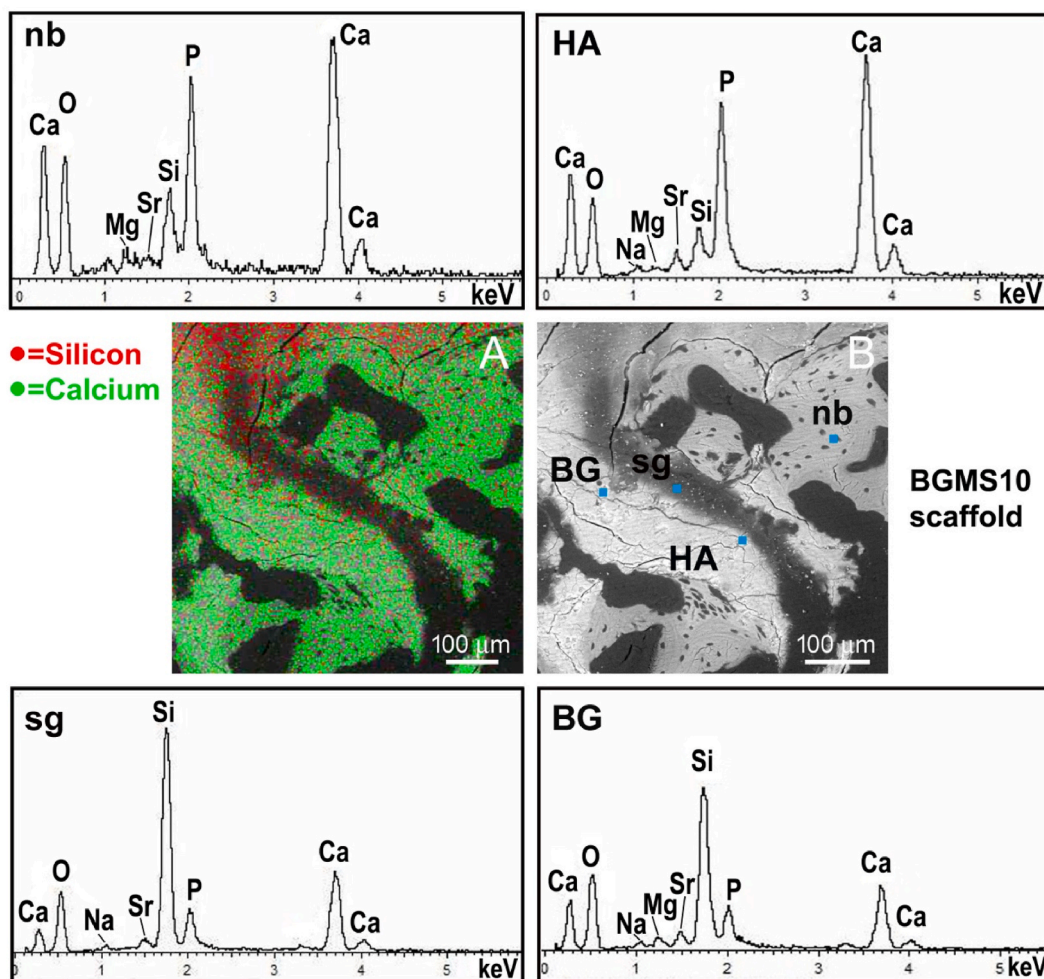


Fig. 11. BGMS10 scaffold: representative SEM micrographs and corresponding X-ray microanalysis results. In detail, the X-EDS map (A) shows the distribution of Silicon, labeling both glass and silica gel (sg), and Calcium labeling hydroxyapatite (HA) (or calcium phosphate-rich phase) and newly-formed bone (nb). The SEM micrograph (B) shows the morphology of the implanted scaffold with the labelled regions: BG, bioactive glass; sg, silica gel; HA, hydroxyapatite; nb, newly-formed bone. Panels show the X-EDS spectra acquired at selected sites indicated by the label (nb, HA, sg, BG), which confirm the elemental composition of these regions. The blue squares denote the EDS analysis locations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the borders of the scaffold, indicating that colonization by mesenchymal stem cells and osteoblasts has supported osteogenic activity. The newly formed hydroxyapatite layer on the surface of the BG, with a composition similar to biological apatite, is therefore functional to provide a substrate for cell adhesion. The numerous bony lacunae observed indicate an active osteogenic phase. Also in this case, the chemical analysis performed via X-EDS, together with the morphological features observable by SEM (see Figs. 9 and 10), allows the identification of silica gel, which adheres to the residual bioglass (clearly visible as whitish in the backscattered electron mode) and to the neo-formed layer of hydroxyapatite. The HA layer, lighter in color than the sg, is also covered by newly-formed bone, and the different regions lack sharp boundaries. On the other hand, HA and newly-formed bone are more easily distinguishable from each other by the morphological viewpoint than by X-EDS analysis about their respective composition. Additionally, these observations are in line with literature data about variations in BSE images, where brighter areas indicate mature highly mineralized bone and darker areas represent less mineralized newly-formed bone [49]. In regions close to the residual BG, traces of strontium and magnesium released from the glass during its dissolution are also detectable. In this regard, the presence of strontium can be beneficial, as it stimulates osteoblasts and inhibits osteoclasts, thereby enhancing osteogenesis [50, 51]. Thus, these data seem to confirm the bioactive and osteoconductive

properties of Bio_MS.

With regard to the BGMS10 scaffold, the porous morphology of the original sponge (Figs. 11 and 12) and the growth of newly-formed bone following the scaffold architecture can be observed. The X-EDS spectra shown in Fig. 11 allow a general identification of the different regions corresponding to the implanted material, HA, and newly-formed bone. However, as seen in Fig. 11, these regions do not exhibit clearly defined boundaries. Their identification therefore also relied on microanalysis, particularly to distinguish between HA and nb, which displayed a similar elemental composition. SEM images at different magnifications (Fig. 12) provided detailed views of the scaffold area and the newly-formed bone integrated into the scaffold architecture.

It has to be underlined that, after 60 days of implantation, both Bio_MS and BGMS10 scaffolds demonstrated significant osteogenic potential, likely due to their sustained release of strontium and magnesium, ions with well-documented biological effects. Among the two experimental glasses, Bio_MS appears to exhibit slightly better biological performance, likely due to its higher calcium content. However, further studies are needed to confirm this point.

The histomorphometric analyses were performed on the SEM images in order to evaluate the Affinity Index (AI), as reported in Table 3 that shows average values measured in the femoral distal epiphyses and tibial proximal diaphyses for each of the three BG scaffolds. All values

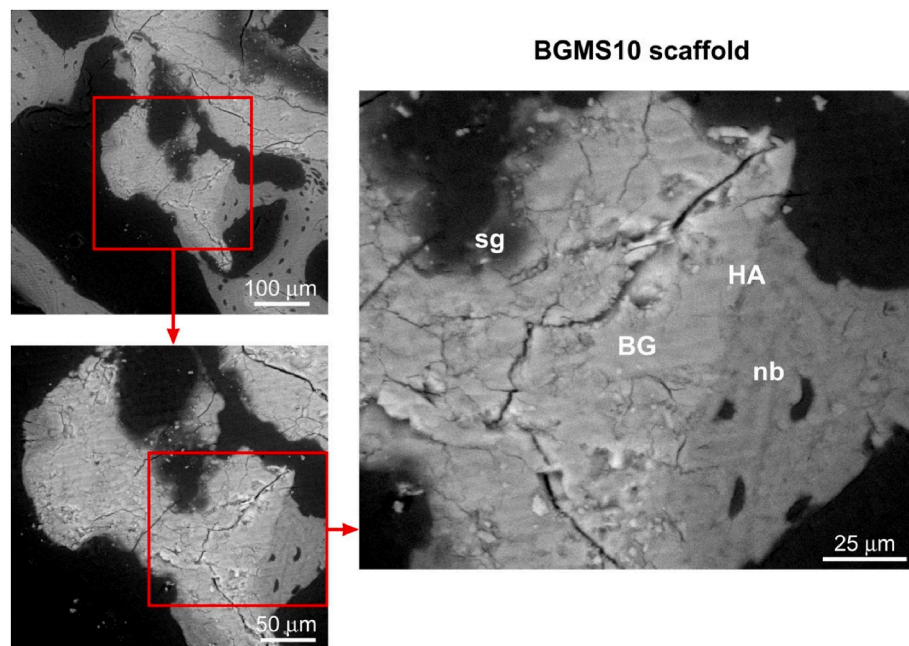


Fig. 12. BGMS10 scaffold: SEM micrographs showing the morphology of the scaffold with newly-formed bone visible. Labelled regions: BG, bioactive glass; sg, silica gel; HA, hydroxyapatite; nb, newly-formed bone. The arrows indicate the enlargements of red squared areas. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3

Affinity Index measured in femoral distal epiphyses and tibial proximal diaphyses.

	45S5	Bio_MS	BGMS10
Femur	25.3 ± 9.9 (n = 4)	34.0 ± 4.8* (n = 4)	15.4 ± 3.6 (n = 5)
Tibia	21.3 ± 8.3 (n = 5)	28.7 ± 10.1 (n = 4)	18.2 ± 7.2 (n = 5)

*p < 0.05 versus BGMS10 Anova test. All values are expressed as mean ± SD.

are higher in the Bio_MS group both in femurs and tibiae and particularly significant with respect to the BGMS10 one in the femurs. No significant differences were observed between femur and tibia for each BG scaffold type.

3.5. Light microscopy histological analysis

From a histological point of view, both at the femoral and tibial level, each BG scaffold shows not-uniform morphology and stainability that reflect the presence of different phases, as shown by the SEM and X-EDS analyses (Fig. 13).

In all samples analyzed, the surface of each BG scaffold appears surrounded by connective tissue, osteoclasts, osteoblasts and newly-formed bone (Fig. 14). A quantitative analysis has not been performed due to the presence of many bubbles that did not allow a clear observation of the entire sections. Nonetheless, as qualitative evaluation, the presence of osteoclasts is more frequently detectable in the BGMS10 samples compared to the 45S5 and Bio_MS ones (in the latter, in particular, they are rarely observable), while it is possible to observe

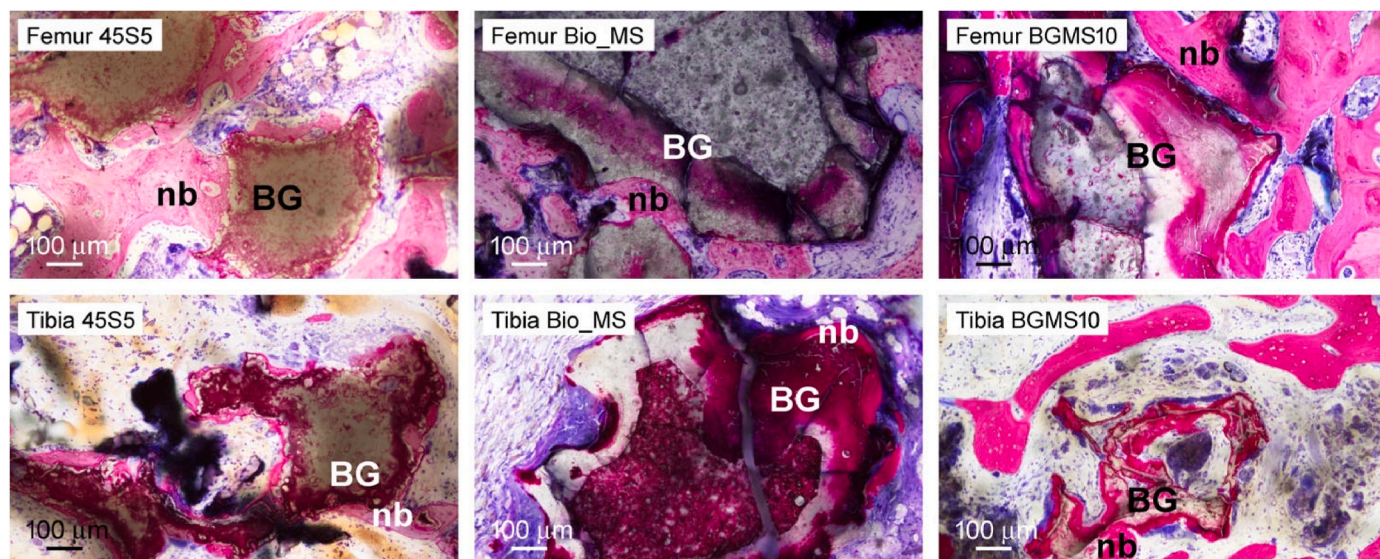


Fig. 13. LM representative images of the 45S5, Bio_MS and BGMS10 scaffolds (BG) both at the femoral and tibial level. Each BG is in contact with the newly-formed bone (nb) stained in pink. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

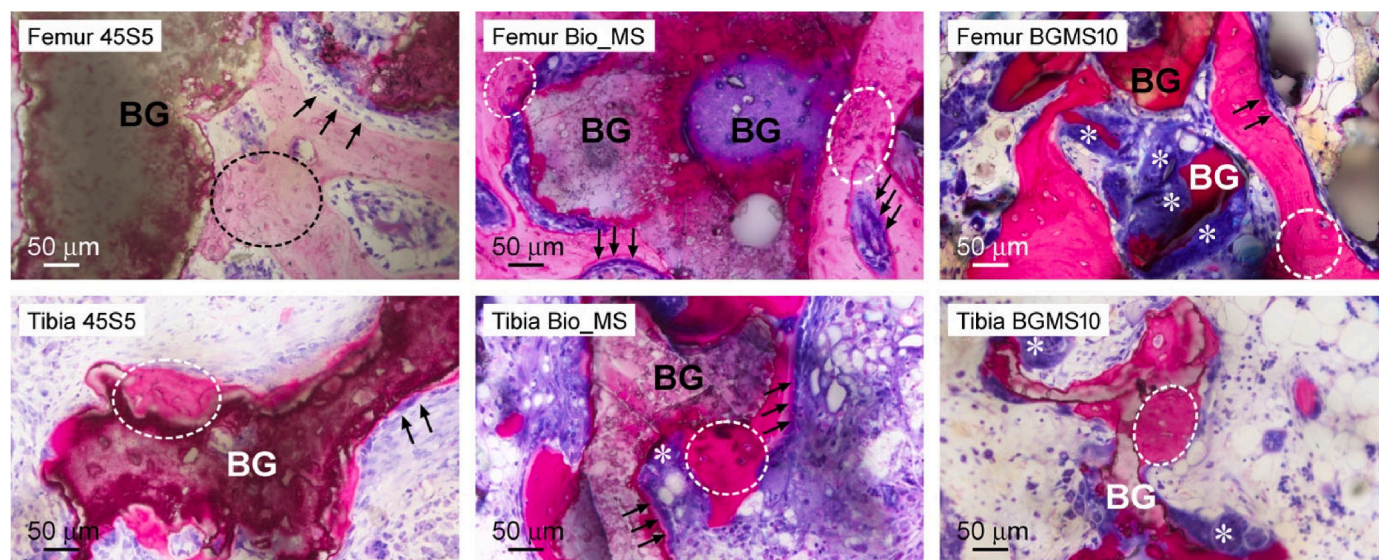


Fig. 14. LM representative micrographs of the 45S5, Bio_MS and BGMS10 scaffolds (BG) both at the femoral and tibial level showing the morphological features of the examined sections. Note the primary bone trabeculae with osteocyte lacunae (circles), osteoblast laminae lining the newly-formed bone (arrows) and osteoclasts (asterisks). Note, also, the absence of cementing lines.

numerous osteoblast laminae lining the newly-formed bone, mostly in the Bio_MS samples compared to the other BG scaffolds. The new-formed bone, with high cellularity, is primary bone; in fact, it does not show cementing lines, a sign of absence of bone remodeling.

4. Discussion

This work aimed to evaluate the regenerative potential of Bio_MS and BGMS10 bioglasses in the form of scaffolds, in terms of angiogenic ability, ionic release and bone regeneration of bone defects, compared to the gold standard bioglass (45S5).

First of all, it should be emphasized that any material/device used for regenerative purposes must have the ability to improve angiogenesis, particularly for all those highly vascularized tissues such as bone. In this regard, in the present work the CAM model was used to evaluate *in ovo* the scaffold's ability in improving the angiogenesis, an essential prerequisite for osteogenesis to occur. Primarily, just four days after implantation on the vascular membrane of CAM, all BG scaffolds showed good ability in stimulating the angiogenic effect, that is demonstrated by the vascular reaction in incrementing the vessel density and the total amount of vessel network. In particular, the two new bioactive glasses (Bio_MS and BGMS10) showed a positive trend with respect to the 45S5 scaffold. On this basis we concluded that the BGs in the form of scaffold can be suitable for a regenerative approach to bone recovery.

As far as BGs regenerative ability is concerned, in a previous study of ours, Bio_MS and BGMS10 in form of granules, produced from melt-quenched powders, were implanted in rabbits for short-term (30 days) and long-term (60 days) evaluation [40]. Based on these findings, we concluded that these bioactive glasses could be promising candidates for bone tissue regeneration and the development of products such as scaffolds for biomedical applications. Therefore, in the present study, we fabricated scaffolds using Bio_MS and BGMS10 powders via the foam replica method and implanted them in rabbit bone, choosing an experimental period of 60 days, on the basis of data from other authors [13]; these authors observed that at 30 days from implantation the bone regeneration is at onset, observing cell attachment, formation of a silica-rich gel layer and the beginning of hydroxyapatite precipitation, indicative of early mineralization and preliminary bone bonding.

In the present work, the produced scaffolds were implanted in both femur and tibia to evaluate their long-term effects; at 60 days after implantation, good evidence of osseointegration and, when present,

significant scaffold degradation emerged. This behavior was observed using scanning electron microscopy and X-EDS spectroscopy. Regarding 45S5 scaffolds, SEM analysis confirmed the well-known bioactivity mechanism, in line with the literature, that is glass network dissolution followed by the formation of a silica-rich gel layer and hydroxyapatite nucleation [52]. It is worth noting that the 45S5 scaffolds are partially crystallized due to the sintering temperature (1050°C); on the other hand, with the other two glasses, it was possible to obtain completely amorphous scaffolds [36,38]. The differential dissolution between the residual glassy phase and the crystalline counterpart in 45S5 scaffolds may result in a relatively thinner and more porous scaffold residue, which may compromise the long-term stability of the scaffold architecture and limit the mechanical support necessary for bone regeneration. Indeed, the 45S5 scaffolds differ from those made of BGMS10 and Bio_MS not only in terms of composition but also in crystallinity, which has a direct impact on the material dissolution. In this respect, although 45S5 is intrinsically slightly more reactive than the other two glasses, it becomes partially crystallized as a result of the applied heat treatment [30,53]. Consequently, in 45S5 scaffolds, a rapid initial ion release is expected - corresponding to the dissolution of the amorphous phase - followed by the persistence of a crystalline phase that is likely to dissolve more slowly than Bio_MS and BGMS10. The latter two materials are expected to exhibit superior biological performance due to their essentially amorphous nature, which is preserved despite the thermal treatment. Furthermore, the new BGs benefit, compared to 45S5, from the presence of specific ions known for their therapeutic effects. SEM analysis of these scaffolds highlighted the presence of hydroxyapatite aggregates that lead to the formation of a continuous layer, suggestive of mineralization. In accordance with Kokubo and Takadama [54], the formation of hydroxyapatite aggregates, progressing to a continuous apatite layer, is widely recognized as a marker of sustained bioactivity, especially when maintained over extended *in vitro* or *in vivo* periods. X-EDS maps and spectra confirmed the co-localization of calcium and phosphorus, validating HA formation [55].

With regard to the differences between Bio_MS and BGMS10, it is important to point out that the network connectivity (NC) of these glasses is different [14]. NC is a very important parameter to understand the dissolution behaviour; specifically, the higher the NC, the less reactive the BG is. If the NC is above 2.6 the glass is no longer bioactive due to its resistance to dissolution; in particular, the NC of BGMS10 is 2.2, whereas the NC of Bio_MS is 2.1, evidencing the difference between

the two BGs [56], and explaining the better performance of Bio_MS (see also the text below, concerning the affinity index).

The different early-stage reaction kinetics are evident from the ion release tests performed at 16 and 48 h. By measuring the ionic concentration in solutions in contact with the three BGs, while the two new glasses continue to release Ca over time, the 45S5 releases only traces, presumably because the Ca released by the 45S5 rapidly reprecipitates on its surface, forming a layer rich in calcium phosphate that quickly evolves into hydroxyapatite. Similarly, Si release from 45S5 is more pronounced than from Bio_MS and BGMS10, further reflecting the rapid dissolution of the residual glass network in the commercial bioglass. Regarding Sr and Mg, their release is slightly higher from BGMS10, which, however, inherently contains a greater amount of Mg. However, it should be hypothesized that the *in vivo* reaction mechanisms - especially over long periods (up to sixty days) - are far more complex.

The X-EDS spectra also revealed the presence of strontium and magnesium in the regions surrounding the implant, as well as in the newly-formed bone. A potential positive effect of these ions is expected, since they are known to promote bone formation. In particular, strontium stimulates osteoblasts (bone-forming cells) and inhibits osteoclasts (bone-resorbing cells), supporting a positive balance for bone growth; magnesium promotes osteoblast proliferation and differentiation, improving the quality of newly-formed bone. In a recent work, Bellucci and coworkers [30] demonstrated that enrichment with strontium and magnesium improved the physical, mechanical, and biological properties of bioactive glasses undergoing thermal treatments, as in the case of the scaffolds investigated in the present study, whose production involved a sintering step. Both of these features are important for promoting bone regeneration.

The authors are currently conducting further research into the findings presented here, specifically regarding the possible molecular mechanisms triggered by the ions released from the scaffold as it dissolves.

As for the bone regenerative potential *in vivo*, the histomorphometric results (Table 3) showed that, in both femurs and tibiae, the Bio_MS scaffold presents a higher osteoconductive ability (greater values of affinity index - AI) with respect to the other bioglasses, in particular with respect to the BGMS10, that on the other hand always shows the lowest AI values, even if only significant in the femur. In addition, the failure to find differences in AI for each BG between femur and tibia, which have different bone architectures and consequently different metabolism (higher in femur trabecular bone, compared with tibial compact one) [57,58], would seem to suggest that the osteogenic response does not depend on the type of bone architecture of the implanted site but rather on the different composition of scaffolds. This finding is partially in contrast with what was found in our previous study with the same BGs employed in the form of granules in rabbit femurs [40]. In fact, the AI did not vary significantly among the three BG granules and the values found at 60 days after implant surgery (same time interval as in this study) were about twice as high as those found with the scaffolds. This difference in AI might be due to the different surface to volume ratio, that is higher in granules with respect to scaffolds, thus allowing granules to be more surrounded by bone, regardless of both composition and ionic release.

Observing the newly-formed bone close to all BG scaffolds (histological results showed in Fig. 14) it is possible to detect the morphological features (i.e., highly cellular primary bone and the adjacent osteoblastic laminae) of the two stages of bone formation, always occurring in sequence during both skeletal organogenesis and bone repair: i.e., first “static” osteogenesis (so called because performed by cords of stationary osteoblasts transforming into globous osteocytes, entrapped in large lacunae in the same place where they differentiated, forming highly cellular primary bone) and, then, “dynamic” osteogenesis (so called because performed by movable osteoblastic laminae differentiated along the bone surfaces of the newly-formed primary bone by static osteogenesis) [59,60]. The presence of osteoblastic laminae

(typical of the second phase of bone deposition, i.e. dynamic osteogenesis), lining the newly-formed primary bone, is particularly abundant in Bio_MS specimens, thus leading to the thickening of the primitive trabeculae formed by static osteogenesis. It can be hypothesized that the abundance of osteoblastic laminae, indicative of a more advanced phase of bone deposition, could be correlated in Bio_MS scaffolds to the higher AI, and consequently a higher osteoconductivity found in this type of BG. This is also in line with the NC parameter (the higher the NC, the less reactive the BG) explaining the better performance of Bio_MS, showing lower NC with respect to BGMS10.

On the contrary, the low AI values recorded in BGMS10 samples could be explained by the presence of many osteoclasts on the scaffold surfaces that prevent the formation of bone directly in contact with the BG. The abundance of osteoclasts could be likely explained, in turn, by the release of Mg from BGMS10 bioglass, whose concentration in Mg is double with respect to Bio_MS one [61]. Although it is well known that Mg is very important in new bone deposition acting on osteoblast differentiation and proliferation [33], it is also true and interesting that several studies [61–64] indicate that the increase in Mg ionic concentration, in experiments both *in vitro* and *in vivo*, enhances differentiation, proliferation and osteoclast function. Moreover, increased Mg levels would also lead to a higher survival rate of osteoclasts, while reducing the osteoblastic surface, leading to the decreased osteoblast differentiation and the reduction in osteoid surface. This evidence too is in line with the observation that the lower the NC the more reactive the BG. It is interesting to underline that this decrease of osteoblastic surface due to Mg concentration increase, also shown by other authors [63], might explain in our findings the presence of many osteoblastic laminae in Bio_MS specimens, where the Mg amount is reduced (almost half) with respect to BGMS10 samples. Furthermore, it is interesting to note that magnesium is not always correlated with osteoblastic or osteoclastic presence. This is highlighted in a recent study of ours [65] in which we observed that, in C57BL/6J male mice fed a magnesium-deprived diet for 8 weeks, the bone mass did not vary compared to control mice.

Another aspect to consider is the absence of cementing lines in the newly-formed bone close to the scaffolds, regardless of the BG employed. It is interesting to compare this situation (primary bone, i.e., bone that has not undergone remodeling, close to the BG scaffolds) with the previous results published by our group in 2023 [40], using the same BGs in the form of granules (instead of scaffolds) implanted *in vivo*. In the previous experiments (using the same long-term implantation at 60 days) many cementing lines were present in the bone surrounding the BG granules, indicating that bone remodeling was already underway, leading to the formation of secondary bone (a more mature bone with respect to the primary one) characterized by mainly lamellar structure containing few ellipsoidal osteocytes. On the contrary, in this study, the newly-formed bone adjacent to the scaffolds has not yet undergone remodeling (in fact, no cementing lines are visible) and is therefore primary bone, containing globous osteocytes, as mentioned above. These differences would suggest that bioglasses in the form of granules are more effective in stimulating bone remodeling and therefore bone turnover. This could be explained by the greater specific surface area of granules compared to scaffolds.

5. Conclusions

In conclusion, the results shown in the present paper strongly indicate that the all three bioglasses show osteoconductive potential, mostly without significant differences, although Bio_MS scaffold could be considered the most promising candidate for bone defect repair in regenerative medicine applications. In fact, having faster ionic dissolution and higher affinity index than the others, Bio_MS scaffold appears to be at a more advanced stage of degradation, anticipating the osteogenesis phase, as evidenced by the presence of abundant osteoblastic laminae close to its surfaces. Diversely, in BGMS10 scaffolds, the sequence of events in response to the BGs appears to be slower, since

their surfaces are still covered by various osteoclasts, whose erosive activity precedes the osteogenic one. All these observations are in line with the stages that temporally follow one another during the repair of bone defects using BGs, *i.e.*, *i*) bioglass dissolution by ion release, *ii*) bioglass erosion by osteoclast activity, *iii*) formation of new primary bone by osteoblastic lamina activity.

The authors, while aware of the need to further investigate the mechanisms of bone recovery following the implantation of bioglasses, nevertheless underline the ability of BG scaffolds (in particular of Bio_MS) to promote bone healing, making them promising materials for regenerative medicine.

CRediT authorship contribution statement

Marzia Ferretti: Conceptualization, Investigation, Methodology, Writing – review & editing. **Francesco Cavani:** Data curation, Methodology, Writing – review & editing. **Alexandre Anesi:** Investigation, Writing – review & editing. **Francesca Chiarini:** Investigation, Writing – original draft. **Roberta Salvatori:** Investigation, Writing – original draft. **Marta Checchi:** Investigation, Writing – original draft. **Ilaria Zanoni:** Investigation, Writing – original draft. **Francesca Paganelli:** Investigation, Writing – original draft. **Devis Bellucci:** Investigation, Methodology, Writing – review & editing. **Valeria Cannillo:** Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Writing – review & editing. **Carla Palumbo:** Conceptualization, Methodology, Supervision, Validation, Writing – review & editing.

Declaration of competing interests

The authors declare the following: no conflict of interests.

Acknowledgements

The authors thank Dr. Marta Benincasa for her technical assistance in the production of iconography. Carla Palumbo acknowledges that the work was funded by FAR-Dept 2022 provided by DBMN-UNIMORE, Modena, Italy. Devis Bellucci received funds from Project FARD-2022 “MATLIFE”, by the Department of Engineering “Enzo Ferrari”, University of Modena and Reggio Emilia, Modena, Italy. The work was also funded by FAR-Dept 2024 (PI: Cavani, Ferretti) provided by DBMN-UNIMORE, Modena, Italy. The bioactive glass Bio_MS (in granules) was patented (Italian patent N. 102019000002229, 2019).

Data availability

Data will be made available on request.

References

- [1] E.H. Schemitsch, Size matters: defining critical in bone defect size, *J. Orthop. Trauma* 31 (Oct. 2017) S20–S22, <https://doi.org/10.1097/BOT.0000000000000978>.
- [2] J.P. Schmitz, J.O. Hollinger, The critical size defect as an experimental model for craniomandibulofacial nonunions - PubMed, *Clin. Orthop.* (1986) 299–308 [Online]. Available: <https://pubmed.ncbi.nlm.nih.gov/3084153/>.
- [3] C.G. Finkemeier, Bone-grafting and bone-graft substitutes, *J. Bone Joint Surg. Am.* 84 (3) (2002) 454–464, <https://doi.org/10.2106/00004623-200203000-00020>.
- [4] A.M. Jakoi, J.A. Iorio, P.J. Cahill, Autologous bone graft harvesting: a review of grafts and surgical techniques, *Musculoskelet. Surg.* 99 (3) (Dec. 2015) 171–178, <https://doi.org/10.1007/S12306-015-0351-6>.
- [5] H. Schliephake, Bone growth factors in maxillofacial skeletal reconstruction, *Int. J. Oral Maxillofac. Surg.* 31 (5) (2002) 469–484, <https://doi.org/10.1054/IJOM.2002.0244>.
- [6] S.W. Choi, J.E. Levine, J.L.M. Ferrara, Pathogenesis and management of graft-versus-host disease, *Immunol. Allergy Clin.* 30 (1) (Feb. 2010) 75–101, <https://doi.org/10.1016/j.iac.2009.10.001>.
- [7] N. Shibuya, D.C. Jupiter, Bone graft substitute: Allograft and xenograft, *Clin. Podiatr. Med. Surg.* 32 (1) (2015) 21–34, <https://doi.org/10.1016/j.cpm.2014.09.011>.
- [8] D. Heimes, et al., Comparison of morbidity-related parameters between autologous and allogeneic bone grafts for alveolar ridge augmentation from patients' perspective-A questionnaire-based cohort study, *Clin. Implant Dent. Relat. Res.* 26 (1) (Feb. 2024) 170–182, <https://doi.org/10.1111/CID.13242>.
- [9] V. Campana, et al., Bone substitutes in orthopaedic surgery: from basic science to clinical practice, *J. Mater. Sci. Mater. Med.* 25 (10) (Sep. 2014) 2445–2461, <https://doi.org/10.1007/S10856-014-5240-2>.
- [10] W. Wang, K.W.K. Yeung, Bone grafts and biomaterials substitutes for bone defect repair: a review, *Bioact. Mater.* 2 (4) (Dec. 2017) 224–247, <https://doi.org/10.1016/j.bioactmat.2017.05.007>.
- [11] B.D. Ratner, A.S. Hoffman, F.J. Schoen, J.E. Lemons, *Biomaterials Science: an Evolving, Multidisciplinary Endeavor*, Request PDF, Third Edition, 2013 [Online]. Available: https://www.researchgate.net/publication/288163931_Biomaterials_Science_An_Evolving_Multidisciplinary_Endeavor.
- [12] M. Böhner, Bone substitute materials, *Encycl. Biomed. Eng.* 1–3 (Jan. 2019) 513–529, <https://doi.org/10.1016/B978-0-12-801238-3.00224-5>.
- [13] F. Baino, C. Vitale-Brovarone, Bioactive glass and glass–ceramic foam scaffolds for bone tissue restoration, *Biomed. Foams Tissue Eng. Appl.* (Jan. 2014) 213–248, <https://doi.org/10.1533/9780857097033.2.213>.
- [14] J.R. Jones, Review of bioactive glass: from hench to hybrids, *Acta Biomater.* 9 (1) (2013) 4457–4486, <https://doi.org/10.1016/j.actbio.2012.08.023>.
- [15] L.L. Hench, R.J. Splinter, W.C. Allen, T.K. Greenlee, Bonding mechanisms at the interface of ceramic prosthetic materials, *J. Biomed. Mater. Res.* 5 (6) (Nov. 1971) 117–141, <https://doi.org/10.1002/JBM.820050611>.
- [16] A.W. Wren, 45S5 bioglass based scaffolds for skeletal repair, in: J. Marchi (Ed.), *Advanced Structured Materials*, vol. 53, Springer International Publishing, 2016, pp. 183–201, https://doi.org/10.1007/978-3-319-44249-5_7.
- [17] I. Ochoa, J.A. Sanz-Herrera, J.M. García-Aznar, M. Doblaré, D.M. Yunos, A. R. Boccaccini, Permeability evaluation of 45S5 bioglass®-based scaffolds for bone tissue engineering, *J. Biomech.* 42 (3) (Feb. 2009) 257–260, <https://doi.org/10.1016/j.jbiomech.2008.10.030>.
- [18] Q.Z. Chen, I.D. Thompson, A.R. Boccaccini, 45S5 bioglass®-derived glass-ceramic scaffolds for bone tissue engineering, *Biomaterials* 27 (11) (Apr. 2006) 2414–2425, <https://doi.org/10.1016/j.biomaterials.2005.11.025>.
- [19] A.R. Boccaccini, J.E. Gough, Tissue engineering using ceramics and polymers, *Tissue Eng. Using Ceram. Polym.* (2007) 1–604, <https://doi.org/10.1533/9781845693817>.
- [20] N. Abbasi, S. Hamlet, R.M. Love, N.T. Nguyen, Porous scaffolds for bone regeneration, *J. Sci. Adv. Mater. Devices* 5 (1) (Mar. 2020) 1–9, <https://doi.org/10.1016/J.JSAM.2020.01.007>.
- [21] D. Bellucci, A. Anesi, R. Salvatori, L. Chiarini, V. Cannillo, A comparative in vivo evaluation of bioactive glasses and bioactive glass-based composites for bone tissue repair, *Mater. Sci. Eng., C* 79 (Oct. 2017) 286–295, <https://doi.org/10.1016/j.msec.2017.05.062>.
- [22] O. Bretcanu, X. Chatzistavrou, K. Paraskevopoulos, R. Conradt, I. Thompson, A. R. Boccaccini, Sintering and crystallisation of 45S5 bioglass® powder, *J. Eur. Ceram. Soc.* 29 (16) (Jan. 2009) 3299–3306, <https://doi.org/10.1016/J.JEURCERAMSOC.2009.06.035>.
- [23] O.P. Filho, G.P. Latorre, L.L. Hench, Effect of crystallization on apatite-layer formation of bioactive glass 45S5, *J. Biomed. Mater. Res.* 30 (4) (Apr. 1996) 509–514, [https://doi.org/10.1002/\(SICI\)1097-4636\(199604\)30:4<509::AID-JBM9>3.0.CO;2-T](https://doi.org/10.1002/(SICI)1097-4636(199604)30:4<509::AID-JBM9>3.0.CO;2-T).
- [24] F.G. Mecca, D. Bellucci, V. Cannillo, Effect of thermal treatments and ion substitution on sintering and crystallization of bioactive glasses: a review, *Materials* 16 (13) (Jun. 2023) 4651, <https://doi.org/10.3390/MA16134651>, 2023, Vol. 16, Page 4651.
- [25] P. Li, Q. Yang, F. Zhang, T. Kokubo, The effect of residual glassy phase in a bioactive glass-ceramic on the formation of its surface apatite layer in vitro, *J. Mater. Sci. Mater. Med.* 3 (6) (Nov. 1992) 452–456, <https://doi.org/10.1007/BF00701242>.
- [26] E. Fiume, J. Barberi, E. Verné, F. Baino, Bioactive glasses: from parent 45S5 composition to scaffold-assisted tissue-healing therapies, *J. Funct. Biomater.* 9 (1) (Mar. 2018) 24, <https://doi.org/10.3390/jfb9010024>, 2018, Vol. 9, Page 24.
- [27] L.L. Hench, The story of bioglass, *J. Mater. Sci. Mater. Med.* 17 (11) (2006) 967–978, <https://doi.org/10.1007/S10856-006-0432-Z>, vol. 17, no. 11, Springer, 2006.
- [28] D. Bellucci, V. Cannillo, G. Ciardelli, P. Gentile, A. Sola, Potassium based bioactive glass for bone tissue engineering, *Ceram. Int.* 36 (8) (Dec. 2010) 2449–2453, <https://doi.org/10.1016/J.CERAMINT.2010.07.009>.
- [29] D. Bellucci, V. Cannillo, A. Sola, Calcium and potassium addition to facilitate the sintering of bioactive glasses, *Mater. Lett.* 65 (12) (Jun. 2011) 1825–1827, <https://doi.org/10.1016/J.MATLET.2011.03.060>.
- [30] D. Bellucci, et al., Enrichment of strontium and magnesium improves the physical, mechanical and biological properties of bioactive glasses undergoing thermal treatments: new cues for biomedical applications, *Ceram. Int.* 50 (24) (Dec. 2024) 52819–52837, <https://doi.org/10.1016/J.CERAMINT.2024.10.135>.
- [31] S.J. Watts, R.G. Hill, M.D. O'Donnell, R.V. Law, Influence of magnesia on the structure and properties of bioactive glasses, *J. Non-Cryst. Solids* 356 (9–10) (Mar. 2010) 517–524, <https://doi.org/10.1016/J.JNONCRYSOL.2009.04.074>.
- [32] R. Wetzel, M. Blochberger, F. Scheffler, L. Hupa, D.S. Brauer, Mg or Zn for Ca substitution improves the sintering of bioglass 45S5, *Sci. Rep.* 10 (1) (Sep. 2020) 15964, <https://doi.org/10.1038/s41598-020-72091-7>, 2020 10:1.
- [33] S.R. Gaviño, et al., Bioactive glasses containing strontium or magnesium ions to enhance the biological response in bone regeneration, *Nanomaterials* 13 (19) (Oct. 2023), <https://doi.org/10.3390/NANO13192717>.
- [34] E. Gentleman, et al., The effects of strontium-substituted bioactive glasses on osteoblasts and osteoclasts in vitro, *Biomaterials* 31 (14) (May 2010) 3949–3956, <https://doi.org/10.1016/j.biomaterials.2010.01.121>.

- [35] P.J. Marie, P. Ammann, G. Boivin, C. Rey, Mechanisms of action and therapeutic potential of strontium in bone, *Calcif. Tissue Int.* 69 (3) (Jan. 2001) 121–129, <https://doi.org/10.1007/S002230010055>.
- [36] D. Bellucci, V. Cannillo, A novel bioactive glass containing strontium and magnesium with ultra-high crystallization temperature, *Mater. Lett.* 213 (Feb. 2018) 67–70, <https://doi.org/10.1016/J.MATLET.2017.11.020>.
- [37] D. Bellucci, et al., Human mesenchymal stem cell combined with a new strontium-enriched bioactive glass: an ex-vivo model for bone regeneration, *Materials* 12 (21) (Nov. 2019) 3633, <https://doi.org/10.3390/MA12213633>, 2019, Vol. 12, Page 3633.
- [38] D. Bellucci, E. Veronesi, M. Dominici, V. Cannillo, A new bioactive glass with extremely high crystallization temperature and outstanding biological performance, *Mater. Sci. Eng., C* 110 (May 2020), <https://doi.org/10.1016/j.msec.2020.110699>.
- [39] M. Barbalinardo, et al., Magnesium and strontium-enriched bioactive glasses: superior biocompatibility and angiogenesis, beyond the gold standard, *Biomater. Adv.* 177 (Dec. 2025) 1–11, <https://doi.org/10.1016/J.BIOADV.2025.214379>.
- [40] A. Anesi, et al., In-vivo evaluations of bone regenerative potential of two novel bioactive glasses, *J. Biomed. Mater. Res.* 111 (8) (Aug. 2023) 1264–1278, <https://doi.org/10.1002/JBM.A.37526>.
- [41] S. Stegen, N. van Gastel, G. Carmeliet, Bringing new life to damaged bone: the importance of angiogenesis in bone repair and regeneration, *Bone* 70 (Jan. 2015) 19–27, <https://doi.org/10.1016/j.bone.2014.09.017>.
- [42] D. Ribatti, Two new applications in the study of angiogenesis the CAM assay: acellular scaffolds and organoids, *Microvasc. Res.* 140 (Mar) (2022), <https://doi.org/10.1016/j.mvr.2021.104304>.
- [43] M. Checchi, et al., Scleral ossicles: angiogenic scaffolds, a novel biomaterial for regenerative medicine applications, *Biomater. Sci.* 8 (1) (Jan. 2020) 413–425, <https://doi.org/10.1039/C9BM01234F>.
- [44] M. Kanazawa, K. Tsuru, N. Fukuda, Y. Sakemi, Y. Nakashima, K. Ishikawa, Evaluation of carbonate apatite blocks fabricated from dicalcium phosphate dihydrate blocks for reconstruction of rabbit femoral and tibial defects, *J. Mater. Sci. Mater. Med.* 28 (6) (Jun. 2017), <https://doi.org/10.1007/S10856-017-5896-5>.
- [45] C.J. Moran, A. Ramesh, P.A.J. Brama, J.M. O'Byrne, F.J. O'Brien, T.J. Levingstone, The benefits and limitations of animal models for translational research in cartilage repair, *J. Exp. Orthop.* 3 (1) (Dec. 2016) 1–12, <https://doi.org/10.1186/S40634-015-0037-X>.
- [46] P. Orth, D. Zurakowski, M. Alini, M. Cucchiari, H. Madry, Reduction of sample size requirements by bilateral versus unilateral research designs in animal models for cartilage tissue engineering, *Tissue Eng. C Methods* 19 (11) (Nov. 2013) 885–891, <https://doi.org/10.1089/TEN.TEC.2012.0699>.
- [47] W. Zhou, R. Apkarian, Z.L. Wang, D. Joy, Fundamentals of Scanning Electron Microscopy (SEM), New York: Springer, New York, NY, 2006, https://doi.org/10.1007/978-0-387-39620-0_1.
- [48] L.L. Hench, Bioceramics: from concept to clinic, *J. Am. Ceram. Soc.* 74 (7) (Jul. 1991) 1487–1510, <https://doi.org/10.1111/j.1151-2916.1991.tb07132.x>.
- [49] R.D. Bloebaum, J.G. Skedros, E.G. Vajda, K.N. Bachus, B.R. Constantz, Determining mineral content variations in bone using backscattered electron imaging, *Bone* 20 (5) (May 1997) 485–490, [https://doi.org/10.1016/S8756-3282\(97\)00015-X](https://doi.org/10.1016/S8756-3282(97)00015-X).
- [50] A.V. Silva, D. dos S. Gomes, R. de S. Victor, L.N. de L. Santana, G.A. Neves, R. R. Menezes, Influence of strontium on the biological behavior of bioactive glasses for bone regeneration, *Materials* 16 (24) (Dec. 2023) 7654, <https://doi.org/10.3390/MA16247654>, 2023, Vol. 16, Page 7654.
- [51] A. Laskus, J. Kolmas, Ionic substitutions in non-apatitic calcium phosphates, *Int. J. Mol. Sci.* 18 (12) (Dec. 2017), <https://doi.org/10.3390/IJMS18122542>.
- [52] L.L. Hench, The story of bioglass®, *J. Mater. Sci. Mater. Med.* 17 (11) (Nov. 2006) 967–978, <https://doi.org/10.1007/S10856-006-0432-Z>, 2006 17:11.
- [53] A.R. Boccaccini, Q. Chen, L. Lefebvre, L. Gremillard, J. Chevalier, Sintering, crystallisation and biodegradation behaviour of bioglass®-derived glass–ceramics, *Faraday Discuss.* 136 (0) (Aug. 2007) 27–44, <https://doi.org/10.1039/B616539G>.
- [54] T. Kokubo, H. Takadama, How useful is SBF in predicting in vivo bone bioactivity? *Biomaterials* 27 (15) (2006) 2907–2915, <https://doi.org/10.1016/j.biomaterials.2006.01.017>.
- [55] H. Wang, J.K. Lee, A. Moursi, J.J. Lannutti, Ca/P ratio effects on the degradation of hydroxyapatite in vitro, *J. Biomed. Mater. Res.* 67 (2) (Nov. 2003) 599–608, <https://doi.org/10.1002/JBM.A.10538>.
- [56] A. Pedone, V. Cannillo, M.C. Menziani, Toward the understanding of crystallization, mechanical properties and reactivity of multicomponent bioactive glasses, *Acta Mater.* 213 (Jul. 2021) 116977, <https://doi.org/10.1016/J.ACTAMAT.2021.116977>.
- [57] G. Marotti, The structure of bone tissues and the cellular control of their deposition, *Ital. J. Anat. Embryol.* 101 (4) (1996) 25–79 [Online]. Available: <https://pubmed.ncbi.nlm.nih.gov/9203871/>.
- [58] M. Ferretti, et al., Interaction among calcium diet content, PTH (1-34) treatment and balance of bone homeostasis in rat model: the trabecular bone as keystone, *Int. J. Mol. Sci.* 20 (3) (2019), <https://doi.org/10.3390/IJMS20030753>.
- [59] M. Ferretti, C. Palumbo, M. Contri, G. Marotti, Static and dynamic osteogenesis: two different types of bone formation, *Anat. Embryol.* 206 (1–2) (2002) 21–29, <https://doi.org/10.1007/s00429-002-0265-6>.
- [60] M. Ferretti, C. Palumbo, Static osteogenesis versus dynamic osteogenesis: a comparison between two different types of bone formation, *Appl. Sci.* 11 (5) (Feb. 2021) 2025, <https://doi.org/10.3390/AP11052025>.
- [61] M. Leidi, F. Dellera, M. Mariotti, J.A.M. Maier, High magnesium inhibits human osteoblast differentiation in vitro, *Magn. Res.* 24 (1) (Mar. 2011) 1–6, <https://doi.org/10.1684/MRH.2011.0271>.
- [62] L. Wu, B.J.C. Luthringer, F. Feyerabend, A.F. Schilling, R. Willumeit, Effects of extracellular magnesium on the differentiation and function of human osteoclasts, *Acta Biomater.* 10 (6) (2014) 2843–2854, <https://doi.org/10.1016/j.actbio.2014.02.010>.
- [63] P.J. Marie, R. Travers, E.E. Delvin, Influence of magnesium supplementation on bone turnover in the normal young mouse, *Calcif. Tissue Int.* 35 (6) (Dec. 1983) 755–761, <https://doi.org/10.1007/BF02405119>.
- [64] Z. Zhao, et al., Capturing magnesium ions via microfluidic hydrogel microspheres for promoting cancellous bone regeneration, *ACS Nano* 15 (8) (Aug. 2021) 13041–13054, <https://doi.org/10.1021/acsnano.1c02147>.
- [65] M. Ferretti, et al., Unexpected absence of skeletal responses to dietary magnesium depletion: basis for future perspectives? *Biomedicines* 11 (3) (Mar. 2023) <https://doi.org/10.3390/BIOMEDICINES11030655>.