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Vertebral bone marrow clot breakthrough: a powerful osteogenic and antibacterial scaffold for spinal fusion surgery

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ABSTRACT

Achieving solid arthrodesis while minimizing postoperative infections remains a critical challenge in spinal fusion surgery. Clotted bone marrow aspirate (BMA) has emerged as promising tools to enhance bone fusion, yet their antimicrobial potential remains underexplored. This study investigates the biological and antimicrobial properties of vertebral body BMA (vBMA) clots as a multifunctional scaffold for spinal fusion. Human vBMA clots were characterized by growth factor and cytokine content via ELISA. Mesenchymal stromal cells (MSCs) were isolated and analyzed for morphology, viability, immunophenotype, and trilineage differentiation potential. The antibacterial activity of vBMA clots was assessed against *Staphylococcus aureus* and *Escherichia coli* strains by monitoring the bacterial concentration over time. Additionally, production of kinocidins (CXCL1, CCL5, CXCL7, CXCL8, CXCL12) was quantified. vBMA clots exhibited high levels of regenerative cytokines, especially PDGF-AB, and contained viable, multipotent MSCs expressing canonical surface markers. Antibacterial assays revealed potent, broad-spectrum bactericidal activity, with >99 % CFU reduction for both pathogens after 24 h of exposure to the clots, independently from the bacterial culture medium used. Notably, kinocidin production significantly increased upon bacterial exposure, particularly in response to *E. coli*, suggesting active immune engagement by clot-resident cells. vBMA clots possess a dual regenerative and antimicrobial function, offering a novel, one step and autologous strategy for enhancing spinal fusion outcomes while reducing infection risk. This bioactive scaffold may represent a paradigm shift in spinal surgery by unifying osteogenic support and localized antibacterial defense in a single therapeutic material.

1. Background

Instrumented spinal fusion surgeries are commonly performed to treat various spinal disorders, including degenerative diseases, traumas-related fractures, and malignancies [1]. A substantial increase in the incidence of these procedures was observed, rising from 64.5 to 135.5 cases per 100,000 adults between 1998 and 2008 [2,3]. The achievement of solid bony arthrodesis is the primary goal of spinal fusion surgery, requiring the formation of new bone between two or more adjacent vertebrae to restore stability to the affected spinal segment [4]. Strategies to promote solid spinal fusion and prevent pseudarthrosis have become key areas of research and investment in modern spine surgery [4]. Despite local autograft, harvested from the surgical site, being the traditional gold standard for bone grafting in spinal fusion surgery, its use is limited by factors such as availability, patient age and comorbidity, and biological quality [5]. In response to the limitations of traditional autografts, a wide range of alternative strategies have been developed and extensively investigated to enhance spinal fusion outcomes [6,7]. These include allografts, synthetic grafts, and bioactive growth factors [6–8]. More recently, autologous cell-based therapies, such as bone marrow aspirate (BMA) in various formulations, have gained attention for their potential to improve spinal fusion rates [9–13]. While these approaches contribute to achieving solid bony fusion, reducing the risk of pseudarthrosis, and ultimately enhancing

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patients' quality of life, spinal fusion procedures are not without other complications [14,15]. One of the most severe complications associated with spinal fusion surgery is surgical site infection, particularly those involving spinal implants [16,17]. Spinal infections pose a unique and difficult challenge due to the anatomical complexity of the spine and its proximity to the central nervous system [17]. If not promptly and effectively managed, these infections can lead to devastating complications, including osteomyelitis, epidural abscess formation, meningitis, and even sepsis, all of which can result in permanent neurological deficits, chronic pain, or fatal outcomes [16–18]. The treatment of deep spinal infections is often prolonged and complex, requiring extensive surgical debridement, prolonged systemic antibiotic therapy, and, in severe cases, implant removal and spinal reconstruction [19]. Despite advancements in surgical asepsis, including bioclean operating environments and antibiotic prophylaxis, the incidence of surgical site infection (SSI) following spinal fusion remains between 1.6 % and 10.3 % [20–25]. Abdul-Jabbar et al. reported that *Staphylococcus aureus* (45.2 %) and *Staphylococcus epidermidis* (31.4 %) are the predominant pathogenic species responsible for SSI, supporting cefazolin as a reasonable prophylactic choice [26]. Although *Escherichia coli* is not the most common pathogen in infections following spinal surgery, a recent clinical study on 96 patients found that *E. coli* is responsible for 23.9 % of early SSI after spinal procedures, making it the second most common pathogen after *S. aureus* [27]. However, the rising prevalence of multidrug-resistant organisms, including methicillin-resistant *S. aureus* (MRSA) strains, has raised significant concerns regarding the efficacy of conventional prophylactic regimens [26,28,29]. Given that 34.3 % of these infections involve methicillin-resistant pathogens, there is an urgent need for alternative or adjunctive antimicrobial strategies to improve infection control in spinal fusion surgery [26,28–30]. Compared to systemic administration, localized antibiotic delivery presents a compelling alternative, as it enables high antimicrobial concentrations directly at the surgical site while minimizing systemic toxicity [26,28,29]. However, the anatomical and physiological characteristics of the spine, including its relatively poor vascularization in certain regions, can compromise the efficacy of systemically delivered antibiotics, further emphasizing the potential benefits of targeted, local antimicrobial therapies [16,17]. Early diagnosis and aggressive intervention are therefore paramount in preventing devastating outcomes. In this context, recently, our research group hypothesized potential antimicrobial properties of a novel BMA formulation from vertebral body, vertebral BMA (vBMA) clot [31]. Beyond their established role in enhancing spinal fusion and promoting bone regeneration in preclinical and clinical studies, we assumed that vBMA clots may also contribute to reducing postoperative infection risk, presenting a promising multifunctional scaffold in spinal fusion surgery [32–35]. By harnessing the biological properties of autologous vBMA clots, this approach could offer both osteogenic and antimicrobial benefits, potentially reducing the reliance on systemic antibiotic prophylaxis and improving overall surgical outcomes [31–35].

In this study we assessed the fundamental biological properties of human vBMA clots to reinforce and expand upon existing literature. By increasing the sample size, this study provides a more comprehensive evaluation of the osteogenic and regenerative potential of vBMA clots, further substantiating their dual role in enhancing spinal fusion, and, for the first time, investigates their early secretory profile immediately after explantation. While previous studies have characterized vBMA clots, our characterization is a prerequisite for subsequent antibacterial activity assays. Building upon this foundation, we evaluate the antibacterial properties of vBMA clots against two bacterial species representatives of the most common Gram-positive and Gram-negative pathogens responsible for spinal infections, specifically *Staphylococcus aureus* and *Escherichia coli*. Given the increasing prevalence of multidrug-resistant bacteria in spinal infections, exploring novel autologous antimicrobial strategies are of paramount importance. To further elucidate the mechanisms underlying the potential antibacterial properties of vBMA clots, we also assessed the presence and production of specific microbicidal proteins known as kinocidins (CXCL1, CCL5, CXCL7, CXCL8, CXCL12). These chemokines play a crucial role in the immune response and may contribute to the observed antimicrobial activity, offering valuable insights into the biological processes involved.

2. Methods

2.1. Human vBMA clot harvest

A total of thirty patients with indications for multilevel (≤5) posterior spinal fusion due to degenerative lumbar spine diseases were screened for inclusion in the study following the provision of written informed consent. The enrolment period extended from April 28, 2023, to February 14, 2025. Eligible participants were adults aged 18 years or older at the time of surgery, presenting with symptomatic degenerative spine disease that necessitated posterior fusion in the lumbar region. Additionally, all participants provided signed informed consent prior to inclusion. Patients were excluded from the study if they had any local or systemic infections, inflammatory or autoimmune disorders, coagulation abnormalities, tumors, substance abuse (alcohol or drugs), pregnancy, or were undergoing chemotherapy, as these factors could interfere with bone regeneration and the surgical process.

A posterior lumbar fusion technique using transpedicular titanium screw/rod instrumentation was performed on all enrolled patients. During surgery, vBMA was harvested from the vertebral pedicle at the time of preparing the pedicle screw insertion site. General anesthesia was administered, and patients were positioned prone. The procedure began with a midline skin incision, which was extended to adjacent levels as needed. Subcutaneous dissection was performed in both cranial and caudal directions beyond the initial skin incision. Subsequently, a subperiosteal muscle dissection was performed to expose the entry points for pedicle screw placement, and vBMA was aspirated. To maximize the number of progenitor cells in the vBMA, a standardized volume of bone marrow was aspirated from each vertebral body, as the aspiration volume directly influences cell concentration. The aspirated vBMA was collected in two sterile containers without anticoagulants and allowed to coagulate under sterile conditions. Meanwhile, the rest of the surgical procedure continued. Following pedicle screw placement, decompression of the cauda equina and nerve roots was achieved through hemilaminectomy and foraminotomy. One portion of the clotted vBMA was applied to the hemilaminae and transverse processes on the contralateral side of the hemilaminectomy, after decortication of the lamina, while the remaining portion was used for the evaluation of biological and antibacterial properties.

2.2. Biological properties of vBMA clot

2.2.1. Characterization of vBMA clot by enzyme-linked immunosorbent assays

The clotted vBMA was placed in culture flask and once it was dissolved (after ~24 h of culture) it was collected and centrifuged to eliminate particulates, and aliquots of were stored at −20 °C. Enzyme-linked immunosorbent assays (ELISA) were employed to quantify the amount of Platelet-Derived Growth Factor AA (PDGF AA) (pg/mL) (Cloud-Clone Corp., USA), PDGF BB (pg/mL) (Cloud-Clone Corp., USA), PDGF AB (pg/mL) (Proteintech, Germany), Interleukin-6 (IL-6) (pg/mL) (Cloud-Clone Corp., USA), IL-1β (pg/mL), Transforming Growth Factor β (TGFβ) (pg/mL) (Proteintech, Germany), Bone Morphogenetic Protein-2 (BMP-2) (pg/mL) (Cloud-Clone Corp., USA), Epidermal Growth Factor (EGF) (pg/mL) (Cloud-Clone Corp., USA), Basic Fibroblast Growth Factor (bFGF) (pg/mL) (Cloud-Clone Corp., USA), according to the manufacturer's instructions. The absorbance was measured at 450 nm using an ELISA reader (Imark Microplate Reader, ELISA-Biorad SRL). Each sample was tested in triplicate.

2.2.2. Isolation of MSCs from vBMA clot

The clotted vBMA was placed in culture flasks containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 μg/mL Plasmocin (Sigma-Aldrich, St. Louis, MO, USA; Lonza, Basel, Switzerland; Gibco Life Technologies, Carlsbad, CA, USA; Invivogen, San Diego, CA, USA) to isolate mesenchymal stem cells (MSCs). The culture flasks were incubated at 37 °C in a 5 % CO2 atmosphere under hypoxic conditions (2 % O2).

2.2.3. MSCs morphology and viability

MSCs isolated from clotted vBMAs were observed twice a week under a light microscope (Nikon Eclipse, Milan, Italy). After 14 days, cell viability was assessed using the LIVE/DEAD® assay (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, MSCs were incubated with calcein-AM (4 μM) and ethidium homodimer-1 (2 μM) for 45 min at 37 °C. Images were captured using a fluorescence microscope (Nikon Eclipse, Milan, Italy) equipped with a digital camera. Green fluorescence indicated live cells, while red fluorescence indicated dead cells. The number of dead cells per cm^2 was quantified. Each sample was tested in triplicate.

2.2.4. MSCs clot flow cytometry

Antigen expression was assessed with FACSCanto II instrument (Becton Dickinson, Franklin Lakes, NJ, USA) and by FACSDiva software 6.0 (Becton Dickinson). Briefly, at passage 1, 0.5–1 $\times$ 10^5 of MSCs for each antigen were washed with Phosphate Buffered Saline (PBS), centrifuged, and incubated in flow cytometry buffer, adding fluorescein isothiocyanate (FITC)-conjugated antibody against CD31, CD34, CD44, CD45, CD90, CD105, CD271. FITC-conjugated nonspecific immunoglobulin G (IgG) was used as control (Bio-Legend, San Diego, CA, USA).

2.2.5. MSCs colony-forming assay

At passage 1, 200 MSCs/cm^2 were plated and cultured for 10 days to assess the number of colony-forming units (CFUs). Cells were fixed in 10 % formalin and stained with toluidine blue. Aggregates with $\geq$20 cells were scored as colonies and counted (Nikon Eclipse, Milan, Italy). Each sample was tested in triplicate.

2.2.6. MSCs osteogenic, adipogenic, and chondrogenic differentiation ability

For osteogenic, adipogenic and chondrogenic differentiation, MSCs were plated at a density of 7 $\times$ 10^3 cells per cm^2 and incubated in DMEM complete medium. After 24 h, osteogenic (Human MSC Osteogenic Differentiation Medium BulletKit™, Lonza, Basel, Switzerland), adipogenic (Human MSC Adipogenic Differentiation Medium BulletKit™, Lonza, Basel, Switzerland) and chondrogenic (Human MSC Chondrogenic Differentiation Medium BulletKit™, Lonza, Basel, Switzerland) medium was added, and MSCs were cultured for 15 days. Osteogenic, adipogenic and chondrogenic cultures were fixed in 10 % formaldehyde or 4 % PFA and subsequently stained respectively with Alizarin Red S (Sigma-Aldrich), 1.8 % Oil Red O (Sigma-Aldrich) and 0.1 % Safranin O (Sigma-Aldrich). Images were captured using a microscope (Nikon Eclipse, Milan, Italy). Each sample was tested in triplicate.

2.3. Antibacterial properties of vBMA clot

The bacterial strains used were *Escherichia coli* ATCC 8739 (Migula, Castellani and Chalmers, American Type Culture Collection 8739) and *Staphylococcus aureus* ATCC 6538P (subsp. aureus Rosenbach, American Type Culture Collection 6538P). To evaluate the antibacterial activity of the vBMA clot, an assay was performed in 12-multiwell plates, in which the clot was directly exposed to the bacterial cells. The inoculum was prepared following overnight bacterial growth (approximately 18 h) in Mueller Hinton Broth (MHB), washed, resuspended in fresh MHB, Tryptic Soy Broth (TSB), or PBS, and diluted to approximately 10^6 CFU/mL. In each well, 1 mL of bacterial inoculum was added, either with or without (control) 1.0 g of the vBMA clot. The plates were incubated at 37 °C with shaking. The antibacterial activity was assessed through serial dilutions and CFU/mL enumeration on Mueller Hinton Agar (MHA) plates of aliquots collected at consecutive experimental time points of 3 h, 6 h, and 24 h. Each experiment was conducted in triplicate.

To better characterize the antibacterial effect of the vBMA clot, tests were also performed using nutrient-rich medium (TSB) and phosphate buffer (PBS).

Furthermore, to evaluate and understand the basis of the antibacterial properties, the production of various kinocidins was quantified in samples with or without vBMA clot that were collected after 24 h of incubation with the bacterial strains. ELISA were employed to quantify the levels of CCL3, CCL5, CXCL1, CXCL8, CXCL7, and CXCL12 (Proteintech, Germany) (pg/mL), according to the manufacturer's instructions. Absorbance was measured at 450 nm using an ELISA reader (Imark Microplate Reader, ELISA-Biorad SRL). Each sample was tested in triplicate.

2.4. Statistical analysis

Statistical analysis was performed with GraphPad Prism software 9.5.1. Data are reported as mean ± standard deviations (SD) at a significance level of $p < 0.05$. After having verified normal distribution and homogeneity of variance, a one-way ANOVA was done for comparison between groups. Finally, the Tukey post hoc multiple comparison test was performed to detect significant differences among groups.

3. Results

3.1. Biological properties of vBMA clot

3.1.1. Characterization of vBMA clot by enzyme-linked immunosorbent assays

The concentrations of growth factors and cytokines detected in the vBMA clot are summarized in Fig. 1. Statistical analysis for these data was not performed as no control clot group was present. Regarding catabolic cytokines, IL-6, IL-1β, and TGF-β are produced by various cell types, including bone marrow-derived MSCs. MSCs are well known for their immunomodulatory and regenerative properties, and the production of these cytokines is one of the key components contributing to these functions. Their baseline levels, in the absence of external inflammatory stimuli, typically range between 10 and 100 pg/mL during the first 24 h of culture, a range within which the values obtained for the vBMA-derived clot are positioned, except for TGF-β, which was undetected in the vBMA clot at the baseline, likely because it typically increases later, playing a role in healing and even fibrosis rather than in acute catabolism responses [36–40]. The results of the immunoenzymatic assays for the anabolic cytokines - BMP-2, EGF, bFGF, and VEGF - further confirmed the presence of these factors within the vBMA-derived clot [41,42]. MSCs can produce these growth factors in a regulated manner, acting through both paracrine and autocrine mechanisms, and play a critical role in supporting long-term tissue regeneration. Similarly, upon activation, platelets provide an immediate release of growth factors, including BMP-2, EGF, bFGF, and VEGF. The quantification of platelet specific growth factor showed high levels for PDGF-AB with value higher than 2000 pg/mL. PDGF-AB is primarily produced by both MSCs and platelets within the vBMA-derived clot, contributing to the recruitment of fibroblasts and endothelial cells to the injury site, thereby promoting wound healing and vascularization [43]. Its high concentration in the vBMA clot further confirms the potential role in enhancing the regenerative and reparative properties of this formulation, as the combination of all these factors, released by both MSCs and platelets, creates an optimal microenvironment for tissue repair and angiogenesis. These findings provide a biochemical profile of the formulation, which will serve as the basis for subsequent antibacterial activity assays.

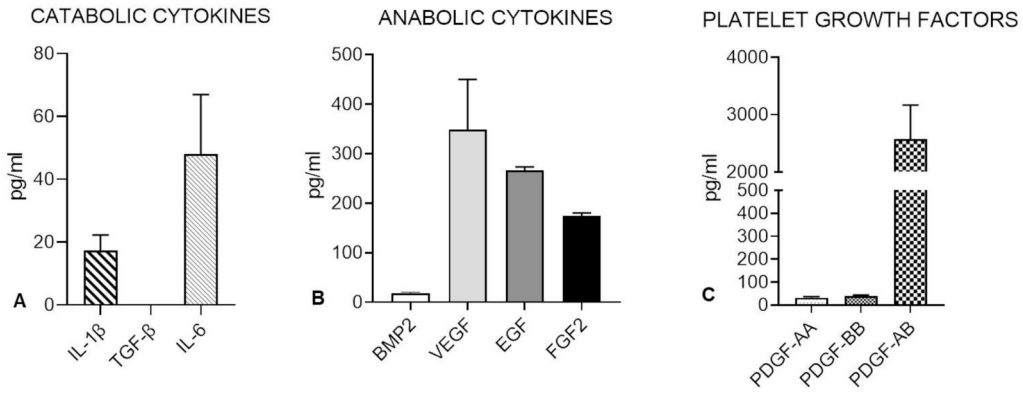

Fig. 1. Quantitative results of ELISA assays performed on dissolved clots after approximately 24 h of culture. Data are presented as mean ± SD. The histogram in Figure A shows the quantification of catabolic cytokines (IL-1β, TGF-β, and IL-6) while Figure B illustrates the release of anabolic cytokines. Figure C represents the quantification of specific platelet growth factors. All experiments were performed in triplicates.

3.1.2. *Characterization of MSCs from vBMA clot*

Microscopic examination using optical microscopy revealed that the cells exhibited a fibroblast-like morphology, a characteristic feature of MSCs (Fig. 2A). The Live/Dead viability assay confirmed a high level of cell viability, with most cells emitting green fluorescence (indicating live cells) and only a small fraction stained red (indicating dead cells) (Fig. 2B).

The CFU assay demonstrated the robust proliferative potential of MSCs, as evidenced by the formation of numerous well-defined colonies (Fig. 2C and D).

To evaluate the differentiation potential of the MSCs, lineage-specific staining assays were performed. Alizarin Red staining revealed prominent calcium deposits, confirming successful osteogenic differentiation (Fig. 2E). Oil Red O staining demonstrated the presence of intracellular lipid droplets, a hallmark of adipogenic maturation (Fig. 2F). Furthermore, Safranin O staining indicated the production of a proteoglycan-rich extracellular matrix, providing strong evidence of chondrogenic differentiation (Fig. 2G).

Flow cytometry analysis confirmed the expression of key MSC surface markers and the absence of hematopoietic markers, reinforcing the immunophenotypic identity of the isolated cells. Specifically, the cells were highly positive for CD44 (99 %), CD90 (97 %), and CD105 (97 %), while showing minimal expression of hematopoietic markers CD31 (5 %), CD34 (4 %), CD45 (4 %), and CD271 (3 %) (Fig. 2H).

3.2. *Antibacterial properties of vBMA clot*

The results of the antibacterial assays in MHB medium are shown in Fig. 3, which illustrates the viable bacterial cells counts, expressed as CFU per mL, after 3 h, 6 h, and 24 h of exposure to the vBMA clot. The results showed that the vBMA clot exhibited high and significant antibacterial activity against both bacterial strains at all tested time points. The kinetics of antibacterial activity over time differed between the two strains. Indeed, a rapid bactericidal effect was observed against *E. coli*, with most of the clot-exposed bacterial cells being below the limit of detection (<LOD) already at the first time tested (3 h). Conversely, the clearance of *S. aureus* cells exposed to the clot was more gradual yet progressive over time, with most samples reaching <LOD after 24 h of exposure to the clot. Despite the different dynamics, after 24 h of bacterial exposure, the clot showed a nearly complete killing against both *S. aureus* and *E. coli* cells, with reduction values exceeding 7 logs compared to control samples.

To investigate whether the antibacterial activity could be dependent on the nutrient availability for the bacterial cells, the same assay was conducted using a richer bacterial medium, i.e., TSB, and phosphate buffer (PBS), each tested with one clot only. The results were consistent with those obtained with MHB, showing >99.99 % of CFU reduction compared to control experiments at 24 h of exposure for both bacterial strains (Supplementary Fig. S1).

Kinocidins levels significantly increased in clots exposed to *E. coli* or *S. aureus* compared to control clot (not exposed to bacteria), regardless of the bacterial culture medium used (Supplementary Fig. S2). This increase indicates a strong activation of the antibacterial response of the cellular components within the clot in the presence of bacteria for all the investigated kinocidins, with different level of significance as shown in Fig. 4. Additionally, all these kinocidins were generally produced at higher levels in clots exposed to *E. coli* than in those exposed to *S. aureus,* especially CXCL-1, whose production and statistical significance compared to the control was a surprising finding. This indicates that the clot-associated kinocidins production is dependent on the bacterial strain tested, and only slightly on the bacterial growth conditions (growth medium composition).

4. Discussion

The results of this study provide valuable insights into the antimicrobial properties of the vBMA clot, highlighting its potential application in spinal fusion surgery as a novel bioactive and multifunctional scaffold. In this context, the term ‘scaffold’ is used in a functional and biological sense, as the vBMA clot constitutes a natural autologous matrix capable of hosting MSCs, platelets, and a variety of bioactive factors, thereby sustaining tissue regeneration and contributing to antibacterial activity.

The characterization of the vBMA clot confirmed a rich profile of growth factors and cytokines, with PDGF-AB being the most abundant. Given its crucial role in recruiting fibroblasts and endothelial cells, this high concentration suggests a strong regenerative potential, which significantly contributes to wound healing and vascularization [44]. Additionally, the presence of other key growth factors such as BMP-2, EGF, bFGF, and VEGF further strengthened that vBMA clot creates an optimal microenvironment for tissue regeneration [45–47]. These findings align with existing literature on the regenerative properties of bone marrow-derived MSCs and platelets, reinforcing their dual function in tissue repair through both direct cellular contributions and paracrine signaling mechanisms [48,49]. In this context, the characterization of MSCs derived from the vBMA clot confirmed their identity and viability. The fibroblast-like morphology, coupled with the high expression of MSC-specific surface markers (CD44, CD90, and CD105) and minimal hematopoietic contamination, underscores the purity of the isolated cell population [50,51]. Moreover, their robust proliferative capacity and successful differentiation into osteogenic, adipogenic, and chondrogenic lineages indicate their multipotency, which is a fundamental property for their application in regenerative medicine [52,53]. These findings further suggest that the vBMA clot not only serves as a

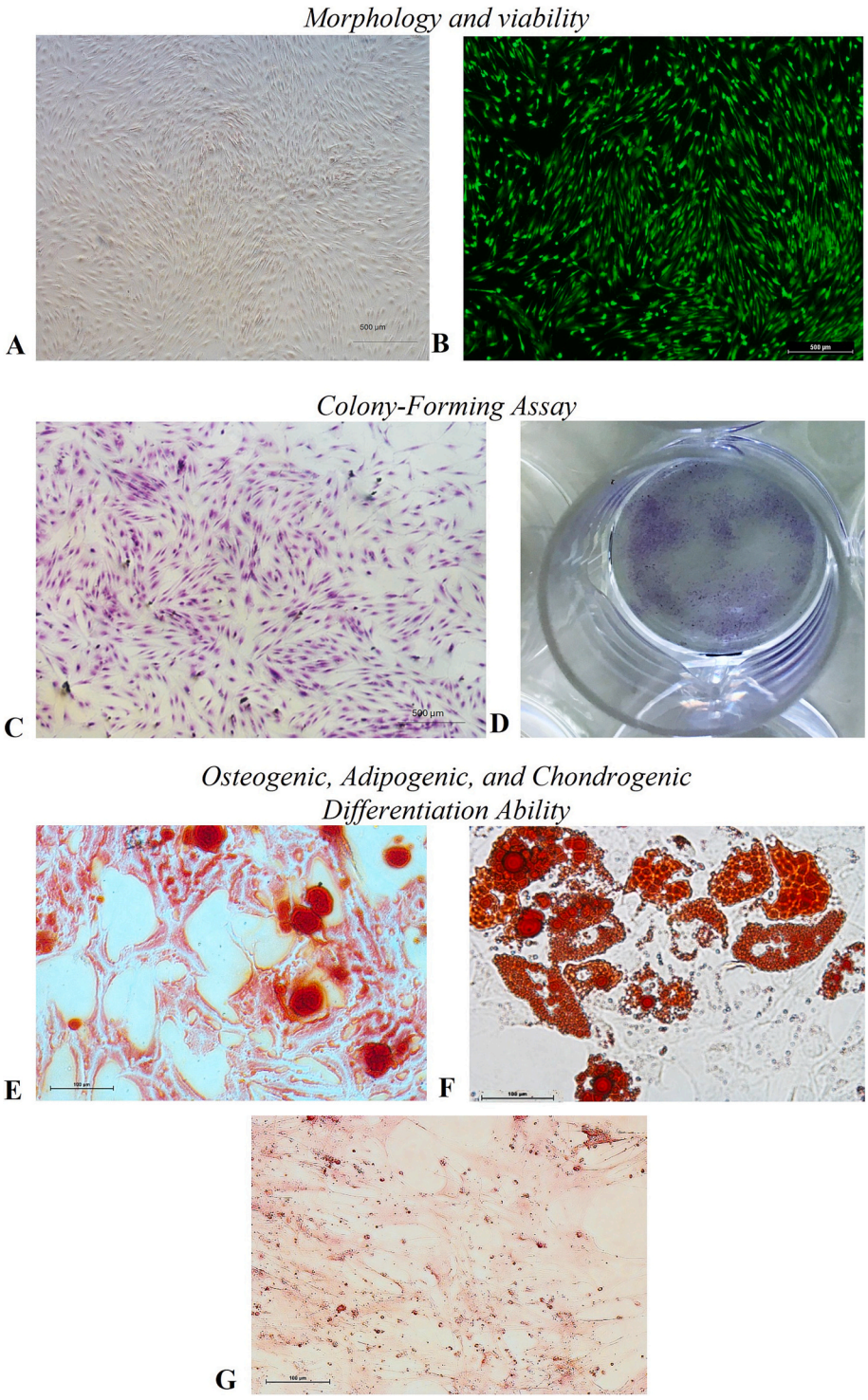

Fig. 2. **A)** MSCs from clotted vBMA at 14 days of culture; the homogenous population of spindle-shaped and plastic-adherent cells. Magnification 4×; scale bar: 500 μm **B)** LIVE/DEAD endogenous cell staining of MSCs from clotted vBMAs after 14 days of culture. Live cells stained in green with calcein-AM. Dead cells stained red with ethidium homodimer-1; magnification 4×; Scale bar: 500 μm; **C)** microscopic images of CFUs stained by toluidine blue. Magnification 4×. Scale bar: 500 μm. **D)** images of CFUs onto 24-well plates observed after 10 days of culture. **E), F)** and **G)** microscopic images of **E)** osteogenic (Alizarin Red S staining, magnification 20×, scale bar: 100 μm), **F)** adipogenic (Oil Red O, magnification 40×, scale bar: 50 μm), and **G)** chondrogenic (Safranin O, magnification 20×, scale bar: 100 μm) differentiation of MSCs from clotted vBMAs.; **H)** Representative flow cytometry graph of MSCs from clotted vBMAs, using fluorescent activated cell sorting (FACS). The MSCs-positive surface CD markers, namely CD44, CD90, and CD105, and the MSCs-negative surface CD markers, namely CD31, CD34, CD271 and CD45, are shown. The independent CD marker antigens were tagged with different fluorochromes. All the MSC-positive CD surface markers demonstrated more than 90 % positivity, and their histograms were considerably shifted to the right compared to their respective isotype controls. All experiments were performed in triplicates.

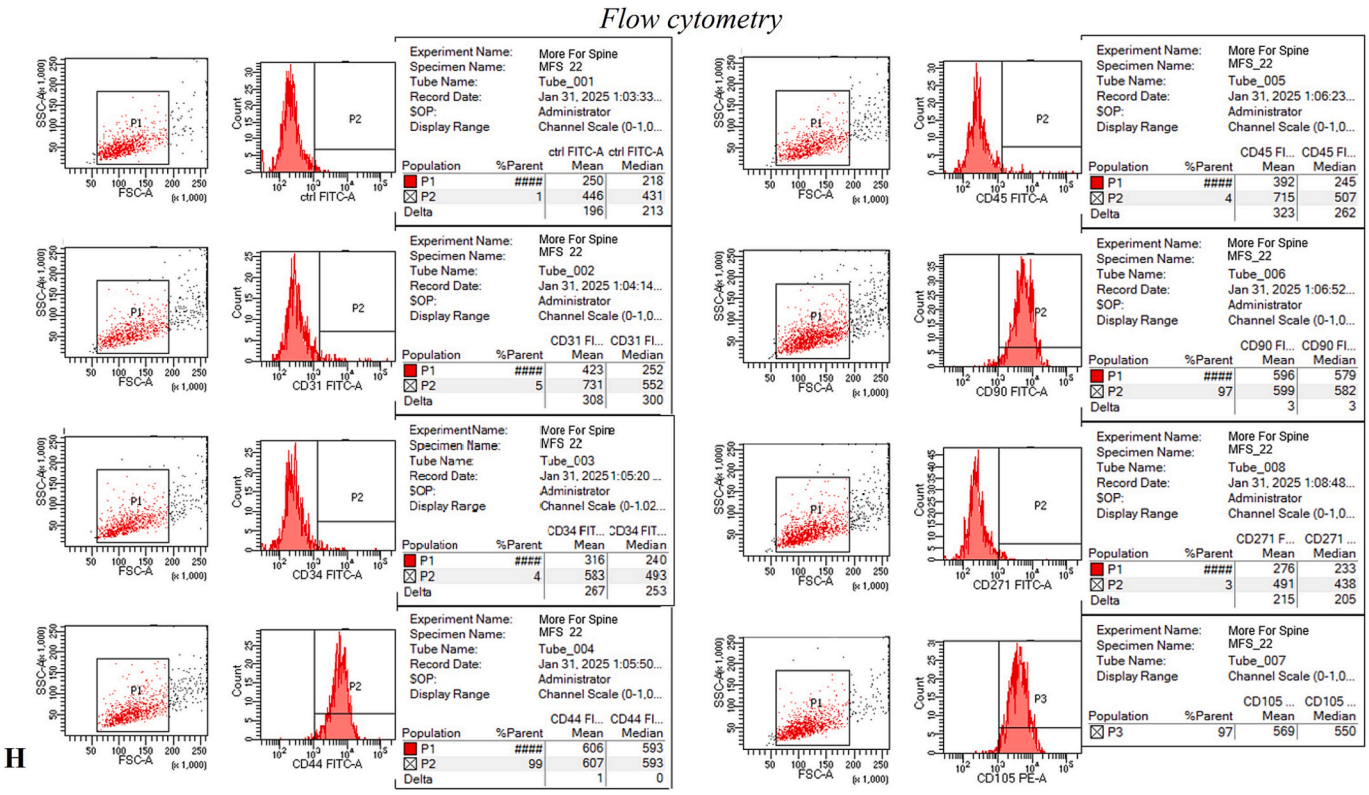

Fig. 2. (*continued*).

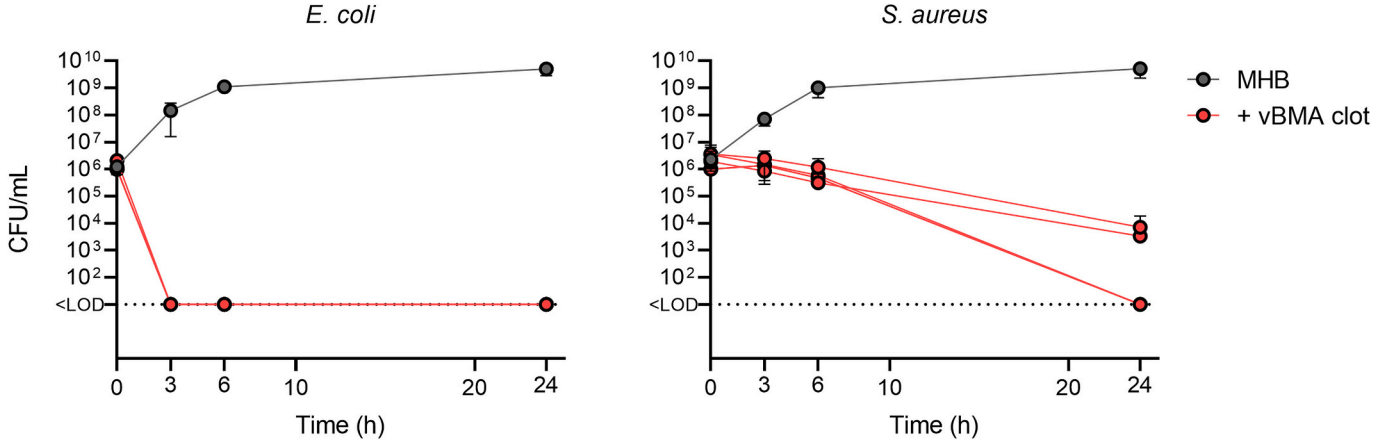

Fig. 3. Antibacterial activity of six different clots against *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 6538P. The graphs show the number of bacterial cells (expressed as colony-forming units, CFU) per mL after 3 h, 6 h, and 24 h of exposure to the vBMA clot in Muller Hinton Broth. The difference between MHB data and + vBMA clot data is statistically significant for all time points ($p < 0.0001$). LOD = limit of detection.

source of bioactive molecules but also provides a viable cellular component capable of contributing to bone regeneration in spinal fusion procedures. It is important to note that MSCs are not the only relevant component within the vBMA clot and do not act in isolation. Other resident cell populations, including hematopoietic stem/progenitor cells, endothelial progenitors, and immune cells, together with soluble growth factors and cytokines, may modulate MSC behavior, thereby enhancing their regenerative and immunomodulatory functions [10,54].

Despite these unique properties of vBMA clot, the central finding of this study lies in the unprecedented investigation of the antimicrobial activity exerted by the vBMA clot, which yielded remarkable results. The antibacterial activity of vBMA clots was clearly demonstrated in the time-course CFU analysis. The clot exhibited a statistically significant bactericidal effect against both *E. coli* and *S. aureus* strains, with bacterial viability drastically reduced at all measured time points compared to control conditions. A striking difference in the kinetics of bacterial clearance was observed between the two species. In the case of *E. coli*, the vBMA clot induced an extremely rapid bactericidal response, with viable bacterial counts dropping below the limit of detection within just 3 h of exposure, and this effect was sustained throughout the subsequent experimental time points, up to 24 h. This indicates that the clot environment either rapidly kills or inhibits the proliferation of *E. coli*, suggesting a highly efficient early antibacterial mechanism. Conversely, the response to *S. aureus* was more gradual but still highly effective. Although complete eradication was not achieved as rapidly as with

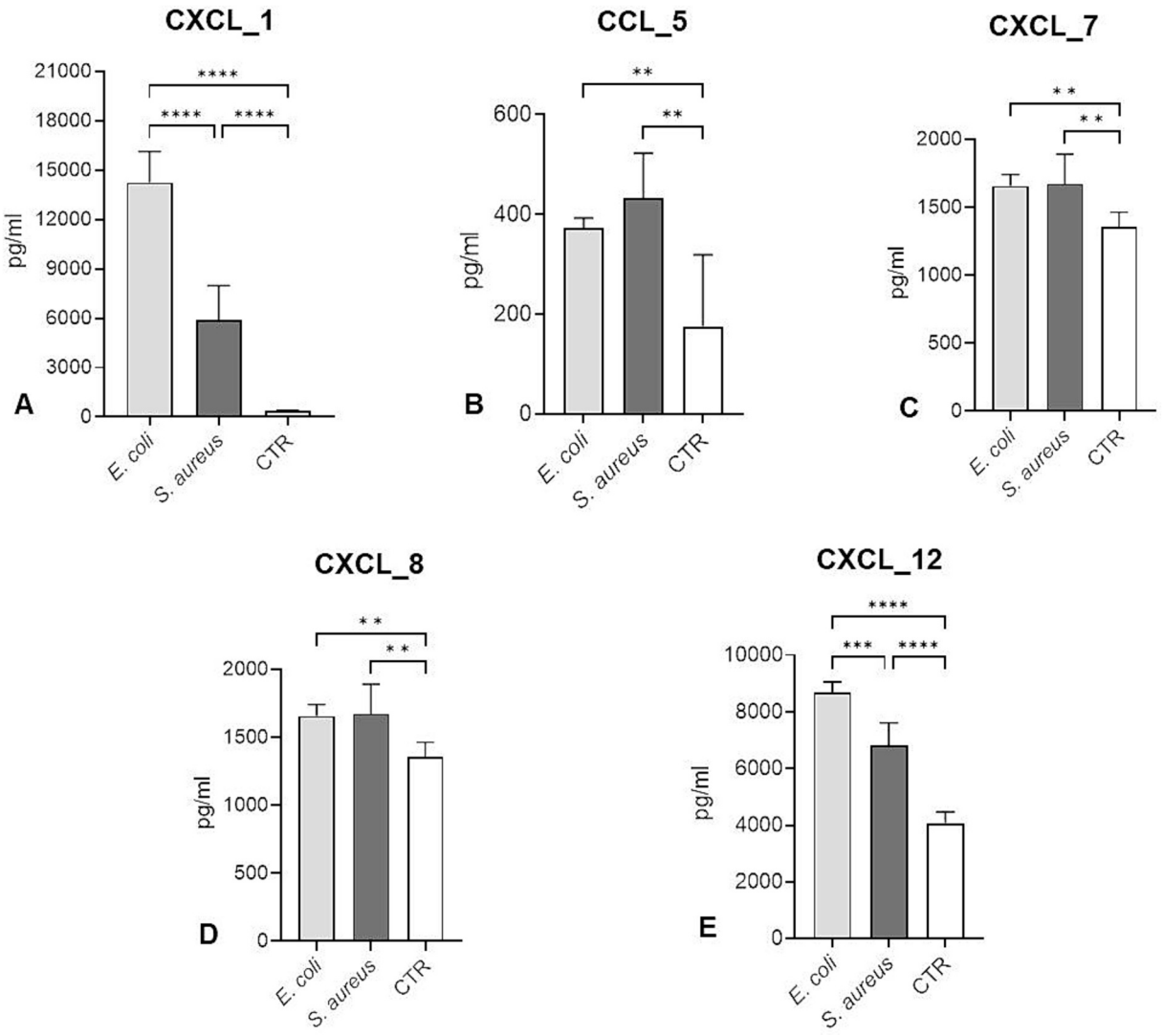

Fig. 4. Quantitative results of ELISA assays performed in samples with or without (CTR) vBMA clot in MHB that were collected after 24 h of incubation with the bacterial strains. Data are presented as mean ± SD. The histogram shows the quantification of **A**) CXCL1, **B**) CCL5, **C**) CXCL7, **D**) CXCL8 and **E**) CXCL12. (**** $p < 0.0001$, *** $p < 0.0005$, ** $p < 0.001$, * $p < 0.01$). All experiments were performed in triplicates.

E. coli, the bacterial load steadily decreased over time, reaching <LOD in most samples after 24 h. This difference likely reflects strain-specific resistance characteristics and interaction dynamics with the clot matrix and its cellular components. Importantly, the robust antibacterial effect was not limited to MHB medium. Similar levels of bacterial clearance, exceeding 99.99 % CFU reduction after 24 h, were also observed when the assay was conducted in both TSB and PBS conditions. These findings suggest that the antibacterial effect of the vBMA clot is not due to nutrient deprivation but more likely to the activity of immune-resident cells, such as neutrophils, monocytes, and activated platelets, and the release of soluble antimicrobial mediators. To further investigate this hypothesis, we analyzed the production of kinocidins within the clot. Kinocidins are chemokines with dual functions: in addition to their canonical role in immune cell recruitment, they possess direct antimicrobial activity against a broad spectrum of pathogens. A significant increase in kinocidin levels was detected in vBMA clots exposed to *E. coli* or *S. aureus* compared to the control condition, which was not exposed to bacteria. Some kinocidins, such as CXCL12, are constitutively expressed in specific tissues like the bone marrow where they help regulate homeostatic neutrophils trafficking [55]. Others, like CXCL7, are not constitutively expressed in the classical sense but may be abundant in the bone marrow microenvironment due to platelet activation and the physiological turnover. Additionally, under specific stimuli from stromal cells, monocytes can also express and release CXCL7, further contributing to its presence and potential functional roles in hematopoiesis and immune cell recruitment [56]. This constitutive expression likely explains the detectable baseline levels observed in the control samples for certain kinocidins. In contrast, other kinocidins such as CXCL1 or CCL2 are inducible and predominantly expressed in response to antimicrobial or inflammatory stimuli [57]. This finding supports the hypothesis that clot-resident cells are immunologically active and capable of mounting a targeted antibacterial response.

The production of CXCL1 was especially pronounced in *E. coli*-exposed clots, with levels significantly higher than those induced by *S. aureus*, indicating a particularly strong pro-inflammatory and neutrophil chemo-attraction in response to bacterial components [57]. Similar trends were observed for CXCL7 and CXCL12, where *E. coli* stimulation resulted in markedly elevated levels compared to both *S. aureus* and control group. These chemokines play crucial roles in neutrophil activation (CXCL1, CXCL7) and immune cell trafficking (CXCL12), suggesting that *E. coli* triggers a more potent innate immune activation within the clot microenvironment [57,58]. CCL5 and CXCL8 also showed significantly increased levels in both bacterial conditions compared to control, though the differences between *E. coli* and *S. aureus*

were less pronounced [58,59]. This could imply that certain aspects of the clot's immune activation are shared between bacterial strains, while others (e.g., CXCL1, CXCL12) are more strain specific. Interestingly, despite the use of different bacterial culture media, kinocidin expression was primarily influenced by the bacterial species rather than growth conditions [59–61]. This indicates that intrinsic bacterial features, such as specific pathogen-associated molecular patterns or virulence profiles, are the main drivers of clot immune activation [59–61]. Overall, these results support the conclusion that the pronounced antibacterial effects of the vBMA clot are not primarily attributable to nutrient competition, but rather to the synergistic action of immune cells embedded within the clot—such as neutrophils, monocytes, and activated platelets—and their release of antimicrobial peptides and kinocidins, which together generate a potent antibacterial microenvironment. The stronger response elicited by *E. coli* may reflect differences in its recognition or higher inflammatory potential compared to *S. aureus*.

The implications of these findings are substantial. The combination of regenerative and antimicrobial properties within a single biological material could represent a paradigm shift in the prevention of post-operative infections in spinal fusion surgery. Current infection management strategies often rely on systemic antibiotics or local antibiotic delivery systems, which may present challenges related to resistance development and limited bioavailability at the surgical site [61]. The use of vBMA clot as a bioactive scaffold could offer a more integrated and sustainable solution by providing both structural support for bone fusion and an intrinsic antimicrobial defense mechanism.

However, while the data presented here are promising, further investigations are required to elucidate the precise mechanisms underlying the antibacterial effects observed. Future studies should aim to explore and elucidate the specific molecular pathways involved and assess the efficacy of vBMA clot against a wider spectrum of bacterial pathogens commonly associated in spinal infections (e.g. *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*), supported by larger patient cohorts to validate these preliminary results with greater statistical strength. In addition, variability in clot composition related to patient-specific factors should be carefully considered. Although gender-related differences were not investigated in the present cohort, a previous study from our group found no significant variability between male and female donors [33]. An ongoing study is currently addressing the impact of age on vBMA clot characteristics. In the present work, potential confounding factors were minimized by excluding patients with conditions known to profoundly alter marrow or coagulation physiology, such as coagulation disorders, malignancies, active infections, prior radio- or chemotherapy, myeloproliferative diseases, chronic steroid or thyroxine use, and immunosuppression. Together, these considerations will be important for optimizing clinical translation of vBMA clots and understanding the potential patient-specific variability in their regenerative and antimicrobial performance.

5. Conclusions

This study demonstrates, for the first time, that vBMA clot possesses both regenerative and antimicrobial properties, making it a highly promising candidate for enhancing the safety and efficacy of spinal fusion surgery. Although several bioactive scaffolds and antimicrobial biomaterials have been explored in spinal surgery, including allogeneic bone grafts, demineralized bone matrix, synthetic ceramics, and antibiotic-loaded carriers, vBMA clots offer a distinctive combination of autologous cellular components together with soluble bioactive factors [62–64]. Unlike antibiotic-loaded materials, which rely on exogenous agents, the antimicrobial effect of vBMA clots is principally mediated by endogenous molecules such as kinocidins, potentially reducing the need for systemic antibiotics. This dual functionality positions vBMA clot as a multifunctional autologous scaffold that simultaneously supports bone regeneration and local infection control, distinguishing it from conventional scaffolds and biomaterials used in spinal fusion procedures. These findings introduce vBMA clot as the first orthobiologic product with demonstrable and potent intrinsic antibacterial activity. Its application could represent a significant advancement in spinal surgery, potentially redefining standard clinical strategies by simultaneously promoting bone healing and preventing infection within a single, biologically active scaffold.

List of abbreviations

BMA	Bone Marrow Aspirate
vBMA	Vertebral Bone Marrow Aspirate
MSC	Mesenchymal Stromal Cell
SSI	Surgical Site Infection
MRSA	Methicillin Resistant *Staphylococcus Aureus*
RCT	Randomized Clinical Trial
ELISA	Enzyme-Linked Immunosorbent Assay
PDGF	Platelet-Derived Growth Factor
IL-6	Interleukin-6
TGFβ	Transforming Growth Factor B
BMP-2	Bone Morphogenetic Protein-2
EGF	Epidermal Growth Factor
bFGF	Basic Fibroblast Growth Factor
DMEM	Dulbecco's Modified Eagle's Medium
PBS	Phosphate Buffered Saline
CFU	Colony Forming Unit
MHB	Mueller Hinton Broth
TSB	Tryptic Soy Broth
MHA	Mueller Hinton Agar
LOD	Limit Of Detection

CRediT authorship contribution statement

Francesca Salamanna: Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Giuseppe Tedesco:** Visualization. **Daniele Ghezzi:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Martina Cappelletti:** Writing – review & editing, Methodology. **Matteo Cianciavicchia:** Methodology, Investigation. **Chiara Alcherigi:** Visualization. **Cristiana Griffoni:** Visualization. **Giovanni Barbanti Brodano:** Writing – review & editing, Supervision. **Alessandro Gasbarrini:** Writing – review & editing, Supervision. **Gianluca Giavaresi:** Writing – review & editing, Supervision. **Maria Sartori:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This is part of a randomized clinical trial (RCT) ongoing at IRCCS – Istituto Ortopedico Rizzoli. Ethical approval was obtained from the local Ethics Committee (Comitato Etico Area Vasta Emilia Centro CE-AVEC) (Protocol MORE_FOR_SPINE; Number 149/2023/Sper/IOR). The study is registered on ClinicalTrials.gov (ClinicalTrials.gov ID: NCT05947175) and carried out in accordance with the principles of the Declaration of Helsinki.

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Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2025.124037.

Data availability

The datasets analyzed during the current study are available from the authors on reasonable request.

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