Multifactorial aetiology defines non-unions, with a biological and a mechanical distortion of the timeline of bone healing.

Research on new advances to increase osteogenesis and promote non-union healing is strongly directed towards new forms of cell products.

Basic science and research on non-union treatments is needed to compile preclinical data on new treatments.

Bone marrow concentration and expanded mesenchymal stromal cells still require extensive clinical research to confirm efficacy in non-union treatment.

Solid preclinical studies, precise cell product definition and preparation, and appropriate ethical and regulatory approvals are needed to assess new advanced therapy medicinal products.

Keywords: ATMPs (advanced therapy medicinal products); bone healing; cell therapy; MSCs (mesenchymal stromal cells) and biomaterials; non-union

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Introduction

Non-union or pseudarthrosis represents the inability of bone to heal after a fracture. Although this is a general definition that is well accepted by most orthopaedic trauma surgeons, it is not a simple concept. The absence of timely bone healing after a fracture, during the expected consolidation process, is associated with the presence of specific signs that confirm that the fracture will not spontaneously heal and therefore may require specific actions to obtain the desired bone healing. As surveys among specialists clearly show, both the consolidated bone fracture and the absence of consolidation producing a non-union or pseudarthrosis both deserve more precise definitions.1,2

Fracture non-union is diagnosed not only based on the absence of consolidation (or the absence of bone bridging and the presence of a fracture line), but rather by the radiological presence of specific signs that indicate the incapability of completing bone healing, with pain and eventually abnormal motion at the fracture site. These specific signs include the cessation of biological reaction at the bone ends, the obliteration of the medullary canal, and the interposition of a fibrous or cartilaginous tissue between the bone ends (Figs 1 and 2).

Interest in non-union treatment research is not only concerned with the insufficient and sometimes ineffective current treatments. It is worth noting that non-unions are also a serious fracture complication, with associated morbidity, repeated hospitalization, significant secondary functional limitations for 40% to 70% of patients,3 and significant resource consumption due to repeated surgeries.4

The rate of aseptic non-union of fractures after acute treatment is consistent among observational and interventional studies and varies from 5% to 10%, after two years of follow-up. Diaphyseal fractures of the humerus, femur and tibia evolve to non-union at three to five-times higher risk than other fracture locations in the same bones.5–8 High-energy trauma, open fractures, bone loss, fracture location, consumption of opioids, analgesics or anticoagulant drugs, are well known to predispose a fracture to delayed union or non-union.9–13 In the case of open fractures, the delayed or non-union may be associated with infection and loss of vascularity.14 Other authors have not found any influence of age, sex, body mass index, general health status (American Society of Anaesthesiology ASA score) or tobacco use on fracture consolidation.9,11,15 Therefore, multifactorial aetiology is involved in non-union, and risk factors associated with the patient, the fracture, and its treatment, may well be the origin of the problem.

In Europe, about one million patients require a surgical bone reconstruction annually. The ‘gold standard’ treatment of non-unions in the orthopaedic trauma field has long been based on the autograft, frequently obtained from the iliac crest. Although little scientific evidence is available, empirical rationale based on the pathophysiology of bone healing over the decades strongly supports its...
Bone autograft is the safest and most effective grafting procedure, since it contains the patient’s mesenchymal stromal cells (MSCs) to enhance osteogenesis and growth factors to enhance osteo-induction, while providing a calcified osteoconductive framework for the new bone to grow into. Although not without complications, these are usually minor (about 20%), though sometimes significantly affecting the donor site and thus the patient (approximately 5%).

Alternatives have also focussed on osteoconduction and osteo-induction methods to stimulate bone healing. These include augmentation with biomaterials or devitalized grafts such as allografts, surgical techniques to optimize the mechanical environment, physical methods to

Fig. 1 Radiographic assessment of a proximal femoral diaphyseal atrophic non-union in (A) anteroposterior and (B) lateral views.

Fig. 2 Intra-operative view of the previously shown femoral atrophic non-union. Note in (A) the exposed non-union, and (B) the non-union after debridement of the fibrous tissue, showing the intramedullary nail.
mechanically induce osteoblastic differentiation (e.g., extracorporeal shock wave therapy or low intensity pulsed ultrasound), and growth factors to stimulate cell growth and differentiation (most studies focussing on bone mor-phogenetic proteins (BMPs)).

A most frequently debated alternative is currently the surgical administration of biological products involving cells. This review will put in perspective non-union research, including preclinical and basic science research, but will extensively focus on cell product therapies such as bone marrow concentration and expanded MSC technologies.

**Definition of non-union and bone healing**

Adequate research on the topic requires clear definitions of non-union and bone healing to confirm the non-union under treatment and the end-point of bone healing. A clear non-union definition is needed to set inclusion criteria in any research study, and different definitions have been proposed. Although timing of the fracture to heal is no longer accepted alone, being highly variable among fractures and patients, a long time without healing suggests a non-union if no biological progression is observed in the fracture site over several months. In this sense, several European trials (EudraCT 2009-017039-16, 2011-005584-24) have considered six months after the fracture, with more than three months without biological progression. Similarly, a recent survey confirms that the majority of surveyed surgeons considered six months as the timeframe to classify a painful delayed union as a non-union. However, and despite the general belief, the most commonly used definition of a non-union in clinical trials today was proposed by the Food and Drug Administration (FDA): ‘A non-union occurs when fracture healing is not completed within 9 months following injury, with absence of progressive signs of healing on serial radiographs over the course of 3 consecutive months’.18

Bone healing or fracture consolidation, particularly in long bones where thick cortices require more time and more biological ‘effort’ to heal, can be radiologically confirmed when bone bridging is observed across the fracture gap, and the fracture line disappears when the continuity of the fractured bone can be ascertained. In long bones, three out of four cortices (meaning two cortices in the anteroposterior (AP) view and two cortices as evaluated in the lateral view) need to be bridged to accept the fracture as healed as per radiological evaluation. These features have been usefully employed by bone healing scores in recent fractures, such as the RUST (Radiographic Union Scale for Tibial fractures)19 or the RUSH (RUS for Hip fractures).20

Yet some differences are encountered in the non-union healing evaluation. Re-modelling may be slow and therefore the resolution of the fracture line may occur much later than bone bridging. Furthermore, the present ‘gold standard’ to assess bone healing or persistence of the non-union is computed tomography (CT) bone evaluation. In this context, a score to assess non-union bone healing after treatment with biomaterials and MSCs has been recently defined and validated by our group,21 considering an intermediate category of bone bridged non-union with still observable fracture line due to slow remodelling, and a good correlation between radiographs and CT in evaluating non-union.

**From basic science to preclinical models of non-union**

Fracture evolution to delayed union and non-union is related to timely bone healing failure. However, the consolidation cascade is highly variable among individuals and fractures. The general frame of the consolidation process is well known, yet the specific sequence, with its drawbacks and regulation, is modulating the prognosis for each bone fracture and each patient and is possibly modified at different moments of the bone healing process.

The cellular events occurring during bone healing are like normal bone embryogenesis, except for the associated inflammation, the lower number of osteoprogenitors in the adult, and the potent mechanical influence that occurs in the adult bone. The bone cells directing bone healing after a fracture are osteoprogenitors from the periosteum or pluripotent cells from the bone marrow, modulated by signalling and transcription factors towards osteoblastic differentiation and osteogenesis.23

New insights of how a disbalance may translate into clinical disorders are attracting research towards non-union biology. Downregulation of effector memory regulatory T cells, effective at suppressing Receptor Activator for Nuclear Factor κ B Ligand (RANKL), was recently observed in patients with tibial fracture delayed unions.24 The biology in the vicinity of the non-union has been also studied, confirming a less favourable environment than other bone locations. In five patients with atrophic non-union, the MSCs at the non-union site and the iliac crest were similar, although the differentiation capability was not evaluated.25 However, Hernigou and Beaujean26 showed in 35 patients that the number of colony-forming units-fibroblastic (CFU-F) both at the non-union site and the iliac crest bone marrow was decreased in patients sustaining the bone healing problem, compared with control donors of bone marrow. The current conclusion is that a biological impairment of the non-union site may relate to different reasons, including vascularity, cell activity and, basically, an unfavourable biological scenario. This may also include decreased local osteogenesis, as concluded by Bajada et al,27 who demonstrated increased levels of the Wnt signalling inhibitor, DKK1, secreted by cells in the
vicinity of the non-union site. This Wnt pathway inhibition could limit BMP-mediated osteoblastic differentiation at the non-union site.

Besides molecular and cellular events, preclinical studies based in animal models are required to evaluate new treatments for the non-union. To create a non-union model, a critical size defect on a long bone is needed. This relates to a defect that does not heal spontaneously and remains unchanged in control animals while properly immobilized by an external fixator, a plate or a nail. The size of the critical defect depends on the animal and the bone.

Reviews of animal models for bone therapies\textsuperscript{28,29} detail the process to design an animal study. The protocols must respect the rules to ensure the well-being of laboratory animals, including the 3Rs (Replacement, Reduction and Refinement) to reduce the number of animals without compromising the quality of the study. This is described in EU Directive 2010/63/EU ‘On the protection of animals used for scientific purposes’, which entered into force in all EU Member States on 1 January 2013. The access to in vivo imaging methods, such as X-rays, CT or magnetic resonance imaging (MRI), should be possible to adequately follow in vivo the regeneration process, and therefore reduce the number of animals.

The non-union experimental model must be as close as possible to human long bone injuries, in term of size and weight-bearing. Large animals such as the sheep, the minipig or the dog may be preferred for this reason. But research in large animals presents drawbacks such as high cost, long time for bone healing, and requirements of appropriate facilities.\textsuperscript{30} Currently, there is no technical alternative to simulate bone regeneration than the long bone critical defect. Before testing an orthotopic therapy in large animals, a preliminary in vitro screening is necessary, followed by in vivo studies on small animals at ectopic sites (subcutaneous tissue or calvaria). The osteo-induction and osteoconduction potential of cells, biomaterials, scaffolds or growth factors under study are then confirmed.

No animal model fully mimics the human injury, and studies are usually performed in healthy animals with a normal tissue environment.\textsuperscript{29} On the contrary, bone defects are often associated in clinical practice with fibrous soft tissues, scars and vascularity impairment. Sheep tibia or metatarsal models have been validated to evaluate bone regeneration in critical size defects of 2.5 cm\textsuperscript{31–33}. The commercial pig grows too fast to be used, but this is not a problem with the minipig, whose bone characteristics approach those of human bone\textsuperscript{34} and even load-bearing is similar,\textsuperscript{28,30} even if more expensive than the sheep. Dog bones are quite similar to human bones, but the feelings of today’s society restrict their use. This concern is even more of a factor for non-human primates.

The design of the study should assess efficacy not only against a placebo, but, rather, should compare new treatments against already validated standard therapies. The number of animals in a placebo group must be as low as possible, even more so if the model has been already validated in the literature. For valid statistical analysis, a minimum number of seven animals per group is usually necessary, with repeated in vivo imaging over a period which goes beyond the expected time of physiological union.\textsuperscript{28}

The operative technique must be very precisely described, particularly regarding the periosteum. Indeed, the simple resection of a bone cylinder leaving the perios- teum in place can regenerate even in a critical size defect, which can distort the results. The osteosynthesis depends on the study design. The external fixator is theoretically ideal due to the absence of material interference with bone regeneration that starts from bone ends or soft tissues. But the stability is imperfect, particularly in cases allowing weight-bearing, and it requires protection to avoid secondary injuries. The locked intramedullary nail may provide stability as in human patients, but it may suppress bone regeneration induced from the medullar cavity and impede the placement of grafts or biomaterials. The plate is probably the best compromise, and produces no nursing problems, although the weight-bearing may require cast protection.\textsuperscript{31} At the end of the study, animals are euthanized and bone explants are collected for processing (imaging, mechanical testing, histology).

Clinical research in non-unions: bone marrow concentration

The percutaneous autologous bone marrow grafting principle is based on osteo-inducing cell activity in the fracture site. It was demonstrated by Paley et al in rabbits in 1986.\textsuperscript{15} These cells correspond to MSCs and are also called colony-forming units-fibroblastic (CFU-F). Connolly et al proposed bone marrow centrifugation to increase the CFU-F rate and tested it in rabbits.\textsuperscript{36} Hernigou and Beaujean further applied this technique to patients.\textsuperscript{26} Bone matrix is synthesized by osteoblasts which originate in CFU-Fs.\textsuperscript{37} Under physiological conditions, there are very few CFU-Fs at the fracture site, and even fewer in patients sustaining a non-union, both at the pseudarthrosis site and at the iliac crest.\textsuperscript{38} This is part of the rationale behind proposing and using procedures to engraf autologous bone marrow, whether concentrated\textsuperscript{39–44} or not.\textsuperscript{22,45–48}

Hernigou et al were the first team to use autologous bone marrow concentration (BMC) in a large cohort of pseudarthrosis after open or closed tibial fractures. They obtained 88% success in 60 tibiae with pseudarthrosis.\textsuperscript{42} With this concentration technique, an increase in the
number of CFU-F was obtained, the starting material with 600 CFU-F/mL showed 2,500 CFU-F/mL after concentration. A significant correlation was found between the CFU-F rate in the BMC graft and bone consolidation. Bone union was obtained when the injected bone marrow contained more than 1,500 CFU-F/mL, at an average in total union was obtained when the injected bone marrow contained more than 360,000 CFU-F in BMC graft and bone consolidation. Bone union was obtained when the injected bone marrow contained more than 360,000 CFU-F/mL, at an average in total union was obtained when the injected bone marrow contained more than 1,500 CFU-F/mL, at an average in total union was obtained when the injected bone marrow contained more than 360,000 CFU-F to obtain 100% success. The timing of the graft was also important, and if the BMC was carried out earlier than 110 days after fracture, the success was 47%. But if the BMC was carried out later than 110 days after the fracture, success increased to 73%. BMC success rate decreased with increasing open fracture severity, and no success was obtained in cases in which the gap was wider than 4 mm. A post-operative fracture gap greater than 4 mm is associated with a high rate of procedures to obtain consolidation. Therefore, a large fracture gap is a contraindication for bone marrow grafting.

Reviewing the literature on autologous bone marrow grafting, one can see a relative homogeneity with nucleated cells numbers, whereas CFU-F numbers show very large variation, confirmed by the variations reported by different teams. Possible explanations may include the automatic process for nucleated cell enumeration (cell counter) instead of cell culture for CFU-F. Variations exist even in the same centre, confirming the difficulties of quantifying CFU-F. However, a precise definition of the injected product, both in research and patient treatment, is of the utmost importance to clarify efficacy.

BMC risks are low. Bone marrow extraction offers very mild complications, if any, but the injected product could carry a risk for the patient. Concerning the infection risk, even if it is possible to have positive bacteriological systematic examinations in a non-union, there is no report of secondary infection in the series. Concerning the oncological risk, a recent study by Hernigou and al. confirmed the absence of increased incidence of oncological pathologies after autologous bone marrow injection. Percutaneous autologous concentrated bone marrow graft is therefore a safe technique that has shown good results for the treatment of delayed union and non-union. Advantages include the intra-operative extraction and injection after concentration, a procedure that can be redone after a few weeks if needed, preserving bone stock and avoiding iliac bone harvesting or surgical exposure complications. Although it is not useful in cases with a large fracture gap or infection history, its results are interesting and need further study, especially CFU-F osteoblastic differentiation capacities, and randomized studies are needed to obtain comparative clinical results.

**Clinical research in non-unions: expanded MSCs**

The rationale behind using expanded MSCs to treat non-unions depends on the cell dose that is required to obtain efficacy. Currently, this cell dose is unknown. The previous approach to cell dose threshold to heal non-unions, as described in the preceding section, was established as 360,000 CFU-F, associating a higher success rate with the higher dose. Those BMC studies inspired MSC expansion to deliver enough cells to the non-union site.

The fate of these cells is also uncertain, but we recently published the results of a Phase I/IIa clinical trial implanting 100–200 x 10^6 MSCs (CD90+, CD63+, CD105+, CD45–) with a cell viability of 97% at release. With these cells, we obtained radiological consolidation at 12 months in 92% of cases, thus confirming the possible efficacy of this cell dose after expansion with this protocol (Figs 3 and 4).

Cell expansion may be efficacious, but this research is much more complex. Regulation 1394/2007 of the European Parliament states that cell and tissue engineering should be considered as ‘advanced therapy’ and requires marketing authorization (pre-market approval), demonstration of quality, safety and efficacy, and post-authorization vigilance. In this context, cells are considered engineered if they have been subjected to substantial manipulation (which occurs when expanded in the Good Manufacturing Practice – GMP – facility to obtain the demonstration of quality and thus the authorization) or if the cells or tissues are not intended to be used for the same essential function or functions in the recipient as in the donor (which can be considered if intended to regenerate bone).

To clinically launch this research, advanced therapy medicinal products, or ATMPs (such as expanded cells) require regulatory and ethical approval (from national regulators or competent authorities and ethical committees). For this, strong preclinical support is needed with adequate research methodology that includes in vitro and in vivo cell assessment, plus safety and efficacy evaluation of the cells in small and large animal models. When the cell product under study (given the cell source, the process and manufacturing technique, and the dose among other standardized parameters) is approved, an adequately designed clinical trial may be launched.

Of course, this complex procedure is very conservative and carries significant difficulties for the performance the clinical research that is required to eventually obtain marketing authorization in Europe. Nevertheless, an hospital exemption clause may facilitate in-hospital research on
the topic and thus autologous therapies may undergo hospital research permission to eventually obtain treatment authorization.

In a 2015 to 2019 brief revision of the literature regarding the use of ATMPs in orthopaedics, we identified a total of 18/59 studies related to bone healing/bone formation after an intervention with an ATMP. Of these, eight were reviews, two consensuses on manufacturing process, one consensus on clinical evidence, five were results from a European survey on the use of ATMP, and only two were

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**Fig. 3** Previously shown femoral atrophic non-union immediately after surgical treatment with expanded mesenchymal stromal cells (MSCs) combined with a Ca-P biomaterial, in (A) anteroposterior and (B) lateral radiographs.

**Fig. 4** Femoral atrophic non-union shown in Figs 1 to 3, surgically treated with expanded mesenchymal stromal cells (MSCs) combined with a Ca-P biomaterial, in (A) anteroposterior and (B) lateral radiographs after 12 months follow-up, confirming bone healing.
clinical trial results. We also revised the clinical trial registries (EudraCT and ClinicalTrial.gov) to identify studies using ATMP for bone healing, specifically for long bone non-unions.

The results of European surveys show an increasing trend in the use of ATMPs for bone regeneration (4% of the total use of ATMPs, as reported in 2015).66 In the musculoskeletal area, the most frequent research focuses on cartilage and maxillary bone. From clinical trial registries, we identified 17/92 (18%) studies using ATMP to heal non-union fractures, from 2007 to 2019 (see Table 1). Twenty four per cent of these studies (4/17) proposed the use of autologous cells while the other 76% proposed the use of allogenic cells. Treatments included percutaneously aspirated autologous bone marrow cells \( (n = 1) \), pre-osteoblast cells from bone marrow \( (n = 2) \), and mesenchymal stem cells from bone marrow \( (n = 9) \). Two trials were withdrawn, four showed an unknown status, and four were completed. Even though more than 15 registered clinical trials were found (after 2010) about bone healing/formation, results were not found in the same proportion.

Authors agree regarding the need to better understand cell behaviour (mechanism of action) and the impact of production processing on the therapeutic response. Another common observation is the heterogeneity of the doses used and the method to count the delivered cells

| Identification | Title                                                                 | Start / Last Update | Status                                           | ATMP                                                                 | Application                                      |
|---------------|----------------------------------------------------------------------|---------------------|--------------------------------------------------|----------------------------------------------------------------------|-------------------------------------------------|
| NCT00424567:  | TRC autologous bone marrow cells for the treatment of appendicular    | 2007 / 2017         | Terminated (closed early for business reasons)   | Aastrom tissue repair cells (TRC): percutaneously aspirated autologous bone marrow cells | Appendicular skeletal fracture non-union          |
| NCT00916981:  | Treatment of refractory non-union fractures by pre-osteoblast cells  | 2009 / 2012         | Completed                                        | Cultured pre-osteoblast cells from bone marrow                      | Pseudarthrosis (non-union fractures of long bones) |
| NCT01206179:  | Effect of bone marrow-derived mesenchymal stem cell transplantation   | 2010 / 2011         | Completed                                        | Autologous mesenchymal stem cell                                   | Pseudarthrosis (non-union fractures of long bones) |
| 2010-019380-11:| Autologous cell therapy of fracture non-union – cell phenotype as a  | 2010 /              | Prematurely ended                                | Autologous mesenchymal stem cell                                   | Pseudarthrosis (non-union fractures of long bones) |
|               | predictor of outcome (09/0278)                                       |                     |                                                  |                                                                      |                                                 |
| NCT01429012:  | Treatment of atrophic non-union fractures by autologous mesenchymal  | 2011 / 2013         | Not yet recruiting                               | Aut. BM-hMSC: autologous bone marrow mesenchymal stem cells         | Pseudarthrosis (non-union fractures)              |
| NCT01435434:  | Mononucleotide autologous stem cells and demineralized bone matrix   | 2011 / 2014         | Unknown                                         | Mononucleotide autologous stem cells                               | Pseudarthrosis (non-union fractures of long bones) |
| NCT01813188:  | Phase II clinical trial of tissue engineering based on the use of    | 2011 / 2017         | Completed                                        | Mononuclear cells from autologous bone marrow                      | Pseudarthrosis (non-union fractures of long bones) |
| NCT01756326:  | A pivotal phase 2b/3, multicentre, randomized, open, controlled study| 2012 / 2018         | Active, not recruiting                           | Autologous osteoblastic cells                                      | Pseudarthrosis (non-union fractures of long bones; hypotrophic) |
| NCT01788059:  | The efficacy of mesenchymal stem cells to stimulate the union in     | 2013                | Unknown                                         | Aut. BM-hMSC: stem cells derived from iliac bone marrow             | Pseudarthrosis (non-union fractures)              |
|               | treatment of non-united tibial and femoral fractures in Shahid Kamyab |                     |                                                  |                                                                      | in tibia and femur                               |
| NCT01842477:  | Evaluation of efficacy and safety of autologous MSCs combined to     | 2013                | Completed                                        | Aut. BM-hMSC: autologous bone marrow mesenchymal stem cells         | Pseudarthrosis (non-union fractures of long bones) |
|               | biomaterials to enhance bone healing (C11-12: OrthocIT)             |                     |                                                  |                                                                      |                                                 |
| NCT02020590:  | A pilot phase 1/2a, multicentre, open proof-of-concept study on the   | 2013                | Active, not recruiting                           | ALLOB®: cultured allogeneic osteoblastic cells                      | Pseudarthrosis (non-union fractures of long bones) |
and yield more homogeneous products globally.

guideline to better harmonize the manufacturing process

Note

healing in non-union (ORTHOUNION)

biomaterial vs iliac crest autograft for bone

of 2 doses of BM autologous H-MSC +

NCT03325504: A comparative study of 2 doses of BM autologous H-MSC+ biomaterial vs iliac crest autograft for bone healing in non-union (ORTHOUNION)

Note. ATMPs, advanced therapy medicinal products; BMSCs, bone mesenchymal stem cells.

which support Phinney et al’s request57 to establish a guideline to better harmonize the manufacturing process and yield more homogeneous products globally.

Conclusions and future directions

Current research on non-union treatment has been fostered by the uncertain efficacy of standard methods based on autograft augmentation. But obviously, treatment requires first an adequate surgical performance that certainly includes consistent pseudarthrosis debridement, including canal permeation, and appropriate bone fixation.

Non-unions are better defined and assessed today due to more specific diagnostic criteria, and different scores have helped to objectively define bone healing after a fracture, or its evolution to non-union, particularly in long bones with thick diaphyseal cortices. Risk factors and non-union epidemiology will help understanding of the patient and fracture characteristics to select treatments. Basic science and experimental models to sustain preclinical research in non-unions are also crucial to launch new treatment options. Major interest is today given to bone marrow concentration procedures and expanded MSC technology, addressing both clinical and regulatory aspects.

When doubts arise about osteo-induction fostered by administered growth factors, due to unclear dose, short-term effect and potential risks, surgically managed strategies such as bone marrow concentration have attracted significant attention. However, the amount of concentration, the exact composition of the re-infused material (cells of different types, growth factors, and other factors), or the association with grafts and/or biomaterials, are not yet clarified and compared. So, it is expected that substantial research may still be performed in the near future on this strategy, with advantages for the surgical management and administration in the operating room.

New options such as cell expansion are complex and expensive, and evidence-based research is definitely required, although the cell dose and product composition may be better defined than in bone marrow concentration. Bioreactors are also under scrutiny to ensure higher cell doses that may be associated with higher efficacy, although still undefined. Adequate cell product characterization (cell surface markers, phenotype, cell doubling), osteo-inductive properties of the product and osteoconductive matrix to deliver it, are all aspects that may modify the efficacy of the final product. Risk factors to the fracture and the patient may orient to autologous or allogeneic treatment research depending on cell reactivity, and
potency assays need to be defined to better understand each patient’s potential to heal with autologous cells. Although this research is not so close to wide clinical application, most research efforts are concentrated today on the biological treatment of non-unions. This biological side needs to be further explored to heal a non-union when a well-performed surgery is not sufficient.

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REFERENCES

1. Bhandari M, Guyatt GH, Swiontkowski MF, Tornetta P III, Sprague S, Schemitsch EH. A lack of consensus in the assessment of fracture healing among orthopaedic surgeons. J Orthop Trauma 2002;16:562–566.
2. Özkân S, Nolte PA, van den Bekerom MPJ, Bloemers FW. Diagnosis and management of long-bone nonunions: a nationwide survey. Eur J Trauma Emerg Surg 2019;45:3–11.
3. Ringburg AN, Polinder S, van Ierland MC, et al. Prevalence and prognostic factors of disability after major trauma. J Trauma 2011;70:916–922.
4. Kanakaris NK, Giannoudis PV. The health economics of the treatment of long-bone non-unions. Injury 2007;38:577–584.
5. Mills LA, Aitken SA, Simpson AHRW. The risk of non-union per fracture: current myths and revised figures from a population of over 4 million adults. Acta Orthop 2017;88:434–439.
6. Fong K, Truong V, Foote CJ, et al. Predictors of nonunion and reoperation in patients with fractures of the tibia: an observational study. BMC Musculoskelet Disord 2013;14:103.
7. Canadian Orthopaedic Trauma Society. Nonunion following intramedullary nailing of the femur with and without reaming: results of a multicenter randomized clinical trial. J Bone Joint Surg Am 2003;85:2093–2096.
8. Ekegren CL, Edwards ER, de Steiger R, Gabbe BI. Incidence, costs, and predictors of non-union, delayed union and mal-union following long bone fracture. Clin Orthop Relat Res 2005;438:221–232.
9. Audigé L, Griffin D, Bhandari M, Kellam J, Rüedi TP. Path analysis of factors for delayed healing and nonunion in 416 operatively treated tibial shaft fractures. Clin Orthop Relat Res 2005;438:221–232.
10. Bhandari M, Tornetta P III, Sprague S, et al. Predictors of reoperation following operative management of fractures of the tibial shaft. J Orthop Trauma 2003;17:353–361.
11. Henley MB, Chapman JR, Agel J, Harvey EJ, Whorton AM, Swiontkowski MF. Treatment of type II, IIIA, and IIIB open fractures of the tibial shaft: a prospective comparison of unreamed interlocking intramedullary nails and half-pin external fixators. J Orthop Trauma 1998;12:1–7.
12. Phieffer LS, Goulet JA. Delayed unions of the tibia. J Bone Joint Surg Am 2006;88:206–216.
13. Zura R, Xiong Z, Einhorn T, et al. Epidemiology of fracture nonunion in 18 human bones. JAMA Surg 2016;151:e162775.
14. Elniel AR, Giannoudis PV. Open fractures of the lower extremity: current management and clinical outcomes. EFORT Open Rev 2018;3:316–325.
15. Adams CL, Keating JF, Court-Brown CM. Cigarette smoking and open tibial fractures. Injury 2001;32:61–65.
16. Caplan AI. Mesenchymal stem cells. J Orthop Res 1999;15:641–650.
17. Hernigou P, Pariat J, Queinnece S, et al. Supercharging irradiated allografts with mesenchymal stem cells improves acetabular bone grafting in revision arthroplasty. Int Orthop 2016;39:1913–1921.
18. FDA. Guidance document for industry and CDRH staff for the preparation of investigational device exemptions and premarket approval applications for bone growth stimulator devices. In: Food and Drug Administration HHS, ed. Vol 63. Rockville, MD: Federal Register, 1998:23292-23293.
19. Whelan DB, Bhandari M, Stephen D, et al. Development of the radiographic union score for tibial fractures for the assessment of tibial fracture healing after intramedullary fixation. J Trauma 2010;68:629–632.
20. Frank T, Osterhoff G, Sprague S, Garibaldi A, Bhandari M, Slobogean GP; FAITH Investigators. The Radiographic Union Score for Hip (RUSH) identifies radiographic nonunion of femoral neck fractures. Clin Orthop Relat Res 2016;474:1396–1404.
21. Gómez-Barrena E, Padilla-Eguiluz NG, Eduardo García-Rey E, et al. Validation of a long bone fracture non-union healing score after treatment with mesenchymal stromal cells combined to biomaterials. Injury: in press.
22. Goel A, Sangwan SS, Siwach RC, Ali AM. Percutaneous bone marrow grafting for the treatment of tibial non-union. Injury 2005;36:203–206.
23. Arvidson K, Abdallah BM, Applegate LA, et al. Bone regeneration and stem cells. J Cell Mol Med 2017;15:778–746.
24. Wang J, Jiang H, Qiu Y, Wang Y, Sun G, Zhao J. Effector memory regulatory T cells were most effective at suppressing RANKL but their frequency was downregulated in tibial fracture patients with delayed union. Immunol Lett 2019;209:21–27.
25. Ismail HD, Phedy P, Kholinne E, Kusnadi Y, Sandhow L, Merlina M. Existence of mesenchymal stem cells in sites of atrophic nonunion. Bone Joint Res 2013;2:112–115.

26. Hernigou P, Beaujean F. Autologous bone marrow graft for patients presenting non unions. Rev Chir Orthop Reparatrice Appar Mot 1997;83:495–504.

27. Bajada S, Marshall MJ, Wright KT, Richardson JB, Johnson WE. Decreased osteogenesis, increased cell senescence and elevated Dickkopf-1 secretion in human fracture non union stromal cells. Bone 2009;45:716–735.

28. Penic M, Domic-Cule I, Grevec D, et al. The rational use of animal models in the evaluation of novel bone regenerative therapies. Bone 2015;70:73–86.

29. Tumedei M, Savadoni P, Del Fabbro M. Synthetic blocks for bone regeneration: a systematic review and meta-analysis. Int J Mol Sci 2019;20.44221.

30. Muschler GF, Raut VP, Patterson TE, Wenke JC, Hollinger JO. The design and use of animal models for translational research in bone tissue engineering and regenerative medicine. Tissue Eng Part B Rev 2010;16:123–145.

31. Viateau V, Guillenin G, Bousson V, et al. Long bone critical-size defects treated with tissue-engineered grafts: a study on sheep. J Orthop Res 2007;25:741–749.

32. Klaue K, Knothe U, Anton C, et al. Bone regeneration in long-bone defects: tissue compartmentalisation? In vivo study on bone defects in sheep. Injury 2009;40: S95–5102.

33. Sarkar MR, Augat P, Shefelbine SJ, et al. Bone formation in a long bone defect model using a platelet-rich plasma-loaded collagen scaffold. Biomaterials 2006;27: 1817–1823.

34.Raab DM, Crenshaw TD, Kimmel DB, Smith EL. A histomorphometric study of cortical bone activity during increased weight-bearing exercise. J Bone Miner Res 1990;5: 741–749.

35. Paley D, Young MC, Fornasier VL, Jackson RW. Percutaneous bone marrow grafting of fractures and bony defects: an experimental study in rabbits. Clin Orthop Relat Res 1989;240:270–280.

36. Connolly J, Guse R, Lippiello L, Dehne R. Development of an osteogenic bone-marrow preparation. J Bone Joint Surg Am 1989;71:684–691.

37. Beresford JN. Osteogenic stem cells and the stromal system of bone and marrow. Clin Orthop Relat Res 1989;240:270–280.

38. Hernigou P, Beaujean F. Bone marrow in patients with pseudarthrosis: a study of progenitor cells by in vitro cloning. Rev Chir Orthop Reparatrice Appar Mot 1997;83:33–40.

39. Connolly JF. Clinical use of marrow osteoprogenitor cells to stimulate osteogenesis. Clin Orthop Relat Res 1998;355:5257–5266.

40. Connolly JF. Injectable bone marrow preparations to stimulate osteogenic repair. Clin Orthop Relat Res 1995;313:8–18.

41. Hernigou P, Mathieu G, Poignard A, Manicom O, Beaujean F, Rouard H. Percutaneous autologous bone-marrow grafting for nonunions: surgical technique. J Bone Joint Surg Am 2006;88:322–327.

42. Hernigou P, Poignard A, Beaujean F, Rouard H. Percutaneous autologous bone-marrow grafting for Nonunions: influence of the number and concentration of progenitor cells. J Bone Joint Surg Am 2005;87:1430–1437.

43. Hernigou P, Poignard A, Manicom O, Mathieu G, Rouard H. The use of percutaneous autologous bone marrow transplantation in nonunion and avascular necrosis of bone. J Bone Joint Surg Br 2005;87:986–992.

44. Galois L, Bensoussan D, Dilignet J, et al. Autologous bone marrow graft and treatment of delayed and non-unions of long bones: technical aspects. Biomed Mater Eng 2009;19:277–281.

45. Connolly JF, Guse R, Tiedeman J, Dehne R. Autologous marrow injection as a substitute for operative grafting of tibial nonunions. Clin Orthop Relat Res 1991;266:259–270.

46. Garg NK, Gaur S, Sharma S. Percutaneous autogenous bone marrow grafting in 20 cases of ununited fracture. Acta Orthop Scand 1993;64:671–672.

47. Sim R, Liang TS, Tay BK. Autologous marrow injection in the treatment of delayed and non-union in long bones. Singapore Med J 1993;34:412–417.

48. Siwach RC, Sangwan SS, Singh R, Goel A. Role of percutaneous bone marrow grafting in delayed unions, non-unions and poor regenerates. Indian J Med Sci 2001;55:326–336.

49. Sugaya H, Mishima H, Aoto K, et al. Percutaneous autologous concentrated bone marrow grafting in the treatment for nonunion. Eur J Orthop Surg Traumatol 2014;24:671–678.

50. LeNail LR, Stanovick J, Fournier J, Splingard M, Domenech J, Rosset P. Percutaneous grafting with autologous bone marrow concentrate for open tibia fractures: analysis of forty three cases and literature review. Int Orthop 2014;38:1845–1851.

51. Bhandari M, Guyatt GH, Swiontkowski MF, Schemitsch EH. Treatment of open fractures of the shaft of the tibia. J Bone Joint Surg Br 2001;83:62–68.

52. Gaebler C, Berger U, Schandelmaier P, et al. Rates and odds ratios for complications in closed and open tibial fractures treated with unreamed, small diameter tibial nails: a multicenter analysis of 467 cases. J Orthop Trauma 2001;15:415–423.

53. Hernigou P, Homma Y, Flouzat-Lachaniette CH, Poignard A, Chevallier N, Rouard H. Cancer risk is not increased in patients treated for orthopaedic diseases with autologous bone marrow cell concentrate. J Bone Joint Surg Am 2013;95:2215–2221.

54. Gómez-Barrena E, Rosset P, Gebhard F, et al. Feasibility and safety of treating non-unions in tibia, femur and humerus with autologous, expanded, mesenchymal stromal cells associated with biphasic calcium phosphate biomaterials in a multicentric, non-comparative trial. Biomaterials 2019;196:100–108.

55. van der Vorst JR, van Dam RM, van Stiphout RS, et al. Virtual liver resection and volumetric analysis of the future liver remnant using open source image processing software. World J Surg 2010;34:2426–2433.

56. Ireland H, Gay MHP, Baldomero H, et al; International Society for Cellular Therapy (ISCT); Tissue Engineering and Regenerative Medicine International Society—Europe (TERMIS-EU); International Cartilage Repair Society (ICRS); International Federation for Adipose Therapeutics (IFAT); European Group for Blood and Marrow Transplantation. The survey on cellular and tissue-engineered therapies in Europe and neighboring Eurasian countries in 2014 and 2015. Cytotherapy 2018;20:1–20.

57. Phinney DG, Galipeau J; MSC Committee of The International Society of Cell and Gene Therapy. Manufacturing mesenchymal stromal cells for clinical applications: a survey of good manufacturing practices at US academic centers. Cytotherapy 2019;21:782–792.