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ABSTRACT

Background: ORTHO-1 is a European, multicentric, first in human clinical trial to prove safety and feasibility after surgical implantation of commercially available biphasic calcium phosphate bioceramic granules associated during surgery with autologous mesenchymal stromal cells expanded from bone marrow (BM-hMSC) under good manufacturing practices, in patients with long bone pseudarthrosis. *Methods*: Twenty-eight patients with femur, tibia or humerus diaphyseal or metaphyso-diaphyseal non-unions were recruited and surgically treated in France, Germany, Italy and Spain with 100 or 200 million BM-hMSC/mL associated with 5–10 cc of bioceramic granules. Patients were followed up during one year. The investigational advanced therapy medicinal product (ATMP) was expanded under the same protocol in all four countries, and approved by each National Competent Authority. *Findings:* With safety

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ATMP
Expanded autologous BM-hMSC
Non-union
Bone tissue engineering

as primary end-point, no severe adverse event was reported as related to the BM-hMSC. With feasibility as secondary end-point, the participating production centres manufactured the BM-hMSC as planned. The ATMP combined to the bioceramic was surgically delivered to the non-unions, and 26/28 treated patients were found radiologically healed at one year (3 out of 4 cortices with bone bridging). *Interpretation:* Safety and feasibility were clinically proven for surgical implantation of expanded autologous BM-hMSC with bioceramic. Funding: EU-FP7-HEALTH-2009, REBORNE Project (GA: 241876).

1. Introduction

Non-union occurs in 5%–20% of long-bone fractures that fail to heal properly after more than six months, causing morbidity, prolonged hospitalization, and considerable resource consumption [1] Non-union is frequently related to impairment of the biological potential of the fracture and biological augmentation is often required [1] The standard augmentation consists of autologous bone grafting surgically obtained from the same patient at a different location, most often from the iliac crest, and transplanted into the reconstruction site [2] The bone autograft fulfils the biological requirements to locally enhance bone regeneration. It contains extracellular matrix (osteoconduction), growth factors (osteoinduction) and cells (responsible for osteogenesis), thus allowing to fill the defect and to regenerate the bone [3] However, its remarkable drawbacks include the limited available amount without causing weakness of the donor structure; the scarce osteoprogenitors available in the harvested area, also depending on the age; the problems to re-harvest autograft because of subsequent fibrous tissue invasion; the associated patient complications (persistent pain, infection, scar, painful gait deriving in abnormality or limp) or even patient refusal to the procedure [4] Besides, the confirmed effectiveness of autograft to regenerate bone is moderate, particularly in complex scenarios as non-unions, with a current radiological success rate (in at least 3 views) of 74% and a combined clinical criteria success rate of 85% [2] Competing technology is not far from this success rate and OP-1 (BMP-7) recipients were found to sustain a 62% radiological healing rate and a clinical success rate of 81% [3] However, limited evidence and heterogenous methodology in the literature suggest that alternative solutions to enhance healing through bone regeneration in complex settings may require different approaches and further research [5] Culture-expanded autologous MSCs associated with biomaterials fulfil the requisites of osteogenesis and possibly osteoinduction (both related to cells), as well as osteoconduction (through the biomaterial), and have attracted significant attention [1,6].

A large variety of biomaterials have been clinically used to provide osteoconduction, although rarely tested in clinical trials. Among these, synthetic biphasic calcium phosphate (BCP) bioceramics composed of hydroxyapatite (HA) and beta-tricalcium phosphate (β-TCP) are currently used in clinical scenarios. Biomaterials approved for clinical use are better candidates for a clinical trial in combination with an Advance Therapy Medicinal Product (ATMP), such as expanded MSC. However, preclinical studies on the association of the selected biomaterial and the cell product under investigation are also required by regulatory agencies before a clinical trial can be launched.

A biphasic material composed of HA/β-TCP in a ratio of 20/80 in weight was chosen after it was implanted in critical size defects in mice [7] It was more efficient than equivalent macro and microstructure of different calcium phosphate bioceramics such as HA, β-TCP, BCP 60/40, 76/24, 63/37 and 56/44 [7,8] for combination with MSC before implantation.

Under the EU-FP7 REBORNE project, the BCP was supplied by Biomatlante (Vigneux de Bretagne, France) under the name of MBCP + ™ (Reference 1502 M + G05), which is CE marked material, Food and Drug Administration 510(k) approved. Under this project, preclinical studies were conducted to analyse the safety and efficacy of its association with MSC, including implantation in critical size defects [9] The *in vivo* experiments confirmed high colonization of the biomaterial by osteogenic cells [10] The association of BCP and BM-MSC did not affect MSC properties, in terms of immune effector cell proliferation, immune effector cells viability, and differentiation into osteoblast-like cells, suggesting that it could be used in bone defects treatment instead of bone allograft or autograft [11] Studies also showed that a minimum dose of cells of 10×10^6 per biomaterial cubic centimeter (cc) induced bone formation [12] Ectopic bone formation was observed under the skin of nude mice using a combined dose of 20 million hMSC per 1 cc of MBCP + granules [13] Mixing MSC with the biomaterial and allowing them to attach on its surface for 60 min consistently improved osteoinduction, if compared with isolated MSC [9].

The dose of BM-hMSC was strategically decided at 20 million BM-hMSC as per experimental studies. In view of the potentially variable requirements of biomaterial to fill the bone defect, the clinical protocol included a range of 100–200 million BM-hMSC to be supplied to the clinical centre and combined with 5–10 cc of BCP (1 or 2 syringes of biomaterial to combine with 1 or 2 syringes of expanded cells containing 100 million BM-hMSC each) (see Fig. 2).

This combination could regenerate a critical size bone defect in the skull of nude mice after 4 weeks [14] and could contribute to healing a critical size defect in long bone of nude rats after 8 and 16 weeks [9].

MSC biodistribution is also a major safety concern for clinical trials. Preclinical studies including qPCR for human Alu sequences proved absence of unwanted MSC homing after IV infusion [15] Subcutaneous implantation of MSCs with the scaffold showed no recirculation or homing in other organs. Moreover, histological analysis of different organs did not show any tumour formation [16].

In such context, this multicentric clinical trial was designed with safety as the primary endpoint, based on preclinical findings. Safety was evaluated as (1) local complication regarding the non-union treatment in the follow-up (FU) and (2) local and general complication in the 12 month FU of patients regarding potential effects of introducing the autologous hBM-MSC combined with BCP. A secondary endpoint was to prove the feasibility of fabricating MSCs using a single standardized protocol across multiple institutions. A tertiary endpoint was to get an estimate of the clinical efficacy that may be achieved using the combination of MSCs and this BCP scaffold, to enable powering of future studies. Clinical efficacy was estimated by the proportion of patients with proven bone healing (defined as 3 cortices with bone bridging out of 4, confirmed by imaging) at 3, 6 and 12 months FU.

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Table 1
Inclusion/Exclusion criteria.

No.	Description
Inclusion criteria	
1.	Age 18 to 65
2.	Both sexes (and efforts were placed to recruit females, if confirmation of negative pregnancy test was obtained the day before surgery and birth control methods were in place in case of fertile female patients)
3.	traumatic isolated closed or open humerus, tibia, or femur diaphyseal or metaphysodiaphyseal fracture status delayed union or non-union at least 3 months from acute fracture
4.	Signed informed consent
5.	Being able to provide the consent
6.	Able to understand and accept the study constraints
7.	Medical health care coverage, in any of the participating countries.
Exclusion criteria	
1.	Pregnancy, breast feeding women and women who are of childbearing age and not practicing adequate birth control
2.	Participation in another therapeutic trial in the previous 3 months
3.	Delayed union or non-union related to iatrogeny
4.	Segmental bone loss requiring specific therapy (like bone transport, large structural allograft, megaprosthesis, etc)
5.	Persisting vascular or neural injury
6.	Other fractures causing interference with weight bearing
7.	Acute persistent chronic bacterial infections such as brucellosis, typhus, leprosy, relapsing fever, meliodosis and tularemia
8.	Visceral injuries of diseases interfering with callus formation (craneoencephalic trauma, etc.)
9.	History of bone harvesting on iliac crest contraindicating bone-marrow aspiration
10.	Corticoid or immunosuppressive therapy more than one week in the three months prior to study inclusion
11.	History of prior or concurrent diagnosis of HIV-, Syphilis, Hepatitis-B- or Hepatitis-C-infection (confirmed by serology or PCR)
12.	History of neoplasia or current neoplasia in any organ
13.	Subject legally protected, under legal guardianship, deprived of their liberty by judicial or administrative decision, subject of psychiatric care, or admission to a health facility
14.	Impossibility to meet at the appointments for the follow up
15.	Insulin dependent diabetes
16.	Obesity (BMI > 30)
17.	Autoimmune inflammatory disease, current treatment by biphosphonate or stopped in the three months prior to study inclusion.

2. Methods

2.1. Study design and participants

ORTHO-1 was a phase I/IIa open, prospective, multicentric, non-comparative interventional clinical trial from the REBORNE EU-funded project (*Regenerating Bone defects using New biomedical Engineering approaches*, FP7 HEALTH-2009-1.4-2, Grant Agreement 241879) designed to evaluate safety and feasibility of autologous expanded Mesenchymal Stromal Cells (MSCs) from Bone Marrow (BM) associated to a bioceramic (MBCP + ™) in patients with long bone delayed unions and non-unions (after a minimum of 3 months from acute fracture). Inclusion and exclusion criteria for patient selection are listed in Table 1.

This was performed in five European centres from four countries, with total recruitment of 30 patients, 28 of whom received treatment (Fig. 1, CONSORT diagram) from March 2013 to February 2015.

During enrolment, the anonymous clinical data and imaging of the eligible patient were forwarded to the other clinical centres. Each patient was only included if no centre was against inclusion and at least two more centres agreed on the inclusion and the treatment. Enrolment of the first four patients was staggered with a minimum interval of two weeks after treatment of the previous patient, to identify any potential early safety problems that may have required terminating the trial. To further standardize the inclusion, patients to be included were those that alternatively would have received bone autograft and/or BMP, thus excluding patients with segmental defects.

Of the 30 recruited patients, one was excluded before bone marrow extraction as hepatitis B virus positive serology was confirmed. A second one was excluded because bone healing was identified before implantation surgery.

Of the 28 patients receiving treatment and analysed under ITT (intention-to-treat), two of them caused early drop-out (one at three months by patient decision, and the second at six months by surgeon decision and further treatment). In the remaining 26 patients, three cases showed protocol deviations (one required a second bone marrow extraction due to insufficient cell material in the first extraction; one required a second bone marrow extraction due to false positive Mycoplasma testing in the cell product in the first expansion; in the third, an oropharyngeal carcinoma was detected at intubation before cell implantation, the patient still receiving the previously cultured cells and followed until six months only due to exitus related to his cancer, ten months after implantation). Two out of 28 cases received 100 million cells in 5 cc of BCP, while 26 received the higher dose of 200 million cells in 10 cc of the biomaterial.

The treatment was performed at mean (range) 27.9 (3.9–163.3) months after the initial fracture and 14.7 (2.1–36.0) months after any previous treatment. Bone injuries that received treatment were located in the femur (11/28), humerus (4/28), and tibia (13/28). The initial fracture was closed in 17/28, and open fractures were seen in 11/28. Most common location was the distal third of the diaphysis (11/28), followed by the middle diaphyseal third (10/28). Fracture characteristics are further displayed in Table 2.

Safety analysis was planned for 12 months with intermediate evaluations at three and six months. A total of 28 patients were evaluated at three months, 27 at six months, and 25 patients were available for the final evaluation at 12 months on ITT.

3. Ethical issues

Four Ethics Committees of clinical research (CPP Tours Région Centre Ouest 1, Tours, France; La Paz Hospital CEIC, Madrid, Spain; Ulm University EC, Ulm, Germany; and Istituto Ortopedico Rizzoli EC, Bologna, Italy) approved the protocol and related documents for all participating clinical centres. As the investigational medicinal product (IMP) was an advanced therapy medicinal product (ATMP) for human use, the final responsibility to authorize the trial was set at the National Competent Authorities (ANSM, France; AEMPS, Spain; PEI, Germany; AIFA, Italy) as per Directive 2001/20/EC and

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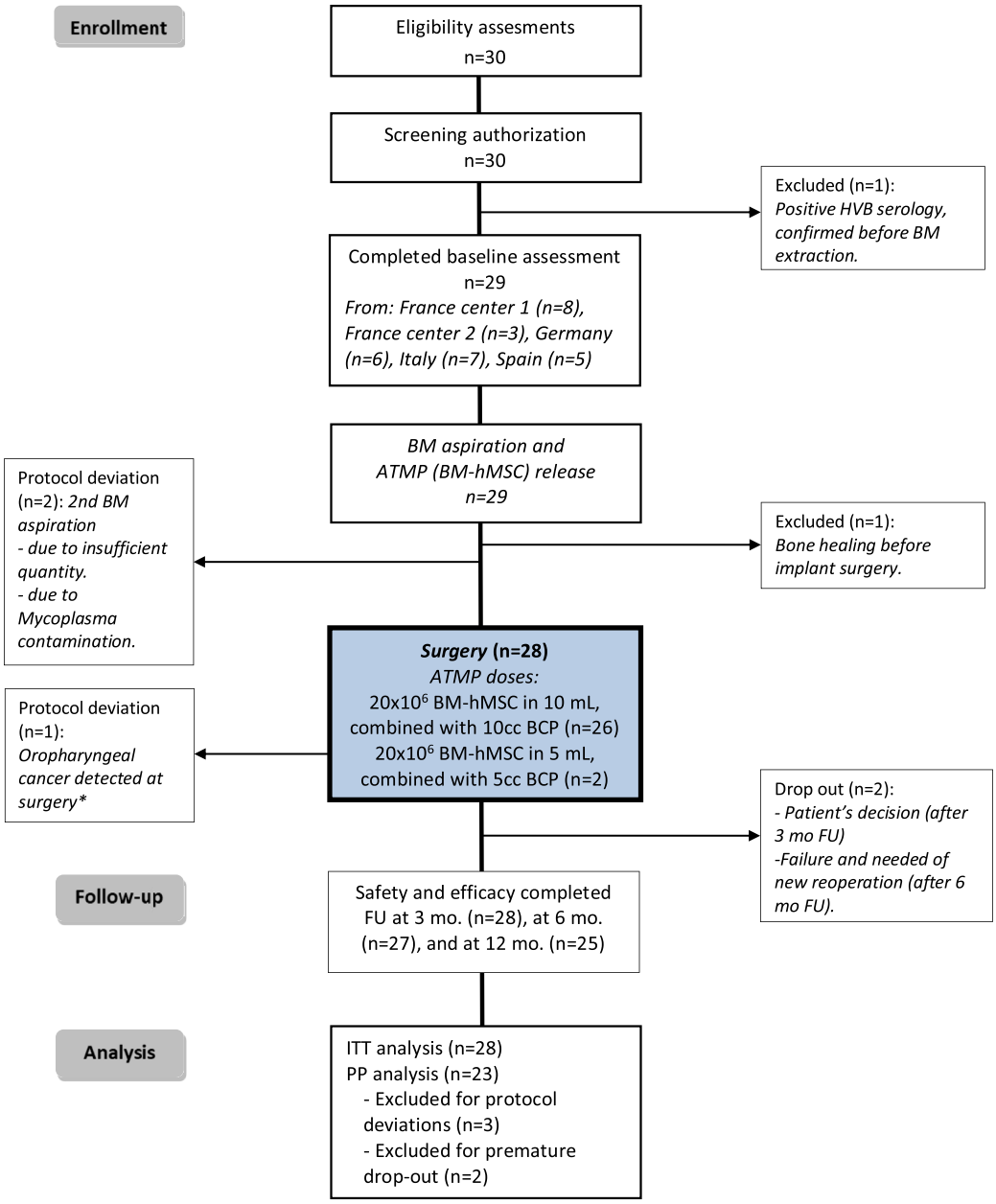

BM: Bone Marrow; ATMP: Advance Therapy Medicinal Product; BM-hMSC: Bone Marrow-derived human Mesenchymal Stromal Cells: BCP: Biphasic Calcium Phosphate bioceramic
** Patient excluded from protocol but treated, FU 6 mo.*

Fig. 1. CONSORT diagram of ORTHO1 clinical trial.

2005/28/EC of the European Parliament. The authorizations were obtained in all participating countries between January and March 2013. The sponsor of the study was INSERM, Paris, France. The EudraCT final number of the trial was 2011-005441-13 (in France, ID RCB number 2011-A00797-34), and the trial was also incorporated to the database ClinicalTrials.gov with the identifier NCT01842477.

4. Procedures

4.1. Bone marrow harvesting

Donation, procurement and testing of the BM were performed in compliance with the European Cells and Tissues Directives; in particular, according to the requirements laid down in Directive 2006/17/EC of 8 February 2006 implementing Directive 2004/23/EC of the European Parliament and of the European Council as regards certain technical requirements for the donation, procurement and testing of human tissues and cells, and applicable national laws. Specifically, patients needed to be negative in serology for Anti-HIV 1-2 Ab, Anti-HCV Ab, HBs Ag, Anti-HBc Syphilis, and negative (not detected by PCR) in HIV NAT, HCV NAT, or HBV NAT. The bone marrow cells were harvested in an operating room under anesthesia, with a trocar by cutaneous puncture from the posterior iliac crest. Bone marrow was harvested by fractions of 2–4 ml in 20 ml syringes heparinized to avoid clotting, then transferred into a bag for transportation prefilled with 5 mL of heparin (1000 IU/ml), and labeled according to the approved protocol. The harvest, in its

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Table 2
Fracture characteristics of ORTHO-1 patients.

Variables	ALL LONG BONES n = 28	
	n	(%)
Open/closed fracture		
Clean wound, wound <1 cm in length (Gustilo 1)	6	(21.4%)
Wound >1 cm in length w/o extensive soft-tissue damage (incl. flaps) (Gustilo more than 1)	5	(17.9%)
Closed fractures	17	(60.7%)
Fracture site		
Proximal metaphysis or metaphyso-diaphysis	1	(3.6%)
Diaphyseal proximal third	4	(14.3%)
Diaphyseal middle third	10	(35.7%)
Diaphyseal distal third	11	(39.3%)
Distal metaphysis of metaphyso-diaphysis	2	(7.1%)
Bone loss/gap		
No	17	(60.7%)
Yes, <50% diaph. Diam	7	(25.0%)
Yes, >50% diaph. Diam	4	(14.3%)
AO diaphyseal fracture classification	**25**	**(80.3%)**
A. Simple	**6**	**(21.4%)**
A1. Spiral	1	(3.6%)
A2. Oblique >30°	1	(3.6%)
A3. Transverse	4	(14.3%)
B. Wedge	**9**	**(32.1%)**
B1. Spiral	1	(3.6%)
B2. Bending wedge	5	(17.9%)
B3. Fragmented w/corner	3	(10.7%)
C. Complex	**10**	**(35.7%)**
C1. Spiral	2	(7.1%)
C2. Segmental	2	(7.1%)
C3. Irregular	6	(21.4%)
AO metaphyseal fracture classification	**3**	**(10.7%)**
A. Simple		
A1. Simple	1	(3.6%)
A2. Wedge	0	(0%)
A3. Complex	2	(7.1%)
Complex fracture	**0**	**(0%)**

primary packaging, was laid out in an isothermal box labeled ac'ording to Directive 2004/23/EC and 2006/17/EC. The transport was done under controlled temperature between 18 and 24 °C if less than 30 min, and at 4 °C with temperature traceability if the transportation time was longer than 30 min.

4.2. Cell product manufacturing process in GMP facilities

The Investigational Medicinal Product (IMP) was composed of human mesenchymal stromal cells from the bone marrow, obtained through a manufacturing process based on plastic adherence, and expanded in culture according to a culture method developed by the REBORNE consortium using 5% human donor platelet lysate produced in Ulm (Germany) and distributed to the other cell therapy units. MSC were defined by a specific immunophenotype expressing the markers CD90, CD73, CD105 and negative for CD14, CD45 and HLA-DR [17] with demonstrated osteogenic properties *in vitro* and *in vivo*. The IMP manufacturing authorization was granted to all five participating GMP facilities (Établissement Français du Sang −EFS- in Toulouse and in Créteil, both in France; Transfusion Medicine Institute of Ulm in Germany; Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico of Milano in Italy; and Cell Production Unit at Hospital Puerta de Hierro-Majadahonda of Madrid in Spain).

An aliquot of starting material was removed to carry out controls including cell count, viability, CFU-F-assay, and sterility. The entire manufacturing process was performed in each manufacturing site, after previous validation in all sites with 22 validation runs confirming the compliance of all the validation batches to the pre-defined specifications. In brief, the culture was performed when the received BM, without any further manipulation, was seeded in alpha-MEM medium with 5% PL and 1U/mL of heparin, at the concentration of 50.000 WBC/cm^2, in a culture chamber. The culture chambers were placed in incubators (5% CO_2, 20% oxygen atmosphere, 95% relative humidity at 37 °C). After 72 h, the supernatant was discarded and replaced by fresh complete medium (alpha-MEM with 5% PL). At days +7 and + 10 of culture, the supernatant was discarded and replaced by complete medium. At +14 day, the confluence was evaluated and, if >50%, the cells were washed with PBS detached and harvested using trypsin (TrypZean solution from recombinant corn − free of animal component− at a concentration of 1×, incubation time from 5 to 10 min). If cell confluence was lower than 50%, an additional medium exchange was performed and cells were harvested at day 17. The harvested cells were then re-seeded at the concentration of 4×10^3 MSC per cm^2 in new culture chambers in alpha-MEM medium with 8% PL. Another medium exchange was performed at day 17. At day 21, the cell culture was washed with PBS and the cells detached and harvested using trypsin. The final product resulting in a dose of 20×10^6 cells per mL was suspended in 5% human albumin up to 10 mL to obtain the ORTHO-1 BM MSC tissue-engineered product. The final active product was packaged for the shipment to the operating room (the delivery contained one or two syringes of 5 mL each). A suitable mode of transportation ensured delivery of the package of BM to the manufacturing site and the expanded cells from the GMP facility to the surgical room within 18 h, and the process was validated for cell viability [10,12].

Each batch of the final product was tested for cell content, immunophenotype, sterility, endotoxins and Mycoplasm before release. Additional quality controls were performed according to each country specific national competent requests.

All the materials and reagents were selected and validated to ascertain their compliance to be used in the manufacturing process, with certificates of analysis of key components included in the IMP for which approval was obtained at each of the National Competent Authorities (NCA) of the participating countries.

4.3. Biomaterial and cell product associated during surgery

The selected calcium phosphate biomaterial (MBCP+, Biomatlante SA, Vigneux de Bretagne, France) was a 100% synthetic CE-marked, FDA (510 k) approved bone substitute, composed of 20% Hydroxyapatite (HA) and 80% beta tricalciumphosphate (β-TCP), in 1−2 mm granules. The material had a total porosity of 73%, constituted of macropores (>100 μm), mesopores of 10−100 μm and micropores (<10 μm) content of about 40%. The crystal size was <0.5−1 μm with a specific surface area around 6 m^2/g. The cell-biomaterial association was performed in the surgical room before implantation (Fig. 2).

Standard operating procedure for mixing autologous MSCs and MBCP + ™ granules in the operating room during surgery. a) Syringes of expanded cells and biomaterial in the surgical setting. b) Combination of both, before implantation.

Preclinical studies with BM-MSC at a concentration of 20×10^6 MSC/mL seeded on 1 cc of MBCP + granules confirmed that less than 5% cells remained in the supernatant or attached to the container after 60 min, and that cells on the BCP granules were capable of forming bone [12].

Microscopy with crystal violet corroborated that cells were attached to the biomaterial [10] within 60 min, and live/dead assay indicated that MSCs were alive on the biomaterial with very few dead cells (Fig. 3a), showing uniform distribution of high cell density on the granules with methylene blue staining (Fig. 3b).

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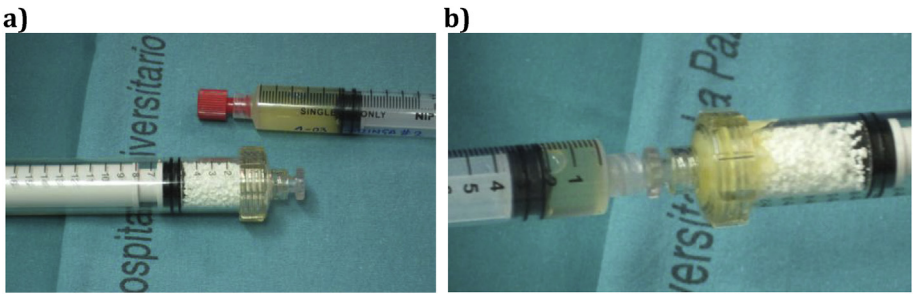

Fig. 2.

Surgical procedure and cell implantation, showing a) attachment of MSCs on the biomaterial after 1 h as prepared in the surgical room (methylene blue staining, magnification ×100, scale bar: 500 μm); b) Viability of MSC on BCP granules after 1 h (live/dead assay, fluorescence microscopy magnification ×50, scale bar: 500 μm; Per protocol, cells were associated to the biomaterial during surgery. Each 5 mL of cell product syringe (cell dose of 20×10^6/mL) was mixed with 5 cc of biomaterial.

4.4. Surgical procedure

The non-union site was surgically prepared through a standard surgical approach adapted to the anatomic location to receive the biomaterial and cell product. This preparation included ablation of necrotic free bone fragments, excision of fibrous tissue and/or decortication of bone ends to bleeding bone. The cell-biomaterial combination product had a pasty consistency and was spread to the full extent of the prepared volume.

5. Outcomes

5.1. Safety

To clinically confirm the preclinical safety studies that observed absence of toxicity or dissemination or histopathological detection of any human tumour development (GLP preclinical study number 110068 from Oncodesign, Dijon, France), a primary safety end-point, defined as detection of local and general complications, was fixed for the clinical trial. This end-point was set at any time in the 12 months of follow-up, but specifically required staggering the first four patients by two weeks, adverse event (AE) reporting at three, six, and 12 months, severe adverse event (SAE) and suspected unexpected and serious adverse reactions (SUSAR) reporting at any time, as required by the regulatory frame (to Eudravigilance - European Union Pharmacovigilance database-, to the competent authorities and to the Ethics Committees). The identification and report of each AE, SAE and SUSAR was made by the physicians/researchers in each clinical centre coordinated by an external Clinical Research Organization who was responsible of monitoring the recorded data into the CRF and to release the final database. The assessment was conducted by the consortium clinical trial research group.

5.2. Feasibility and early efficacy

Feasibility was assessed in treatment preparation (BM harvest, cell expansion and delivery to the surgical room) and administration (combination with the biomaterial and implantation) in a multicentric international setting (both for cell production and implantation).

Early efficacy was clinically and radiologically evaluated at 3, 6 and 12 months follow-up (X-Rays and CT imaging was performed per protocol during the FU of the clinical trial). Clinical efficacy (Yes/No) was considered when all the three following criteria were met: Radiological bone bridging in 3 out of 4 cortices, on at least ¾ views; pain less than 3 (Likert scale from 0 to 10); no further surgical intervention in the callus site (as nail or plate replacement, or replacement of all components of the previous surgery). The radiological bone bridging was assessed by an external adjudication committee using blinded images.

6. Histology

Two of the cases requiring secondary surgical procedure (screw extraction in locked nails) provided bone biopsies from the non-union site about four months (111 days) and about eight months (309 days) after cell product implantation. Bone cylinders were percutaneously obtained under fluoroscopy at the time of secondary surgery. These bone biopsies were fixed in formaldehyde 10% and processed for decalcified histology as previously described

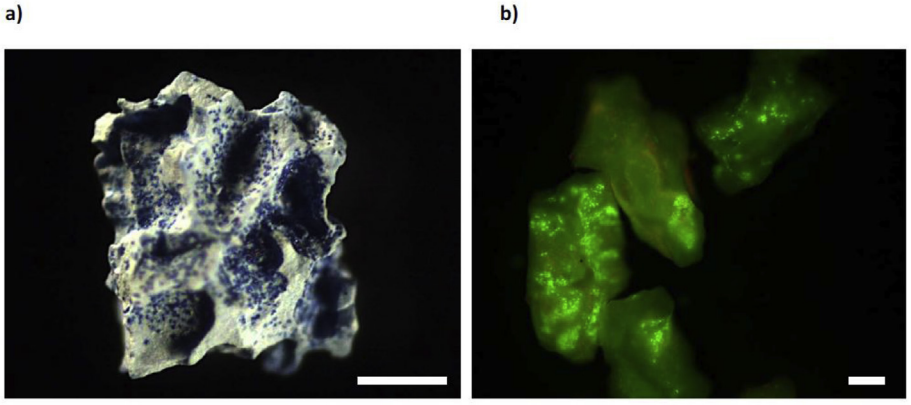

Fig. 3.

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Table 3
ORTHO-1 adverse events.

Patient id	FU days	Description of the Adverse Event (AE)	Severity	Status*	IMP relationship	AE treated	Duration (days)	Outcome*
502	-27	Fever after BM aspiration	Mild	Ceased	Definitely not	No	5	Recovered
201	0	Ophthalmic migraine	Mild	Ceased	Definitely not	No	0	Recovered
401	8	Bacterial tonsillitis	Moderate	Ceased	Definitely not	Yes	5	Recovered
103	**17**	**Superficial infection related to musculocutaneous vascularized flapin surgical wound ****	**Mild**	**Ceased**	**Definitely not**	**Yes**	**17**	**Recovered**
404	31	Common cold	Mild	Ceased	Definitely not	Yes	7	Recovered
502	34	Superficial infection of wound at the operation site	Moderate	Ceased	Definitely not	No	7	Recovered
402	**42**	**Fatigue failure of implant****	**Severe**	**Ceased**	**Definitely not**	**Yes**	**2**	**Recovered**
104	**56**	**Superficial infection related to musculocutaneous vascularized flapin surgical wound ****	**Mild**	**Ceased**	**Definitely not**	**Yes**	**7**	**Recovered**
201	77	Rhinitis	Mild	Ceased	Definitely not	Yes	7	Recovered
401	82	Allergic reaction to unknown component	Severe	Ceased	Probably not	Yes	1	Recovered
104	94	Superficial infection related to musculocutaneous vascularized flapin surgical wound	Mild	Ceased	Definitely not	Yes	68	Recovered
101	100	Tendinitis in affected leg	Moderate	Ceased	Definitely not	Yes	11	Recovered
101	108	Influenza	Mild	Ceased	Definitely not	Yes	9	Recovered
105	111	Intervention to change distal locking screws	Mild	Ceased	Definitely not	Yes	1	Recovered
206	117	Foot numbed	Mild	Ceased	Probably not	No	7	Recovered
404	143	Bacterial sinusitis	Moderate	Ceased	Definitely not	Yes	38	Recovered
404	143	Trigeminal neuralgia	Moderate	Ceased	Definitely not	Yes	38	Recovered
402	157	Breakage of proximal locking screw	Moderate	Ceased	Definitely not	No	4	Recovered
203	176	Keratoseseborrheique	Mild	Ceased	Definitely not	No	0	Recovered
406	**188**	**Gastric bleeding****	**Moderate**	**Ceased**	**Definitely not**	**Yes**	**4**	**Recovered**
201	302	Diarrhea	Mild	Ceased	Definitely not	Yes	4	Recovered
103	309	Intervention to remove proximal screw nail dynamization	Mild	Ceased	Definitely not	Yes	1	Recovered
201	335	Vertigo	Mild	Ceased	Definitely not	Yes	2	Recovered

IMP: Investigational medicinal product
*Status/Outcome at the end of the study; **Serious adverse event properly reported (Requiring in-patient hospitalization).

[12,13] Thin sections were stained with hematoxylin/eosin and Masson trichrome. Immunohistology was performed to identify macrophages with human CD68 primary antibody (mouse anti-human, 1/100, MCA1815T, AbdSerotec, Oxford, UK), revealed by secondary antibody (goat anti-mouse biotinylated, 1/400, E0433, Dako, Les Ulis, France), counterstained using Gill's hematoxylin solution and covered with cover slips. Sections were viewed using the Nanozoomer 2.0 Hamamatsu slide scanner (NanoZoomer; Hamamatsu, Photonics, Hamamatsu City, Shizuoka Prefecture, Japan).

7. Statistical analysis

Descriptive statistics were performed using STATA software version 12 (StataCorp. 2011).

8. Role of funding source

Institutional public funding obtained in a competitive European Union public call sustained all preclinical and clinical research. The funding source did not influence any of the results or had any responsibility in the study design, collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the paper for publication. The corresponding author confirms that he had full access to all the data in the study and had final responsibility for the decision to submit for publication.

9. Results

9.1. Safety

No adverse event, or SAE, or SUSAR, were identified in relationship with the IMP. Particularly, no tumorous condition or cell-related overgrowth was detected in any patient after cell implantation.

A total of 19 adverse events (AE) were reported (Table 3), 15 of them were considered mild to moderate (fever, ophtalmic migraine, tonsillitis, superficial wound infection, rhinitis, sinusitis, flu, trigeminal neuralgia, vertigo, diarrhea, breakage of locking screws requiring screw change, screw removal, tendinitis, residual valgus knee deformity). A total of four severe adverse events were reported requiring hospitalization, also with full recovery (Appendix). These included superficial infection of the wound in two patients that previously had musculocutaneous vascularized flaps in previous surgeries to treat the initial fracture, leaving a severe atrophic scar and a compromised skin in the surgical approach. Both required hospitalization to undergo intravenous antibiotic treatment and both fully resolved without sinus tract, drainage or surgical intervention into the non-union site. Apparently unrelated gastric bleeding occurred in a patient 188 days after surgery who fully recovered four days after hospital admittance. Finally, one patient required intramedullary nail exchange (without opening the fracture site) due to fatigue failure of the implant, 42 days after cell implantation in which no nail modification was originally performed (the nail was apparently stable although in place for the previous seven months).

Bone biopsies after histopathological evaluation confirmed bone formation surrounding the BCP granules with the attached, expanded MSCs delivered into the non-union site at surgery.

Lamellar bone and osteoid tissues were found at the vicinity of the BCP granules. Multinucleated giant cells labeled by the anti-body CD68 and TRAP staining were primarily located surrounding the BCP biomaterial. These cells were considered osteoclasts with a pivotal role in bone regeneration, as demonstrated in preclinical studies [13,18].

9.2. Feasibility and early efficacy

After appropriate protocol validation in all the participating production centres, multinational authorization was obtained. This fact reinforces the feasibility of autologous cell expansion as a viable clinical, large-scale option. Descriptive cell values, along the process of expansion and at release of the cell product in the treated cases, are included in Table 4.

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Table 4
Cell expansion and final cell product.

Variables	Mean ± n/N	SD (%)	(Min-	Max)
BM aspiration				
Aspirated bone marrow volume	50.4 ±	18.6	(22.5-	90.0)
WBC concentration (x10^6/mL)	26.1 ±	11.3	(11.2-	61.0)
Seeded				
No. of 2-chamber Cell stack layer seeded	3.5±	1.7	(2.0-	7.0)
Total volume of seeded BM (mL)	9.7 ±	5.3	(2.6 -	23.8)
Total No. seeded WBC (x10^6)	221.6 ±	106.1	(127.0 -	445.2)
Viability of starting material (%)	96.5 ±	2.9	(87.2–	100)
Clonogenicity: (CFU–F/10^6 MSC seeded)	47.0±	33.0	(4.0–	122.0)
Microbialtesting (No Growth)	28/28	(100%)		
Reseeded at P0				
Culture duration ofp0 (days)	13.6 ±	1.1	(10.0-	15.0)
No. of harvested MSC (x10^6)	161.1 ±	136.6	(16.8-	500.0)
Viability (%)	95.8 ±	4.4	(80.2-	100.0)
Clonogenicity: (x10^3 CFU-F/10^6 MSC seeded)	198.4±	140.0	(0.0-	500.0)
No. 2-chamber cell stack seeded	5.7±	1.7	(3.0-	8.0)
Microbial testing at P0 (No Growth)	28/28	(100%)		
Reseeded at P1				
Culture duration of p1 (days)	6.7 ±	0.8	(5.0-	9.0)
No. of MSC harvested (x10^6)	352.7 ±	128.9	(95.2-	690.0)
Viability (%)	97.5 ±	2.3	(91.8 -	100.0)
Clonogenicity: (x10^3 CFU-F/10^6 MSC seeded)	162.3±	158.8	(2.0-	500.0)
Microbial testing at P1(No Growth)	28/28	(100%)		
Additional Quality control data				
Flow cytometry:				
Acquisition of CD3 performed (n = 13)	0.0 ±	0.1	(-0.3 -	0.4)
Acquisition of MHC class I performed (n = 19)	98.1 ±	3.6	(85.7-	100.0)
Karyotyping performed w/Findings	0/22	(0%)	–	
Mycoplasma screening w/Findings	0/22	(0%)	–	
Endotoxin test performed	0/19	(0%)	–	
MSC production release criteria				
$CD90^+$ (%)	99.1 ±	1.6	(92.2 -	100.0)
$CD73^+$ (%)	97.9 ±	2.8	(89.9 -	100.0)
CD105+ (%)	97.6 ±	2.6	(86.7 -	99.9)
$CD45^+$ (%)	0.1 ±	0.1	(0.0 -	0.5)
HLA DR (%)	0.8 ±	1.4	(0.0 -	6.0)
Viability (%)	97.5 ±	2.3	(91.8 -	100.0)

Treatment preparation and delivery was successfully performed in 28 patients from 4 countries, 5 GMP cell production centres and 5 clinical centres, and this confirms the feasibility of the treatment application in different settings. Following the definition of radiological bone healing (bridging of 3 out of 4 cortices), 26/28 (92%) of the treated bone injuries were considered consolidated at 12 months post-implantation surgery.

9. Discussion

First accomplishment in this trial included the set-up of identical GMP fabrication procedure at 5 sites in four countries, with strict release and safety screening criteria of culture expanded cells that meet the definition of MSCs, together with a consortium of treatment centres to deliver and evaluate treatment of long-bone fracture non-unions. Second, this non-comparative trial achieved fabrication and treatment delivery of 20 million BM-hMSC per mL (100–200 million MSC in 5–10 mL) in 28 attempts (two patients needed to be re-aspirated, and the process repeated), confirming feasibility and demonstrating the absence of adverse events using a total dose of cells (100–200 million MSC) that is higher than the dose generally used in prior studies.

This high dose safely, successfully, and consistently obtained clinical and radiological consolidation of femoral, tibial, and humeral non-unions. The dose of MSCs provided to a site may be a critical variable in the success of therapy. A relationship between cell dose and clinical success has been previously proven in other settings, such as the concentration of marrow derived nucleated cells using density separation [19] This may also be the case with expanded MSCs. The biological properties of the cells in a defect site may also have an impact on bone regeneration. At least one study has shown that MSCs generated from cells harvested from a non-union site, [20] have lower proliferative capability than MSCs that are fabricated using cells harvested from native bone marrow [21] The minimal required dose is currently unknown. Therefore, the appropriate number of cells may require more studies. Current uncertainty may justify the underreporting of results from many registered clinical trials.

This study accomplished its primary and secondary goals of demonstrating the feasibility and the safety of MSCs as a treatment alternative for fracture non-union.

Feasibility was demonstrated by establishing a consortium for multicentric production of MSCs using a common standard operating procedure (SOP) for generating a culture expanded MSC product at five GMP facilities from four European countries. The established SOP included rigorous safety screening for the end product based on cell content, immunophenotype, sterility, endotoxins and karyotype. Based on intent to treat, MSCs were successfully fabricated in 93% of patients on the first attempt. Two patients who failed in the initial fabrication were successfully served by a second aspiration and fabrication procedure.

Safety was demonstrated in 28 patients, in whom no adverse events could be attributed directly or indirectly to the use of MSCs in their care.

While this study was not designed as a test of clinical efficacy, 26 of 28 patients demonstrated radiographic union during follow-up (bridging 3 of 4 cortices). This represents a rate of clinical success that is higher than most series of this type and severity, and provides reassurance that MSCs at the used dose (20 million cells per 1 cc of graft material) may provide therapeutic benefit.

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The total dose of 200 million cells is higher than most prior studies. The use of this dose was a strategic decision to minimize the risk that the selected dose would be sub-therapeutic, while also enhancing the strength of our safety assessment for MSCs. Prior studies of the use of culture expanded MSCs have often been equivocal in cell dose and outcomes, as previously reviewed [1,6].

The choice of a biphasic calcium phosphate ceramic was also strategic. We believe that many biomaterials can successfully attach cells and help to deliver them into the injury. We selected to combine cells and material in the surgical room to isolate the cell production technology issues from the potential biomaterial influence in cell expansion. To play it safe, we selected a well-tested biphasic ceramic in clinical use with proved cell attachment properties and osteoconductive properties that are appropriate to support a positive MSC effect on bone healing. Furthermore, we used a particulate material to better adapt to the non-union gap. A major future challenge for any biomaterial proposal combined with ATMP would be to further undergo into comparative clinical trials, to prove superiority about the current gold standard for bone healing augmentation, such as bone autograft.

In conclusion, this study provided feasibility evidence of GMP, multicentric, equivalent cell production of expanded BM-hMSC at a dose up to 200 million cells. Furthermore, it proved multicentric feasible and safe surgical delivery of this cell product in combination with 5–10 cc of BCP to treat long-bone fracture non-union, without adverse events related to the cell product. Bone healing obtained through this method and through a multicentric collaboration may help in the near future to compare the efficacy of this strategy with that of current clinical standards such as autograft, to determine the appropriate cell dose, or to examine the relative efficacy of MSC delivery in alternative biomaterial scaffolds.

Conflicts of interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Conceived and designed the protocol

EGB, PR, PH, FG, NB.

Performed and reported the clinical work (surgery, data collection, reporting)

EGB, PR, PH, FG, NB, JS, CE, GC, EGR, JCA, JCRS, CHFL, DMD, MHL, CAS

Contributed to the development, validation and preparation of the cell product

HS, HR, LS, RMG, RG, MTR, RL, NC, SF, MD, MNF, JRC, TM, EV

Analysed the data, wrote the paper

EGB, NPE, PR, PH, FG, NB JS, CE, GC, HR, LS, RMG, RG, CP, MD, HS, PL

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.biomaterials.2018.03.033.

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