Injury 51 (2020) S63–S73

Contents lists available at ScienceDirect

Injury

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

Early efficacy evaluation of mesenchymal stromal cells (MSC) combined to biomaterials to treat long bone non-unions☆

Enrique Gómez-Barrena[a,*], Norma Padilla-Eguiluz[a], Philippe Rosset[b], Florian Gebhard[c], Philippe Hernigou[d], Nicola Baldini[e], Helène Rouard[d,f], Luc Sensebé[f,g], Rosa-María Gonzalo-Daganzo[h], Rosaria Giordano[i], Eduardo García-Rey[a], José Cordero-Ampuero[j], Juan Carlos Rubio-Suárez[a], Marta Dominguez García-Simón[a], Julien Stanovici[b], Christian Ehrnthaller[c], Markus Huber-Lang[c], Charles Henri Flouzat-Lachaniette[d], Nathalie Chevallier[d,f], Davide Maria Donati[e], Benedetta Spazzoli[e], Gabriela Ciapetti[e], Sandrine Fleury[f,g], Manuel-Nicolás Fernandez[h], José-Rafael Cabrera[h], Cristina Avendaño-Solá[h], Tiziana Montemurro[i], Carmen Panaitescu[k], Elena Veronesi[l], Markus Thomas Rojewski[m], Ramin Lotfi[m], Massimo Dominici[l], Hubert Schrezenmeier[m], Pierre Layrolle[n]

[a] *Servicio de Cirugía Ortopédica y Traumatología, Hospital Universitario La Paz-IdiPAZ and Facultad de Medicina, Universidad Autónoma de Madrid, P° Castellana 261, 2804, Madrid, Spain*
[b] *Service de Chirurgie Orthopédique et Traumatologique 2, Hôpital Trousseau, Université François-Rabelais de Tours, CHU de Tours, Tours, France*
[c] *Department of Traumatology, Hand-, Plastic-, and Reconstructive Surgery, Center of Surgery, University of Ulm, Ulm, Germany*
[d] *Orthopaedic Department, Hôpital Henri Mondor, InsermU955 and UPEC (University Paris-Est, Créteil), Créteil, France*
[e] *Laboratory for Orthopaedic Pathophysiology and Regenerative Medicine, Istituto Ortopedico Rizzoli, and Dept. of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy*
[f] *Établissement Français du Sang, Paris, France*
[g] *STROMA Lab, UMR5273-INSERM U1031, Toulouse, France*
[h] *Servicio de Hematología, Hospital Universitario Puerta de Hierro-Majadahonda, and Universidad Autónoma de Madrid, Madrid, Spain*
[i] *Cell Factory, Center for Cellular Therapy and Cryobiology, Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milano, Italy*
[j] *Servicio de Cirugía Ortopédica y Traumatología, Hospital Universitario La Princesa and Facultad de Medicina, Universidad Autónoma de Madrid, Madrid, Spain*
[k] *Victor Babes University of Medicine and Pharmacy, Timisoara, Romania*
[l] *Laboratory of Cellular Therapies, Department of Medical and Surgical Sciences for Children & Adults, University - Hospital of Modena and Reggio Emilia, Modena, Italy*
[m] *Institut for Transfusion Medicine, Ulm University, and Institute for Clinical Transfusion Medicine and Immunogenetics Ulm, German Red Cross Blood Transfusion Service and University Hospital Ulm, Ulm, Germany*
[n] *INSERM U957, Lab. Pathophysiology of bone resorption, Faculty of Medicine, University of Nantes, Nantes, France*

ARTICLE INFO

Article history:
Accepted 15 February 2020

Keywords:
Non-union
Long Bone
Treatment
MSC
Bioceramics
Efficacy
Clinical Consolidation
Radiological Consolidation

ABSTRACT

Background and study aim: Advanced therapy medicinal products (ATMP) frequently lack of clinical data on efficacy to substantiate a future clinical use. This study aims to evaluate the efficacy to heal long bone delayed unions and non-unions, as secondary objective of the EudraCT 2011-005441-13 clinical trial, through clinical and radiological bone consolidation at 3, 6 and 12 months of follow-up, with subgroup analysis of affected bone, gender, tobacco use, and time since the original fracture.

Patients and methods: Twenty-eight patients were recruited and surgically treated with autologous bone marrow derived mesenchymal stromal cells expanded under Good Manufacturing Practices, combined to bioceramics in the surgical room before implantation. Mean age was 39 ± 13 years, 57% were males, and mean Body Mass Index 27 ± 7. Thirteen (46%) were active smokers. There were 11 femoral, 4 humeral, and 13 tibial non-unions. Initial fracture occurred at a mean ± SD of 27.9 ± 31.2 months before recruitment. Efficacy results were expressed by clinical consolidation (no or mild pain if values under 30 in

☆ This paper is part of a Supplement supported by The Orthopaedic Surgery and Traumatology Spanish Society (SECOT).

* Corresponding author.
E-mail addresses: egomezb@salud.madrid.org, enrique.gomezbarrena@uam.es (E. Gómez-Barrena).

https://doi.org/10.1016/j.injury.2020.02.070

VAS scale), and by radiological consolidation with a REBORNE score over 11/16 points (value of or above 0.6875). Means were statistically compared and mixed models for repeated measurements estimated the mean and confidence intervals (95%) of the REBORNE Bone Healing scale. Clinical and radiological consolidation were analyzed in the subgroups with Spearman correlation tests (adjusted by Bonferroni).

Results: Clinical consolidation was earlier confirmed, while radiological consolidation at 3 months was 25.0% (7/28 cases), at 6 months 67.8% (19/28 cases), and at 12 months, 92.8% (26/28 cases including the drop-out extrapolation of two failures). Bone biopsies confirmed bone formation surrounding the bioceramic granules. All locations showed similar consolidation, although this was delayed in tibial non-unions. No significant gender difference was found in 12-month consolidation (95% confidence). Higher consolidation scale values were seen in non-smoking patients at 6 ($p = 0.012$, *t*-test) and 12 months ($p = 0.011$, *t*-test). Longer time elapsed after the initial fracture did not preclude the occurrence of consolidation.

Conclusion: Bone consolidation was efficaciously obtained with the studied expanded hBM-MSCs combined to biomaterials, by clinical and radiological evaluation, and confirmed by bone biopsies, with lower consolidation scores in smokers.

Introduction

Non-union represents a serious fracture complication due to associated morbidity, repeated hospitalization, and significant resource consumption. Its incidence is highly variable but may represent over 5% of long-bone fractures. Fracture evolution to delayed union and non-union is related to timely healing failure. Although the criteria are debatable, certain consensus has been reached in defining the non-union if bone healing is not obtained after more than nine months since the fracture, with more than 3 months without bone healing progression [1]. The physiological mechanism of bone healing [2], supported by current treatments, is capable of solving over 90% of bone injuries. In case of failure to complete bone healing, pain and disability subsequently impair the patient's quality of life and limit patient's work activities.

Complex fractures, associated to severe traumatic injuries such as traffic accidents [3], frequently produce secondary limitations, ranging from 40 to 70% of patients [4]. Functional capacity and quality of life in survivors of severe injuries often do not recover even one year after trauma, female gender and comorbidity being predictors of long-term disability.

The development of alternative solutions to enhance healing through bone regeneration [5], particularly in complex settings such as non-unions, is a challenging aim with modest results and scarce clinical confirmation so far [6]. Treatment based on culture-expanded autologous mesenchymal stromal cells (MSC) associated with biomaterials fulfils the requisites of osteogenesis, osteoinduction and osteoconduction. However, the literature and the declared clinical trials show not only a highly variable methodology, but also underreported results [6]. At this point, data may be insufficient to define and support the clinical application of regenerative medicine solutions in patients, and more clinical information on efficacy is required to understand treatment indications.

In this context, a phase I/IIa open, prospective, multicentric, non-comparative interventional clinical trial (EudraCT 2011-005441-13) was recently completed to evaluate safety and feasibility as primary endpoints [7]. This trial evaluated an Advanced Therapy Medicinal Product –ATMP– (autologous expanded human Mesenchymal Stromal Cells –hMSCs– from Bone Marrow –BM–) combined to a CE-marked bioceramic in patients with long bone fractures status delayed union and non-union. The ATMP (between 100×10^6 and 200×10^6 expanded hBM-MSC) was produced in manufacturing centers approved by the National Competent Authority of each participating country, following the same Good Manufacturing Practice (GMP) protocol. Primary outcome results confirmed that no severe adverse events were considered related to the ATMP. No tumorous condition or cell related overgrowth was detected in patients after cell implantation. From the cell production perspective, feasibility evidence of GMP, multicentric, equivalent cell production of expanded BM-hMSC was confirmed and reported [7]. However, efficacy as a secondary endpoint and related to patient and fracture characteristics may offer important information to better understand the possibilities of this treatment.

This study aims to evaluate the efficacy (secondary objective) to heal patients with long bone delayed unions and non-unions (minimum of 3 months after the acute fracture) in the EudraCT 2011-005441-13 clinical trial. Efficacy was evaluated through the analysis of clinical and radiological bone consolidation at 3, 6 and 12 months of follow-up, with subgroup analysis uncovered by the clinical trial.

Patients and methods

Twenty-eight patients were recruited and surgically treated (Fig. 1) with GMP expanded hBM-MSCs combined to MBCP+^{TM}, a 100% synthetic CE-marked (Biomatlante, Vigneux, France) bone substitute composed of 20% Hydroxyapatite (HA) and 80% beta tricalcium phosphate (ß-TCP), in 1-2 mm granules. The cell-biomaterial association was performed in the surgical room before implantation. Treatment was performed within the ORTHO1 clinical trial (EudraCT 2011-005441-13) incorporated to the database ClinicalTrials.gov with the identifier NCT01842477. Four Ethic Committees of clinical research (La Paz Hospital CEIC, Madrid, Spain; CPP Tours Région Centre Ouest 1, Tours, France; Ulm University EC, Ulm, Germany; and Istituto Ortopedico Rizzoli EC, Bologna, Italy) approved the protocol and related documents for all participating clinical centers. As an ATMP for human use, the trial was authorized by the National Competent Authorities (AEMPS, Spain; ANSM, France; PEI, Germany; AIFA, Italy) as per Directive 2001/20/EC and 2005/28/EC of the European Parliament. The CONSORT diagram and the inclusion and exclusion criteria have been published with the primary outcome of the clinical trial [7].

Patients and trauma characteristics

The mean age of the 28 treated patients was 39 ± 13 years, 57% were males, and the mean Body Mass Index (BMI) was 27 ± 7. Thirteen cases (46%) reported active use of tobacco, with a mean of two packs daily, and two more were former smokers. There were 11 femoral, 4 humeral, and 13 tibial non-unions in the study.

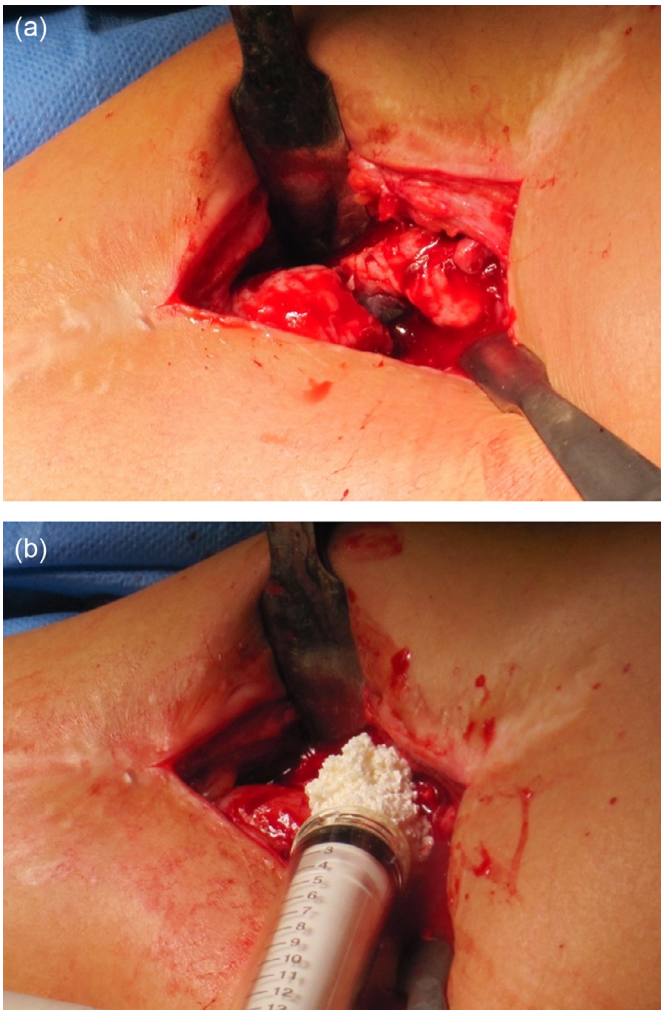

Fig. 1. (a) surgical preparation of the nonunion site including ablation of necrotic free bone fragments, excision of fibrous tissue and/or decortication of bone ends to bleeding bone; (b) combined biomaterial – cell product with a pasty consistency, fixed to the full extent of the prepared area as for spongy bone.

The original fracture originated from a traffic accident in 67% of the cases (19/28), and 40% (11/28) were open fractures, 8 in the tibia and 3 in the femur. The original open fracture was less severe with wounds under 1cm (Gustilo I) in 4 tibial and 2 femoral non-unions. The open fracture was more severe with wounds larger than 1cm (Gustilo II, III) in 4 tibial and 1 femoral non-union. No significant differences were found among affected bones for demographic and trauma characteristics (Table 1).

Regarding the fracture site, distal third of the diaphysis was the most common fracture location for the femur (4/11) and the tibia (7/13), followed by the middle third of the diaphysis in femur (3/11), humerus (2/4) and tibia (5/13). Fractures in the proximal third of the diaphysis occurred in 1 tibia (1/13), humerus (2/4) and femur (1/11). Metaphysis or metaphysodiaphyseal regions (2 distal and 1 proximal) were involved in 3 femur non-unions. The AO diaphyseal fracture classification (A Simple/B Wedge/C Complex), for femur was 3/3/2, for humerus 1/0/3, and for tibia 2/6/5. The AO metaphyseal fracture classification (A Simple/B Wedge/C Complex) of the 3 femoral cases was 1/0/2.

The initial fracture occurred at a mean ± SD of 27.9 ± 31.2 months before recruitment, and the last operation occurred at a mean ± SD of 14.9 ± 9.9 months before cell therapy. Previous treatments included 5 cases with intramedullary nail (N), 3 cases with plate (P) and 3 cases with external fixation (EF) at the femur.

Table. 1
Patient and trauma characteristics.

Variables	ALL LONG BONES $n = 28$		FEMUR $n = 11$				HUMERUS $n = 4$				TIBIA $n = 13$				P value
	Mean±n	SD(%)	Mean±n	SD(%)	(Min-	Max)	Mean±n	SD(%)	(Min-	Max)	Mean±n	SD(%)	(Min-	Max)	
Demographics															
Age	39.8±	13.4	42.4±	14.9	(19.1-	64.9)	46.5±	8.5	(37.6-	57.9)	33.9±	11.8	(19.0-	53.3)	0.307†
Height (cm)	172.1±	9.7	171.2±	12.4	(151.0-	189.0)	171.8±	10.9	(160.0-	186.0)	173.1±	7.5	(162.0-	188.0)	0.882†
Weight (Kg)	81.7±	23.3	75.6±	20.1	(44.0-	98.0)	86.0±	21.6	(68.0-	114.0)	85.5±	26.8	(62.0-	162.0)	0.945†
BMI	27.4±	7.3	25.5±	5.0	(17.4-	30.9)	28.8±	3.9	(24.1-	33.0)	28.7±	9.6	(21.2-	57.4)	0.897†
Male sex	16	(57.1%)	7	(63.6%)			2	(50%)			7	(53.8%)			0.897‡
History of smoking (yes)	*15*	*(53.6%)*	*4*	*(36.4%)*			*2*	*(50%)*			*9*	*(69.2%)*			0.294‡
No. Packs p/day	2.0±	3.0	3.0±	4.7	(0.5-	10.0)	3.5±	3.5	(1.0-	6.0)	1.3±	2.2	(0.3-	7.0)	0.653†
Duration (years)	19.4±	13.6	26.3±	14.9	(10.0-	45.0)	35.0±	7.1	(30.0-	40.0)	12.9±	10.2	(2.0-	30.0)	0.327†
No. Packs p/year	47.5±	99.0	15.0±	7.1	(5.0-	20.0)	27.0±	4.2	(24.0-	30.0)	69.0±	130.1	(0.0-	365.0)	0.557†
Stop smoking (yes)*	2	(7.1%)	1	(9.1%)	1/4	(25.0%)	0	(0%)			1	(11.1%)			–
History of the accident															
Months from acute fracture	27.9±	31.2	38.3±	44.8	(6.8-	163.3)	25.5±	26.5	(7.9-	64.7)	19.8±	12.9	(3.9-	40.0)	0.597†
Months from last operation	14.7±	9.9	15.9±	10.6	(2.1-	36.0)	20.5±	16.8	(8.7-	32.4)	14.0±	9.1	(3.4-	26.9)	0.593†
Mechanism of action															0.006‡
Crushing	7	(25.0%)	2	(18.2%)			0	(0%)			5	(38.5%)			
Direct impact	10	(35.7%)	8	(72.7%)			0	(0%)			2	(15.4%)			
Fall	9	(32.1%)	1	(9.1%)			4	(100%)			4	(30.8%)			
Torsion	2	(7.1%)	0	(0%)			0	(0%)			2	(15.4%)			
Traffic injury (Yes)	*19*	*(67.9%)*	9	*(82%)*			*1*	*(25%)*			9	*(69.2%)*			0.451‡
Vehicle occupant	6	(21.4%)	4	(36.4%)	4/9	(44.4%)	0	(0%)	0/1	(0%)	2	(15.4%)	2/9	(22.2%)	
Passenger 2 wheels	10	(35.7%)	4	(36.4%)	4/9	(44.4%)	0	(0%)	0/1	(0%)	6	(46.2%)	6/9	(66.7%)	
Pedestrian	3	(10.7%)	1	(9.1%)	1/9	(11.1%)	1	(25%)	1/1	(100%)	1	(7.7%)	1/9	(11.1%)	

* *Years w/o smoking for Femur = 0.5 years (n = 1) and, for Tibia = 11 years (n = 1)* †*Kruskall-Wallis test* ‡ *Fisher's exact test*

In the humerus, 1 case was treated by cast alone and 3 with nails. In the tibia, there were 2N, 5P and 6EF cases. A total of 57% (16/28) cases had history of osteosynthesis changes (8 femurs, 2 humerus, and 6 tibias) and 36% (10/28) had history of locking screw removal (5 femoral, 1 humeral, 4 tibial fractures). The main reported complication after initial fixation was neurological injury (6/28 cases), 4 of them in the femur, and infection (7/28 cases), 6 of them in the tibia. No significant differences were found among affected bones for initial treatment of the fracture.

General anaesthesia was used in 70% of the cases (8 femoral, 4 humeral, 7 tibial non-unions), and spinal anaesthesia in the rest. About the surgical technique to treat the non-union, decortications were performed in all femoral and humeral non-unions, and in the majority of tibias (all but one, 92%). Excision of fibrous tissue was also required in all tibias, 8 femurs and 2 humerus. Finally, ablation of necrotic bone was reported in 5 femurs, 1 humerus, and 9 tibias. Eight treated patients (8/28, 29%) did not need changing of the fixation when treating the non-union. The case-mix of techniques N/P/EF (nail/plate/exfix) after changing the fixation during non-union treatment in 20 cases was 5/2/0 in the femoral non-unions, 1/2/0 in the humerus, and 5/2/3 in the tibia. The final N/P/EF techniques in the 28 cases was 7/4/0 in the femur, 2/2/0 in the humerus, and 7/3/3 in the tibia.

Efficacy evaluation

A secondary end-point for early efficacy was set for the trial, to confirm bone consolidation in the treated non-unions. Clinical and radiological evolution was followed at 3, 6, and 12 months. Clinical evaluation of pain under full weight-bearing was performed at 3, 6, and 12 months through the visual analog scale (VAS), considering clinical consolidation if pain was under the threshold of 30/100 [8]. Radiological evaluation with radiographs (anteroposterior and lateral views) was performed at 3, 6, and 12 months. Imaging with CT sections was also performed at 3 and 6 months to more accurately assess bone bridging, particularly in case of hardware interference.

In the study radiographs and CT sections, four cortical views were evaluated for each bone (medial and lateral cortices in anteroposterior radiographic views and coronal and/or transverse CT sections, anterior and posterior cortices in lateral radiographic views and sagittal and/or transverse CT sections). The REBORNE bone healing scale was estimated following this formula: REBORNE score= (Internal cortical value + External cortical value + Anterior cortical value + Posterior cortical value) / (4 * number of evaluated cortices). The value of each cortical is assigned following this rule: 1 point if fracture unchanged, 2 points if callus but non-continuous, 3 points if callus continuous but fracture still apparent, 4 points if callus with same density as cortical, and 0 points if cortical was non-interpretable or non-visible. The maximum consolidation received a score of 16 (over 16 possible points), with a value of 1.0. The non-union was healed by bone consolidation if the score was at or above 11 points (11/16) with a value of 0.6875.

Imaging was initially evaluated for consolidation by the participating clinicians and incorporated in the case report forms (CRF). However, to avoid any evaluation bias, all the images were sent to the coordinating investigator and an adjudication committee was set with three experienced orthopaedic surgeons to adjudicate each consolidation.

Bone biopsies were done only in two cases that required screw removal, at the time of this surgical procedure, upon the recommendation of Ethical Committees to avoid other invasive evaluation.

Efficacy results were expressed by clinical consolidation, with values under 30 in the VAS scale meaning no or mild pain, and by radiological consolidation at adjudication with a REBORNE score over 11/16 points (value of or above 0.6875).

Statistical analysis

Data were described and analyzed using STATA software version 12 (StataCorp. 2011. Texas, USA.) by the non-union anatomical site. Differences in frequency were evaluated by the Fisher's exact test. Comparison of means were performed with the Student's t test, Mann-Whitney test, ANOVA (one way) test, or Kruskal-Wallis test, as deemed appropriate, with 95% of significance level. Mixed Models for Repeated Measurements (MMRM) were generated to estimate the mean and confidence intervals (95%) of the REBORNE Bone Healing scale. Consolidation (under 30 in VAS scale and more than 0.6875 in the REBORNE scale) were analyzed in the following subgroups: affected bone (femur, humerus, tibia), gender (male, female), history of smoking habit (yes, no), and months since acute fracture. Within these, Spearman correlation tests (adjusted by Bonferroni) were performed, correlating the VAS pain scale with the REBORNE radiological scale.

Results

Efficacy endpoint

Clinical and radiological consolidation was observed in all 25 patients finishing the follow-up of one year (1 patient died due to unrelated disease after six months, with the non-union healed), and in 26 out of 28 patients that were treated and evaluated under ITT until end of follow-up or study drop-out. Two patients were interpreted as failure. One of these patients voluntarily abandoned the study at three months to request treatment (plate removal) in a different hospital. The second patient was considered a failure at six months and the non-union required a new intervention (new decortication and hardware exchange, adding bone marrow concentrate) by the surgeon in charge.

Clinical consolidation evaluated by the VAS scale (threshold of 30 out of 100) was 24/28 (85.7%) at 3 months (mean ± SD scale value 20.8 ± 20.7, range 0.0-76.0), 24/27 (88.9%) at 6 months (13.3 ± 15.0, range 0.0-50.0), and 25 out of 25 patients that completed the follow-up at 12 months (6.6 ± 7.4, range 0.0-30.0). While all locations showed average VAS scale values under the 30/100 threshold, pain was lowest in the later follow-up, and at any follow-up visit when the humerus was treated.

Progression of radiological consolidation, estimated by the REBORNE bone healing scale (threshold of 0.6875, or 11/16 points) for 3, 6, and 12 months, was summarized in Fig. 2. The mean ± SD REBORNE score changed from 0.62 ± 0.08 (range 0.45–0.83) at 3 months, to 0.78 ± 0.09 (range 0.56–1) at six months, and to 0.89±0.09 (range 0.71–1) at 12 months.

At 3 months, the radiological healing rate was 25.0% (7/28 cases), while at 6 months it had raised to 67.8% (19/28 cases including the extrapolation of one failure and one drop-out not previously healed). At one year, the clinical healing rate was 92.8% (26/28 cases including the drop-out extrapolation of two failures not healed before 3 and 6 months, and one patient with protocol deviation who suffered from unrelated exitus before 12 months but who was previously healed at 6 months). Fig. 3 displays typical cases of treated non-unions in the femur, humerus, and tibia. The variation between the VAS scale and the REBORNE scale produced a Rho = -28% ($p = 0.000$), including all follow-up visits after surgery.

Bone biopsies

After histopathological evaluation, bone biopsies confirmed bone formation surrounding the bioceramic granules with the attached, expanded MSCs delivered into the non-union site at surgery (Fig. 4). Lamellar bone and osteoid tissues were found in

the vicinity of the BCP granules. Multinucleated giant cells labeled by the antibody CD68 and TRAP staining were primarily located surrounding the BCP biomaterial. These cells are considered osteoclasts with a pivotal role in bone regeneration, as demonstrated in preclinical studies [9,10].

Subgroup analysis

Analysis per bone (femur, humerus, tibia, as seen in graphs displayed in Fig. 5) confirmed that all locations showed consolidation at one year, although this was delayed in tibial non-unions (Rho_{tibia} = -28%, Rho_{femur} = -34%). The mean ± SD score of the VAS/REBORNE relation in femoral non-unions progressed from 24.1 ± 20.6/0.64 ± 0.06 at 3 months, to 12.8 ± 14.1/0.79 ± 0.09 at 6 months and to 6.9 ± 8.9/0.88 ± 0.09 at 12 months. In humeral non-unions, at 3 months the VAS/REBORNE relation was 5.0 ± 10.0/0.65 ± 0.05, at 6 months 0 ± 0/0.83 ± 0.09, and at 12 months 0 ± 0/0.94 ± 0.06. In tibias, the VAS/ REBORNE score relation changed from 23.18 ± 22.3/0.62 ± 0.10 at 3 months, to 18.7 ± 16.2/0.75 ± 0.11 at 6 months, and to 7.5 ± 6.3/0.87 ± 0.11 at 12 months. At six months, every humerus and all but one femur were consolidated, while four tibias were not yet seen as consolidated.

For male gender, the mean ± SD score for the VAS/REBORNE relation was 15.9 ± 19.8/0.62 ± 0.07 at 3 months, 11.6 ± 14.8/0.77 ± 0.09 at 6 months, and 7.1 ± 8.4/0.89 ± 0.08 at 12 months. For female gender, the VAS/REBORNE relation was 27.3 ± 20.9/0.64 ± 0.09 at 3 months, 16.2 ± 15.8/0.79 ± 0.10 at 6 months, and 5.9 ± 6.3/0.87 ± 0.10 at 12 months. Specific subgroup analysis per gender (Fig. 6) showed no significant gender difference in consolidation at any time, although the correlation between VAS scale and REBORNE scale was 15% higher in females.

Consolidation was also compared in smoking patients (Rho = -35%) versus non-smokers (Rho = -19%) (Fig. 7). For smoking patients, the mean ± SD score of the VAS/REBORNE relation was 24.0 ± 22.4/0.61 ± 0.06 at 3 months, 13.8 ± 15.4/0.73 ± 0.07 at 6 months, and 7.1 ± 9.4/0.85 ± 0.07 at 12 months. For non-smoking patients, the VAS/REBORNE relation was 17.5 ± 19.2/0.66 ± 0.08 at 3 months, 12.8 ± 15.3/0.83 ± 0.10 at 6 months, and 6.1 ± 4.8/0.93 ± 0.09 at 12 months. Significant differences were found at 6 months (more consolidation scale value in non-smoking patients, $p = 0.012$, t-test) and at 12 months (more consolidation also in non-smoking patients, $p = 0.011$, t-test).

Finally, longer time elapsed after the initial fracture did not preclude the occurrence of consolidation (Rho = 0.019, $p = 0.729$) (Fig. 8).

Discussion

This study proved efficacy, by means of clinical and radiological consolidation, in femoral, humeral and tibial non-unions surgically treated with a combination of bioceramics and expanded autologous hBM-MSCs. Although clinical consolidation (no or mild pain) was early seen (more than 80% at 3 months), radiological consolidation estimated by a bone healing score was 25% at 3 months, 67% at 6 months, and only at 12 months, a 92% radiological consolidation was confirmed.

Long bone non-union treatment is always a challenge, due to the frequent multifactorial underlying cause [11–16]. While surgical treatment by means of appropriate fixation is frequently successful in case of hypertrophic non-unions, biological support is strictly needed when the non-union is not hypertrophic, confirming a certain limitation to regenerate bone at the injury site. The clinical standard biological support, the iliac crest autograft, is seldom evaluated for efficacy, while downsides are being recently stressed [17]. Its use as a control to new proposals of biological enhancement [18] provides some efficacy data. When evaluated upon rigorous radiological criteria (3 out of 4 cortices), the autograft efficacy was set at 74% consolidation at 9 months [19]. In the same publication, the general adjudication of radiological consolidation was considered 84% with autograft, when only one cortical bridging was interpreted as consolidation. This confirms the limited clinical evidence in autograft efficacy evaluation, and the difficulties to interpret efficacy without rigorous methods.

Current alternative biological augmentation to treat non-unions provide efficacy in studies of osteoinduction, particularly by bone morphogenetic protein –BMP- clinically available in forms 2 and 7 as recombinant human BMP [18,19], and studies on osteogenesis through different cell therapy techniques [5,6]. The literature on the mentioned topics very seldom provides clinical and radio-

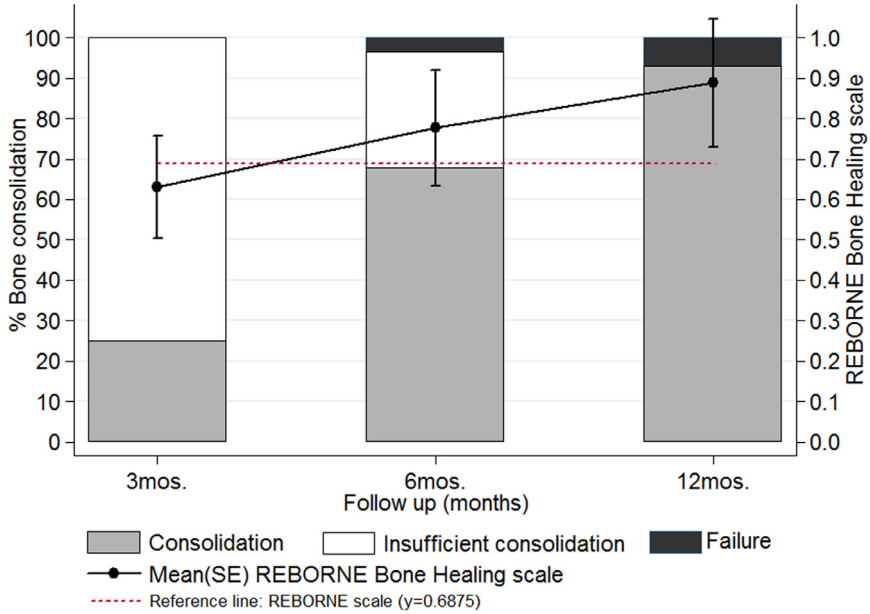

Fig. 2. Efficacy results, mean values (with confidence interval 95%) of radiological consolidation for all cases based on the REBORNE consolidation scale (threshold of consolidation at 0.6875, or 11/16 points) after adjudication of bone consolidation on imaging for 3, 6 and 12 months.

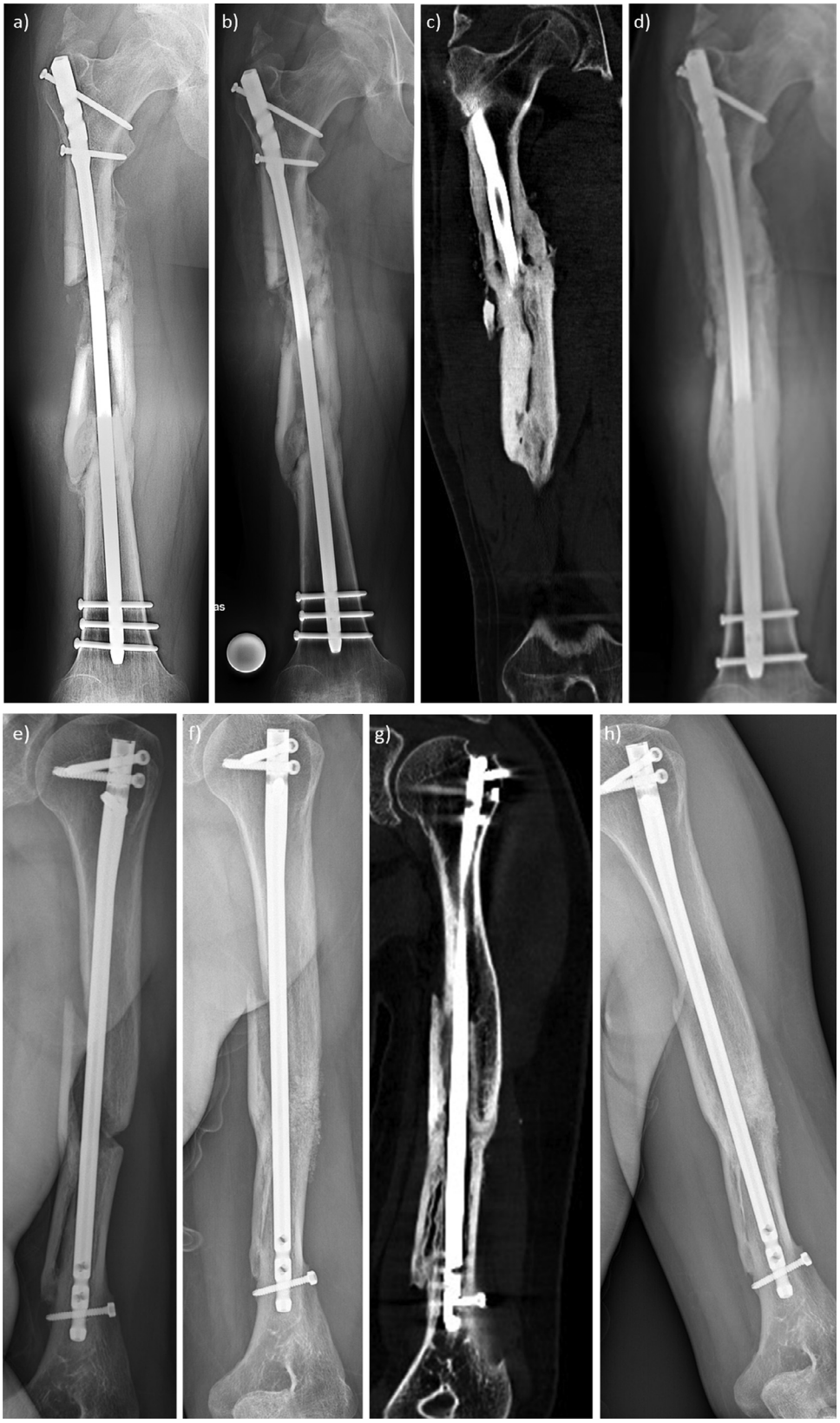

Fig. 3. Radiographs and CT sections of clinical cases in the trial; (a) femur nonunion AP view prior to the treatment; (b) same femur postop (c) femur treated nonunion coronal section at 6 months; (d) femur treated nonunion AP view at 12 months; (e) humerus nonunion AP view prior to the treatment; (f) same humerus postop; (g) humerus treated nonunion coronal section at 6 months; (h) humerus treated nonunion AP view at 12 months; h) tibia nonunion AP view prior to the treatment; (i) same tibia postop; (j) tibia treated nonunion coronal section at 6 months; (k) tibia treated nonunion AP view at 12 months.

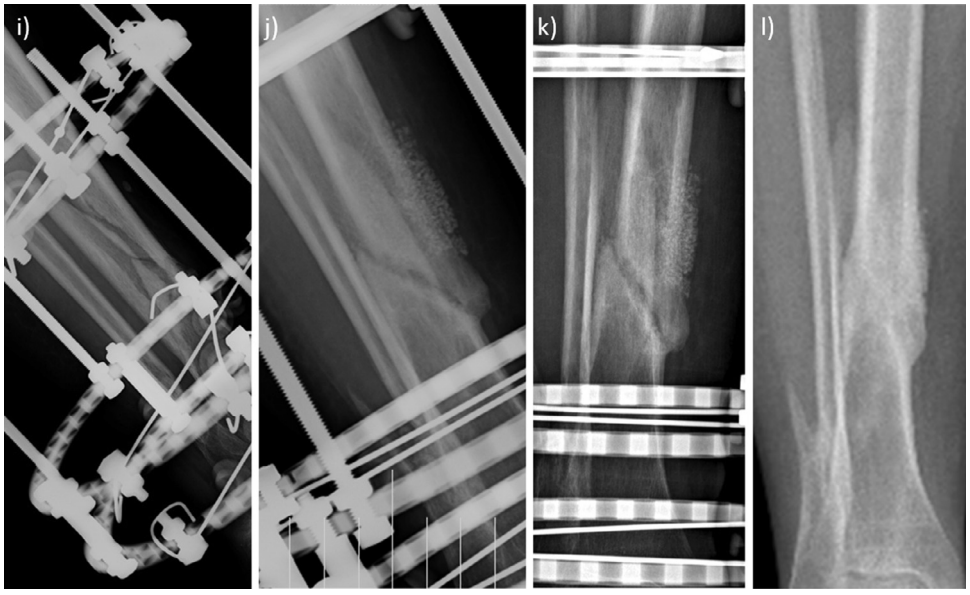

Fig. 3. Continued

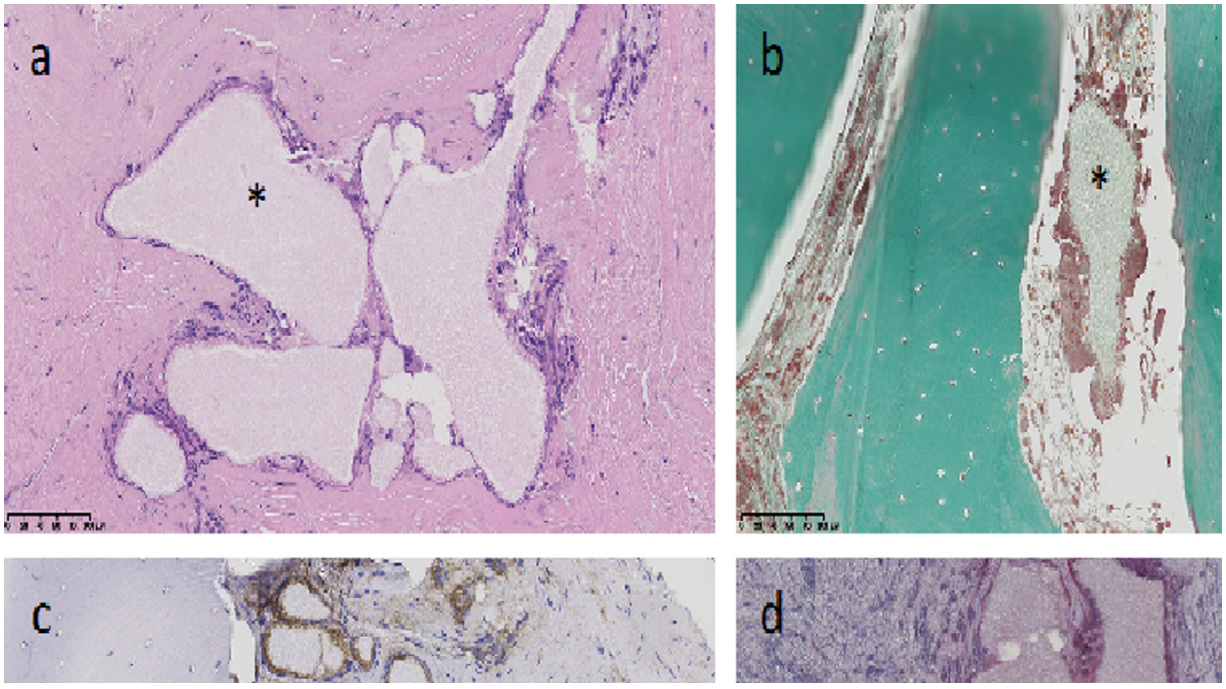

Fig. 4. Histology of bone biopsies from the callus formed after 8 months, (a) hematoxylin-eosin, (b) Masson trichrome, (c) immunohistochemistry with CD68, (d) TRAP staining (objective x20, * shows the bioceramic granules).

logical results in well controlled cases. Non-union treatment with rhBMP-7 has confirmed an efficacy of 81% healing at 5 months, without structured radiological evaluation but confirmed at 5 years [19], while a different study on BMP-7 confirmed only 63% healing at 9 months, after a rigorous radiological evaluation [18]. Result variability may originate in the case mix but also in the radiological evaluation. A recent review of percutaneous injection of autologous BM from different authors and techniques showed a general union rate of 82.4% (range 57–94%) with a mean time to achieve solid union of 4.8 months (range 2.5–8.1)[20]. Difficulties to standardize the included non-unions, the treatment, and the evaluation partly justify wide efficacy ranges.

Discrepancy of clinical and radiological consolidation is a frequent finding. In our study, clinical consolidation was early obtained, but radiological consolidation was not evident. This fact has prompted the use of radiological scales, mostly oriented and validated for fracture healing evaluation [21]. Very seldom scales are applied to non-union healing. Union rates were clinically and radiologically assessed [20] in a series of patients with failed femoral shaft aseptic non-union, treated with percutaneous autologous bone marrow grafting [22]. Radiographic union was diagnosed in this series with a score $\geq$10 according to the Radiographic Union Scale in Tibial fractures (RUST), although the RUST was initially designed for tibial fractures with intramedullary fixation [23], and detected in 8/16 patients (50%) at one year follow-

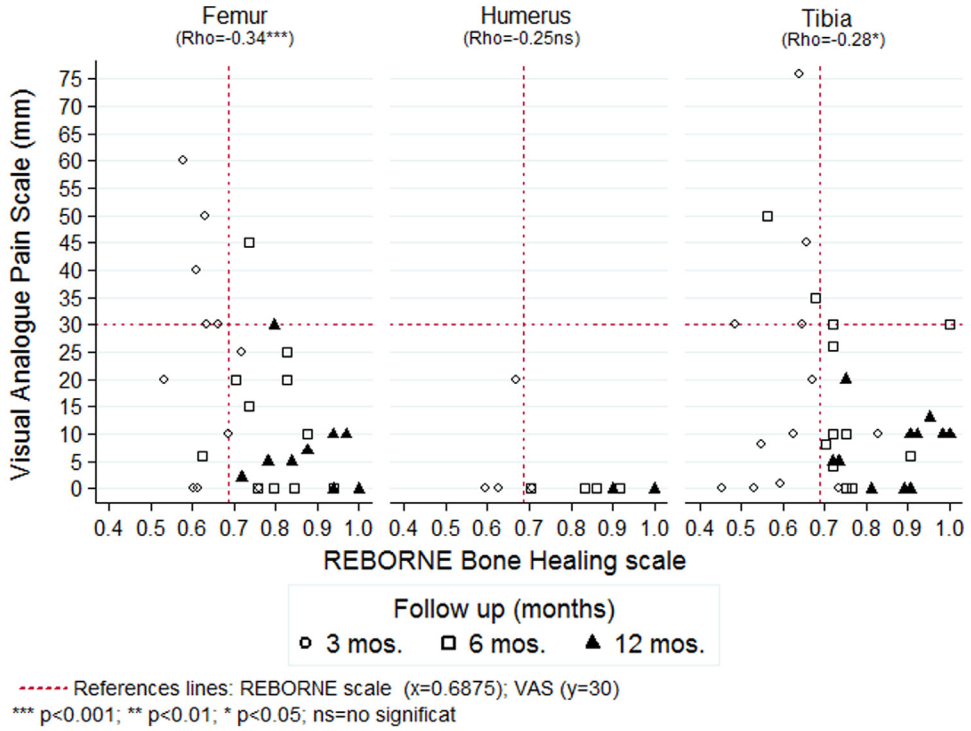

Fig. 5. Subgroup analysis for anatomical site of the nonunion including femur, humerus and tibia (values from pain VAS –*y* axis- and mean REBORNE consolidation scale values –*x* axis-) at 3, 6, and 12 months follow-up. Note clinical consolidation is considered below the VAS value of 30, and imaging consolidation pass the REBORNE score value of 0.6875.

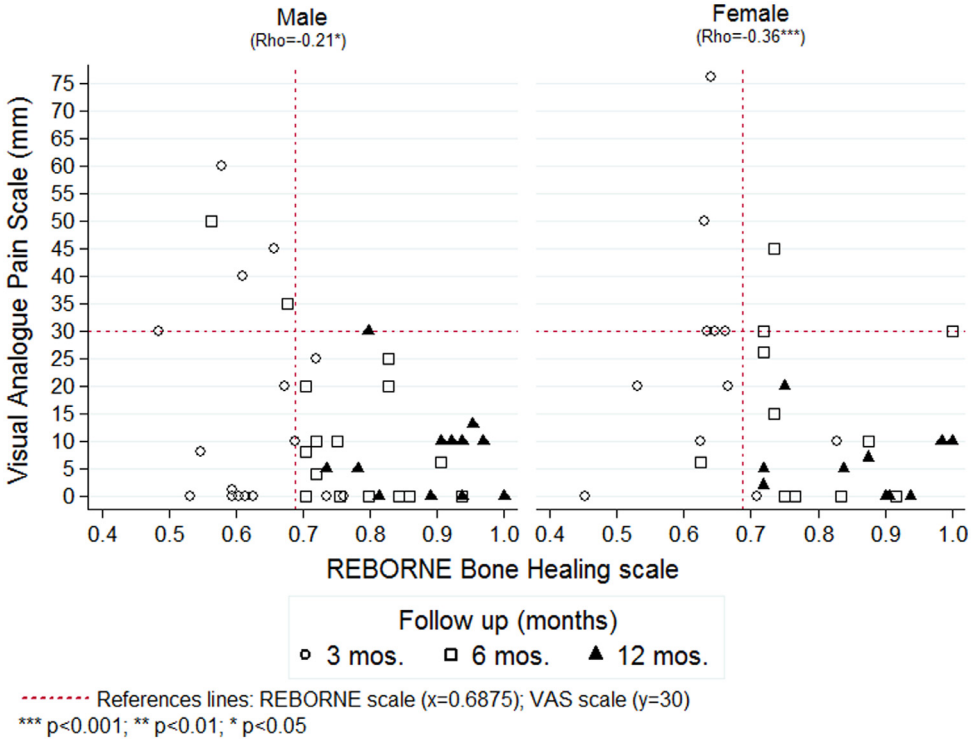

Fig. 6. Subgroup analysis for gender including nonunions treated in male and female patients (values from pain VAS –*y* axis- and mean REBORNE consolidation scale values –*x* axis-) at 3, 6, and 12 months FU. Note clinical consolidation is considered below the VAS value of 30, and imaging consolidation pass the REBORNE score value of 0.6875.

up. Bone marrow grafting significantly increased the radiographic score with early bone formation up to union at an average of 5.0 ± 1.75 months in healed patients, and also increased the local bone production even in patients who did not heal. Our results confirm early bone formation, but the advantage of expanded cells seems more related to the progression towards union also in those patients that were not early healed. In agreement to those authors and the original publication of Hernigou et al. [22], efficacy to consolidate may be related to the number of available osteoprogenitors, expanded cells ATMPs providing higher numbers. Underreporting of trials about non-unions treated with expanded MSC [6] suggest that more information is required to adequately

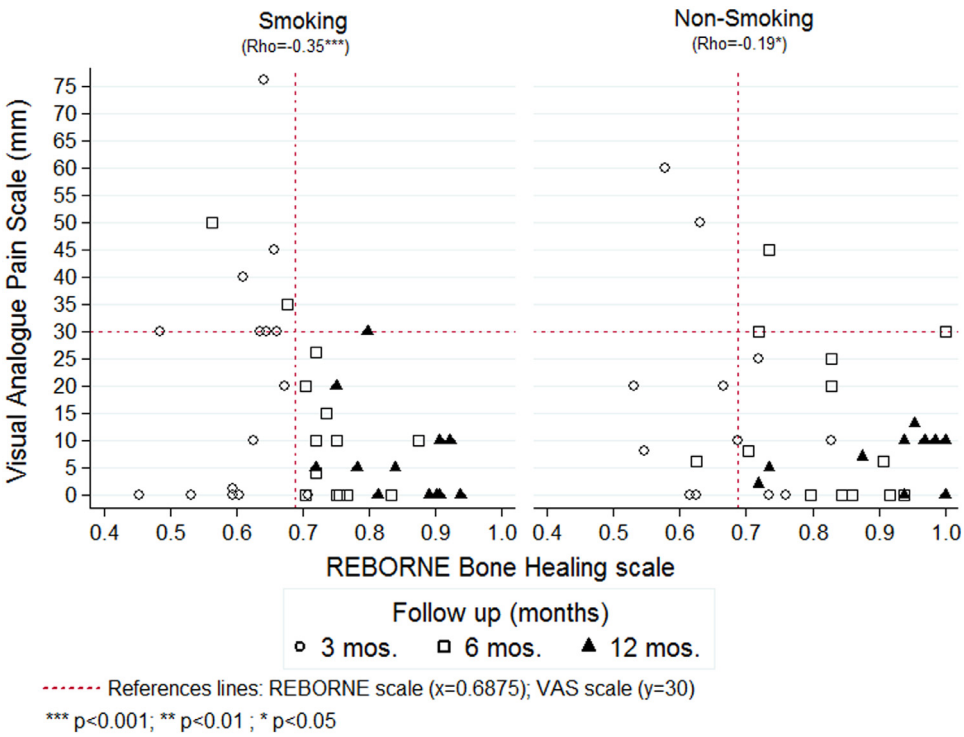

Fig. 7. Subgroup analysis for tobacco use including nonunions treated in non-smokers and smoker patients (values from pain VAS –*y* axis- and mean REBORNE consolidation scale values –*x* axis-) at 3, 6, and 12 months FU. Note clinical consolidation is considered below the VAS value of 30, and imaging consolidation pass the REBORNE score value of 0.6875.

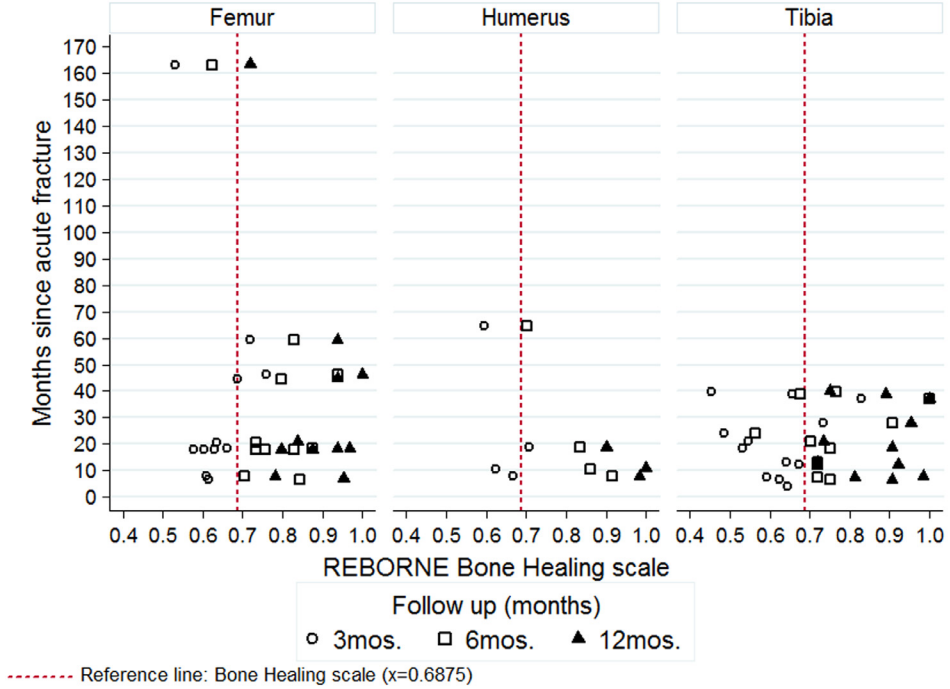

Fig. 8. Analysis of time since initial, acute fracture (in months, y axis) on consolidation at 3, 6, and 12 months FU (mean REBORNE consolidation scale values). Note imaging consolidation is considered pass the REBORNE score value of 0.6875 (*x* axis).

frame the role of expanded MSCs in non-union healing, but our results are encouraging.

Timing to establish consolidation is an important issue about efficacy. We confirmed through rigorous radiological evaluation at 12 months a well-established bone healing in 90% of the cases, but uncertain at 6 months (67%) and improbable at 3 months in many cases (only 25% healed). Intermediate results to speak about early efficacy also require appropriate radiological evaluation. Bone marrow injection obtained consolidation at about 5 months of 50% of non-unions [20], but the rest were not healed at 12 months. Other treatment options, such as BMP-7, also claim early healing of 5.2 average months (range 3-10) in 81% aseptic non-unions

[19], although unfortunately the radiological evaluation was not based on a scale. Using cortical bridging as the radiological criteria [18] at 9 months, 75% of the tibial non-unions treated with BMP-7 were healed if considering bone bridging in one view, and 63% if considering bridging in 3 out of 4 views. When non-union healing incorporated different treatments (femoral non-union after intramedullary nail treated with plate and bone grafting), 7.2 months (range 5-11) were needed [24]. When different bones (tibia, femur, humerus) were treated by different techniques [25], the average time to healing in the successful cases averaged 6 months (range 4-8). These authors also reported earlier healing in the humerus (average 16 weeks, range 6-36) [26], congruently with our series where the humeral non-unions healed significantly earlier than femoral and tibial non-unions. The affected bone may then impact in the non-union treatment efficacy and time to heal, as shown in our results. In the upper limb, other reports [27] confirm that healing may occur under 6 months, while femur [24] and tibia [18] frequently heal above 6 and even 9 months.

Another factor considered in our study that may impact efficacy is gender difference that could justify more disability observed in females after severe accidents [4]. This was not confirmed in our study, and other authors [19] also found similar efficacy in treating males and females. Chronicity of the non-union, measured in time since the original fracture, did not influence efficacy in our study, which is a consistent finding with that of Papaniagiotu et al. after a multilevel regression analysis [19].

Smoking habits have been associated with delayed consolidation of tibial open fractures [28]. Tobacco use has been reported as a risk factor for non-union, either after open or close fractures [29], although this association did not consider infection in the model and tobacco influence could be overestimated [30]. Our data confirmed that tobacco use influenced but did not jeopardize final consolidation in our patients. This is in accordance with cell studies confirming the presence of osteoprogenitors and its capacity to undergo osteoblastic differentiation despite tobacco use [31].

Our study sustains several limitations. First is the study design, where a comparative, randomized study with controls would offer higher evidence. This comparative randomized study is already on its way [32]. The variability related to the fracture and to the patient is a limitation, as the trial evaluates an autologous treatment with different osteogenic potential of individual patients. And finally, the number of cases is short, given the fact that this is an early trial. More cases are required to ascertain efficacy, particularly in some subgroups, and this can be considered an exploratory trial for efficacy.

Conclusion

Bone consolidation was efficaciously obtained with the tested expanded hBM-MSCs combined to biomaterials, by clinical and radiological evaluation, better defined at 12 months. Bone regeneration was confirmed by bone biopsies. No difference was observed among the affected bones to reach consolidation, although it was slower in tibial non-unions. Lower consolidation scores were seen in smokers at 6 and 12 months. No influence was detected by the gender or by the timing since the original fracture.

Author statement

Conceived and designed the protocol: EGB, PR, PH, FG, NB.

Managed the research project, contributed to ethical and regulatory approval: PL, CP, 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, MDGS, 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.

Analyzed the data, wrote the paper: EGB, NPE.

Revised and approved the paper: All.

Declaration of Competing Interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no financial support for this work that could have influenced its outcome. The authors do not communicate any conflict of interest about this work and manuscript.

Acknowledgement

This research received funding from the European Union's Seventh Framework Programme (FP7/FP7-HEALTH-2009) with the REBORNE Project (under G.A. 241876), and the European Union's Horizon 2020 Programme (H2020-SC1 2016-2017), with the ORTHOUNION Project (under G.A. 7333288).

References

[1] . 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. Rockville, MD, USA: Federal Register; 1998. p. 23292–3. editor.
[2] Arvidson K, Abdallah BM, Applegate LA, Baldini N, Cenni E, Gomez-Barrena E, et al. Bone regeneration and stem cells. J Cell Mol Med 2011;15(4):718–46.
[3] Iwakura T, Miwa M, Sakai Y, Niikura T, Lee SY, Oe K, et al. Human hypertrophic nonunion tissue contains mesenchymal progenitor cells with multilineage capacity in vitro. J Orthop Res 2009;27(2):208–15.
[4] Ringburg AN, Polinder S, van Ierland MC, Steyerberg EW, van Lieshout EM, Patka P, et al. Prevalence and prognostic factors of disability after major trauma. J Trauma 2011;70(4):916–22.
[5] Gomez-Barrena E, Rosset P, Muller I, Giordano R, Bunu C, Layrolle P, et al. Bone regeneration: stem cell therapies and clinical studies in orthopaedics and traumatology. J Cell Mol Med 2011;15(6):1266–86.
[6] Gomez-Barrena E, Rosset P, Lozano D, Stanovici J, Ermthaller C, Gerbhard F. Bone fracture healing: cell therapy in delayed unions and nonunions. Bone 2015;70:93–101.
[7] Gomez-Barrena E, Rosset P, Gebhard F, Hernigou P, Baldini N, Rouard H, et al. Feasibility and safety of treating non-unions in tibia, femur and humerus with autologous, expanded, bone marrow-derived mesenchymal stromal cells associated with biphasic calcium phosphate biomaterials in a multicentric, non-comparative trial. Biomaterials 2019;196:100–8.
[8] Jensen MP, Chen C, Brugger AM. Interpretation of visual analog scale ratings and change scores: a reanalysis of two clinical trials of postoperative pain. J Pain 2003;4(7):407–14.
[9] Gamblin AL, Brennan MA, Renaud A, Yagita H, Lezot F, Heymann D, et al. Bone tissue formation with human mesenchymal stem cells and biphasic calcium phosphate ceramics: the local implication of osteoclasts and macrophages. Biomaterials 2014;35(36):9660–7.
[10] Miron RJ, Bosshardt DD. OsteoMacs: Key players around bone biomaterials. Biomaterials 2016;82:1–19.
[11] Douras P, Tosounidis T, Giannoudis PV. Application of the 'diamond concept' with fast bone marrow aspirate concentration for the treatment of medial malleolus non-union. Injury 2018;49(12):2326–30 Dec.
[12] Cho JW, Kim J, Cho WT, Kim JK, Song JH, Kim HJ, Masquelet AC, Oh JK. Circumferential bone grafting around an absorbable gelatin sponge core reduced the amount of grafted bone in the induced membrane technique for critical-size defects of long bones. Injury 2017;48(10):2292–305 Oct.
[13] Tarchala M, Engel V, Barralet J, Harvey EJ. A pilot study: Alternative biomaterials in critical sized bone defect treatment. Injury 2018;49(3):523–31 Mar.
[14] Yılmaz O, Özmeriç A, Alemdaroğlu KB, Celepli P, Hücümenoğlu S, Ö Şahin. Effects of concentrated growth factors (CGF) on the quality of the induced membrane in Masquelet's technique - An experimental study in rabbits. Injury 2018;49(8):1497–503 Aug.
[15] Stella M, Santolini E, Autuori A, Felli L, Santolini F. Masquelet technique to treat a septic nonunion after nailing of a femoral open fracture. Injury 2018;49(4):S29–33 DecSuppl.
[16] Cano-Luís P, Andrés-Cano P, Ricón-Recarey FJ, Giráldez-Sánchez MA. Treatment of posttraumatic bone defects of the forearm with vascularized fibular grafts. Follow up after fourteen years. Injury. 2018;49(2):S27–35 SepSuppl.
[17] Hernigou P, Desroches A, Queinnec S, Flouzat Lachaniette CH, Poignard A, Allain J, et al. Morbidity of graft harvesting versus bone marrow aspiration in cell regenerative therapy. Int Orthop 2014;38(9):1855–60.

[18] Friedlaender GE, Perry CR, Cole JD, Cook SD, Cierny G, Muschler GF, et al. Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions. J Bone Jt Surg Am Vol 2001:S151–8 **83-A Suppl 1**(Pt 2).
[19] Papanagiotou M, Dailiana ZH, Karachalios T, Varitimidis S, Vlychou M, Hantes M, et al. RhBMP-7 for the treatment of nonunion of fractures of long bones. Bone Jt J 2015;97-b(7):997–1003.
[20] Guimaraes JA, Duarte ME, Fernandes MB, Vianna VF, Rocha TH, Bonfim DC, et al. The effect of autologous concentrated bone-marrow grafting on the healing of femoral shaft non-unions after locked intramedullary nailing. Injury 2014;45(5):S7–s13 **Suppl**.
[21] Whelan DB, Bhandari M, McKee MD, Guyatt GH, Kreder HJ, Stephen D, et al. Interobserver and intraobserver variation in the assessment of the healing of tibial fractures after intramedullary fixation. J Bone Jt Surg Br 2002;84(1):15–18.
[22] 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 Jt Surg Am Vol 2005;87(7):1430–7.
[23] Whelan DB, Bhandari M, Stephen D, Kreder H, McKee MD, Zdero R, 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(3):629–32.
[24] Choi YS, Kim KS. Plate augmentation leaving the nail in situ and bone grafting for non-union of femoral shaft fractures. Int Orthop 2005;29(5):287–290.
[25] Babhulkar S, Pande K, Babhulkar S. Nonunion of the diaphysis of long bones. Clin Orthop Relat Res 2005;431:50–6.
[26] Babhulkar S, Babhulkar S, Vasudev A. Recalcitrant aseptic atrophic non-union of the shaft of the humerus after failure of surgical treatment: management by excision of non-union, bone grafting and stabilization by LCP in different modes. Injury 2017;48(2):S33–43 **Suppl**.
[27] Giannotti S, Trombi L, Bottai V, Ghilardi M, D'Alessandro D, Danti S, et al. Use of autologous human mesenchymal stromal cell/fibrin clot constructs in upper limb non-unions: long-term assessment. PLoS One 2013;8(8):e73893.
[28] Adams CI, Keating JF, Court-Brown CM. Cigarette smoking and open tibial fractures. Injury 2001;32(1):61–5.
[29] Hernigou J, Schuind F. Smoking as a predictor of negative outcome in diaphyseal fracture healing. Int Orthop 2013;37(5):883–7.
[30] Hernigou J, Schuind F. Comment on Hernigou and Schuind: smoking as a predictor of negative outcome in diaphyseal fracture healing. Int Orthop 2013;37(5):983.
[31] Bidula J, Boehm C, Powell K, Barsoum W, Nakamoto C, Mascha E, et al. Osteogenic progenitors in bone marrow aspirates from smokers and nonsmokers. Clin Orthop Relat Res 2006;442:252–9.
[32] Gomez-Barrena E, Padilla-Eguiluz NG, Avendano-Sola C, Payares-Herrera C, Velasco-Iglesias A, Torres F, et al. A multicentric, open-label, randomized, comparative clinical trial of two different doses of expanded hbm-mscs plus biomaterial versus iliac crest autograft, for bone healing in nonunions after long bone fractures: study protocol. Stem Cells Int 2018;2018:6025918.