Autologous stem cells as a promising therapeutic approach for augmentation of alveolar bone

Cecilie Gudveig Gjerde

Thesis for the degree of Philosophiae Doctor (PhD)
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Dedication

This thesis is dedicated to the two persons who really have taught me about strength and courage: my husband Harald and my niece Liv Cecilie

Scientific environment

The work comprising this thesis was conducted at the Department of Clinical Dentistry (IKO), Faculty of Medicine, University of Bergen, Norway in collaboration with the Research and Development-Clinical Trial Unit at Haukeland University Hospital and was part of large collaborative projects financed by the EU Commission, Trond Mohn Foundation, the Research Council of Norway and Helse Vest funds.

The data analysed in study I, were obtained from the Registry Data of patients treated at Haukeland University Hospital, Bergen, Norway.

Study II and III were conducted as part of the EU FP7 Reborne project no. 241879.

The bone marrow aspirations were performed at the Research and Development-Clinical Trial Unit, Haukeland University Hospital, Bergen, Norway.

The stem cells were expanded at Institute for Clinical Transfusion Medicine and Immunogenetics Ulm, Red Cross Blood Service Baden-Württemberg—Hessen and Institute for Transfusion Medicine, University Hospital Ulm, Ulm, Germany.

The surgical intervention was undertaken at the Section of Oral and Maxillofacial Surgery at IKO. Cell viability tests and μCT analyses were performed at the Research Laboratoy in IKO.

Histology of the biopsies was performed at the INSERM, UMR 1238, PHY-OS, Laboratory of Bone Sarcomas and Remodeling of Calcified Tissues, Faculty of Medicine, University of Nantes, Nantes, France.

The main supervisor was Professor Kamal Mustafa, with Professor Trond Berge and Professor Annika Rosén as co-supervisors.

Abbreviations

αMEM	Alpha modified Eagle ´s medium
βFGF	Beta fibroblast growth factor
βTCP	Beta tricalcium phosphate
μCT	microcomputed tomography
2D	Two Dimensional
3D	Three Dimensional
ALP	Alkaline phosphatase
ATMP	Advanced therapy medicinal products
BBM	Bovine bone material
BCP	Biphasic calcium phosphate
BM	Bone marrow
BM MNC	Bone marrow mononuclear cells
BM MSC	Bone marrow-derived MSC
BMP	Bone morphogenetic proteins
BMP2	Bone morphogenetic protein 2
BMP7	Bone morphogenetic protein 7
BM WBC	Bone marrow white blood cells
BV	Bone volume
CaP	Calcium phosphate

CBCT	Cone beam computed tomography
CDM	Chemically defined media (serum-free)
CT	Computed tomography
DA	Degree of anisotropy
DAPI	4’,6-diamidino-2-phenylindole
DBBM	Deproteinized bovine bone material
DMEM	Dulbecco´s modified Eagle medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
FBS	Fetal bovine serum
FDA	US food and drug administration
FD	Fractal dimension
FGF-ß	fibroblast growth factor beta
GBR	Guided bone regeneration
GF	Growth factors
GMP	Current Good Manufacturing Practice
HA	Hydroxyapatite
HLA-DR	Human Leukocyte Antigen – DR isotype
HPD	Human platelet derivatives

IGF	Insulin like growth factor
iPSC	Induced pluripotent stem cells
ISCT	International Society for Cellular Therapy
ISQ	Implant stability quotient
ITEP	Injectable tissue engineered bone
MAXILLO-1	Name of the clincal trial
MBCP+	Biomatlante syntetic bone graft substitute
MHC	major histocompatibility complex
MNC	Mononuclear cells
MSC	Mesenchymal stromal/stem cells
MSC P0	MSC passage 0
MSC P1	MSC passage 1
OHIP-14	Oral Health Impacts Profile
OHRQoL	Oral health-related quality of life
PC	Platelet concentrate
PDGF	Platelet derived growth factor
PFA	Paraformaldehyde
Ph Eur	European Pharmacopoeia
PL	Platelet lysate
PROM	Patient reported outcome measures

PRP	Platelet rich plasma
PTFE	Polytetrafluoroethylene
QoL	Quality of Life
REK	Regional ethical committee
rhBMP2	recombinant human BMP2
RO1	Region of interest
SMI	Structural model index
SPSS	Statistical Package for the Social Sciences
TO	Time point 0
T1	Time point 1
Tb.Sp	Trabecular separation
Tb.Th	Trabecular thickness
TCP	Tricalcium phosphate
TGF-β	Transforming growth factor beta
TV	Total volume
VAS	Visual Analog Scale
VO1	Volume of interest
WBC	White blood cells

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ABSTRACT

The current gold standard for reconstructive bone surgery is based on autologous bone grafts. However, the risk of complications at both the donor and recipient sites is considerable. There is therefore a need to explore alternative methods of bone regeneration which will restore a defect to full functionality and meet esthetic demands. Nowhere is this a greater challenge than in reconstruction of defects in the orofacial region.

Preliminary data, from limited in vitro and in vivo studies, indicate that bone marrow-derived MSC have potential application in bone tissue regeneration. However, interpretation of these studies is complicated by lack of conformity with respect to cell type (expanded or native), culture medium, source of growth factors, expansion time, cell dose and other variables. Moreover, biopsies are required to confirm the osteogenic capacity of the implanted cells and this has not been done routinely. In most studies to date, follow-up has been limited to radiographs, which do not allow differentiation between bone tissue formed by the implanted cells and by the native cells from the border of the osseous defect. The question also remains as to whether the presence of any new bone should qualify as clinical success, or whether a successful outcome requires evidence of new bone formation at the center of the regenerated area. With respect to culture and expansion of MSC for bone tissue engineering, a further issue has arisen, namely the exclusion of animal-derived products from culture medium, requiring a human-derived source of growth factors to replace FBS.

The work presented in this thesis was undertaken in order to develop and validate each step in a standardized protocol for expanding autologous MSC in vitro in a GMP-compliant facility (Study II). The expanded MSC produced by this protocol were then applied in a phase I/II clinical trial of restoration of the mandibular alveolar ridge in 11 patients. The surgery was carried out by one experienced oral surgeon (Study III). The same surgeon also undertook the post-operative follow-up, with standardized patient evaluations at each appointment.

Bone regeneration was confirmed in all 11 patients, as evidenced by radiographs and biopsies taken at installation of all 21 dental implants. All the implants osseo-integrated. All patients considered the outcomes to be satisfactory, with minimum pain and no morbidity.

In a retrospective study of 59 patients who had undergone advanced alveolar ridge reconstruction in accordance with the current gold standard procedure, using autologous bone grafts (Study I), patient satisfaction and OHRQoL among participants was favorable. Despite their overall satisfaction with the outcome, these patients reported significant pain and morbidity. Furthermore, procedures based on autologous grafts from the iliac crest require substantial resources including hospitalization and sick leave.

The following conclusions are drawn from this series of studies. Firstly, a standard protocol has been established for GMP expansion of autologous human MSC, using PL as a source of growth factors instead of FBS. Secondly, fresh autologous MSC can be manufactured, expanded and applied in bone regeneration, despite considerable geographic distance between the cell production facility and the clinical center. Thirdly, this protocol was successfully applied for alveolar ridge bone regeneration in 11 patients, with clinical outcomes comparable to those achieved using grafted autologous bone, recovered surgically from a second site. Although patient satisfaction with the new protocol was no different from the standard approach, those treated according to the new protocol reported low pain and morbidity. The results of the comprehensive trial confirm that bone marrow mesenchymal stem cells can successfully promote bone regeneration, with no unexpected adverse events and minimal pain. Hence, this novel augmentation procedure warrants further investigation. It has the potential to form the basis of a new therapeutic approach which may challenge the current gold standard.

List of Publications

This thesis is based on the following publications:

Study I: Gjerde C, Shanbhag S, Neppelberg E, Mustafa K, Gjengedal H. Patient experience following iliac crest-derived alveolar bone grafting and implant placement. Int J Implant Dentistry 2020; 6:4. DOI: 10-1186/s40729-019-0200-8.

Study II: Rojewski M, Lofti R, Gjerde C, Mustafa K, Veronesi E, Ahmed A, Wiesneth M, et al. Translational of a standardized manufacturing protocol for mesenchymal stromal cells: A systematic comparison of validation and manufacturing data. Cytotherapy 2019; 21:468-482.

Study III: Gjerde C, Mustafa K, Hellem S, Rojewski M, Gjengedal H, Yassin M, Feng X, et al. Cell therapy induced regeneration of severely atrophied mandibular bone in a clinical trial. Stem Cell Res Ther 2018; Aug 9;9(1):213 DOI: 10.1186/s13287-018-0951-9.

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1. INTRODUCTION

Bone is the framework of our body, gives us a back bone which enables us to stand up straight, makes us able to move our muscles, serves as a reservoir for ions, contains the blood forming organ in its marrow, and protects vital organs such as heart and brain. The human skeleton is a work of art and the skull has a beautiful shape that I personally find inspiring. But the solidity of bone is, like civilization, not durable in the face of disease and trauma. It is therefore crucial that when there is loss or damage we find ways to re-establish normal form, including esthetics and function. This has been the ultimate goal of the research presented here from its inception. As with all grand goals, there have been many small steps along the way. This introduction will provide a background to the material and underlying skeleton supporting the research, which has been provided by a multidisciplinary team.

Facial defects - congenital, traumatic or as a result of disease – tend to be highly visible and can have a pronounced negative effect on quality of life (QoL), on the ability to speak and eat, on self-esteem and on social interactions (1). Reconstruction of such defects, to restore full function and meet high esthetic demands, is very challenging. In many cases, ideal reconstructive goals, such as a complete return to original form and function, are not completely achieved (1). A critical-size defect of the cranial vault is likely to sustain less biomechanical force than a critical-size defect of the mandible. Although both the cranium and mandible have excellent blood supply, bacterial contamination is much more of an issue in the mandible. Hence, the 3- dimensional (3D) construct providing structural support for the reconstruction must meet the biomechanical demands and provide an appropriate environment for regeneration.

The gold standard for reconstructive bone surgery today is autologous bone grafting, which fulfills basic criteria for an ideal implant: histocompatibility, non-immunogenic, osteogenic, osteoinductive and osteoconductive. There are, however, disadvantages associated with this procedure, both at the donor and recipient sites (2-5). See section 1.3 and Table1.

1.1 Components of bone tissue

The functional integrity of bone is maintained by three different committed cell types: osteoblasts, osteocytes and osteoclasts (6), embedded in a highly complex matrix comprising a mineralized (hydroxyapatite) and a non-mineralized component. The non-mineralized, organic component contains collagens, glycoproteins, proteoglycans and sialoproteins, which have essential roles in control of growth and differentiation of osteoblasts, osteocytes and osteoclasts, and in bone remodeling (6-9). Bone development and bone regeneration are complex regulated processes involving a wide range of different growth and transcription factors, which coordinate the interaction of cells and matrix in response to external or internal stimuli (6, 10-13). Bone can be separated into the osteoblast lineage, i.e. the bone-forming axis (MSC, pre-osteoblasts, mature osteoblasts, bone-lining cells and osteocytes), and the osteoclast lineage, i.e. the bone-resorbing arm (macrophages, osteoclasts, and multinucleated giant cells, all derived from bone marrow hematopoietic stem cells) (6). Throughout life, bone is continuously shaped, reshaped and repaired to maintain its structural properties and its role in mineral homeostasis. This occurs through two separate mechanisms: bone resorption and bone formation, coordinated by osteoclasts and osteoblasts respectively (14). This process is called remodeling and achieves complete regeneration of the adult skeleton every 10 years. The purpose of this remodeling is not entirely clear, although in bones that are load bearing, it most likely serves to repair fatigue damage and to prevent excessive aging and its consequences (15).

Bone resorption may also be associated with injury or lesions, followed in turn by bone regeneration/repair. Maintaining the balance depends on osteoblastic activity to form new bone, and on osteoclastic activity to remove excess bone. This balance is tightly controlled and any disruption may lead to bone disease, such as osteoporosis (16).
The remodeling cycle is completed when an equal amount of resorbed bone has been replaced. Osteoclasts undergo apoptosis; the mature osteoblasts form bone-lining cells or differentiate into osteocytes. The balance is kept and maintained until the next time remodeling is initiated (14).

1.2 Bone regeneration and healing of bone grafts

Bone regeneration is a highly efficient and tightly regulated process, the result of a continuous interplay between growth factors and cytokines for both initiation and regulation of the remodeling process (11, 12).

The three factors essential for bone formation and bone mineralization are multipotent precursor cells, an ample blood supply and mechanical support (12). After grafting, bone remodeling is dependent on re-establishing vascularization (17), to ensure transport of growth factors, hormones, cytokines and metabolites (18).

Today, autologous bone grafting is the gold standard for osteogenic bone replacement in osseous defects (19-22). Bone is the most common tissue transplanted, second only to blood transfusion. In orthopedics, neurosurgery and dentistry, more than 500,000 bone grafting procedures are undertaken annually in the United States and 2.2 million worldwide. (23, 24).

Following transplantation, autologous bone grafts fill substance deficits and induce bone tissue formation at the defect site. Chips, larger pieces and even blocks several centimeters in size can be harvested. Depending on donor site, size, shape and quality these grafts exhibit some initial stability.

However, clinical application of autologous bone transplants is limited by considerable donor site morbidity, which increases with the amount of bone harvested. Common complications of harvesting are bleeding, hematoma, infection, and chronic pain. Other disadvantages of autologous bone grafting include insufficient amounts of graft material available, particularly in children and for revision of reconstructive procedures; the likelihood of significant postsurgical morbidity at the donor site (i.e. rib, fibula, iliac crest), such as infection, pain, hemorrhage, muscle weakness, and nerve injury; increased surgical time and blood loss; and additional cost (2, 3, 13). Studies of autologous grafts report considerable reduction in grafted bone after bone reconstruction, corresponding to 36%–44% after 1–5 years (25, 26). Some studies have

reported reduction of bone volume, evaluated using CT scans, to be 47.5% within 6 months of transplantation (27).

Three critical factors are intimately involved in the physiology of graft incorporation and survival: osteogenesis, osteoinduction, and osteoconduction. Osteogenesis is the ability of a graft to synthesize new bone, either through cells within the donor graft or the recipient bone. A graft with higher osteogenic potential has the greatest potential to form new bone. This potential largely relies on the viability of cells, both the osteoprogenitor and the supportive cells, within the graft. Osteoinduction is the process of stimulating mesenchymal stem cells at the recipient or graft site to differentiate into osteoblasts. In osteoconduction, a scaffolding is provided to enhance migration of recipient site MSC, capillaries, and tissue to produce bone (13, 17, 28-31).

Graft incorporation, or take, is a complex process, dependent on variables within the recipient site and the graft. The rate and degree of incorporation are largely dependent on the osteogenic, osteoinductive, and osteoconductive properties of the graft. The process of bone graft incorporation involves an initial hemorrhage and hematoma within and around the graft: this serves to nourish the graft until distinct capillaries and vasculature develop. There is an inflammatory response to the initial surgical intervention, resulting in ingrowth of granulation tissue into the graft. This revascularizes the tissue and transports osteoprogenitor cells into the graft. Once revascularization is complete, viable cells within the graft and the recipient osteoprogenitor cells begin to resorb the old bone and form new bone (13, 17, 32). The process of new bone formation and old bone resorption within bone grafts is called creeping substitution. After new bone is formed, it is mineralized and remodeled (29).

Table 1 Advantages and disadvantages of the most commonly used materials for bone grafting (33-36).

Material	Advantages	Disadvantages
Autograft	Osteogenic Osteoconductive Osteoinductive No immunoreaction	Increased patient morbidity: pain and infection at donor site, nerve damage Not a standardized product Two operation areas Longer operation time Lack of vascularization Limited quantity and availability Graft resorption
Allograft	Osteoconductive Osteoinductive Ready availability Easy handling No donor site morbidity	Lacks osteogenicity and vascularization Relatively higher rejection risk Risk of disease transmission High cost Limited mechanical properties
Alloplastic Graft	Osteoconductive Ready availability Easy handling No donor site morbidity Safe Modifiable in terms of resorption	Lack of osteogenic properties Limited mechanical properties Long healing time High cost
Xenograft	Osteoconductive High availability No donor site morbidity Very little resorption	Lack of osteogenic properties Risk of immunogenicity Very little resorption Limited mechanical properties Long healing time High cost

1.3 Tissue engineering and regenerative medicine

The basic premise of tissue engineering or regenerative medicine is the provision of a new construct to replace lost tissue. Tissue engineering is an interdisciplinary, translational field which applies the principles of bioengineering to the development of biological substitutes which restore, maintain, or improve tissue function (37). The traditional triad of tissue engineering attempts to replicate the intrinsic properties of autograft reconstructions (18, 38), i.e. adequate osteo-competent cell transfer, a structured scaffold to maintain space and provide osteoconduction, and growth factors to induce adjacent mesenchymal osteogenesis (19, 38, 39). A multidisciplinary approach combining bioscience, bioengineering, biomaterials science, and clinical science is thus evolving in the attempt to find workable constructs simulating the body's ability to produce the desired regenerated tissue (40).

1.3.1 Scaffolds

Tissue in the craniomaxillofacial region is varied in composition and in its simplest form consists of a matrix and different cell types (37). The matrix represents a 3D structure, or scaffold, for cells, providing a specific environment and architecture for a given functional purpose (41). The structure also serves as a reservoir for fluids, nutrients, cytokines, and growth factors. When these concepts are applied to tissue engineering to restore function or regenerate bone tissue in the craniofacial skeleton, the scaffold acts as a temporary matrix, or template, for cell proliferation, extracellular matrix deposition, bone regeneration, and remodeling until the mature bony tissue is regenerated (42). During this process, the scaffold also acts as a template for vascularization (43).

An ideal scaffold for MSC transfer and tissue engineering is a bioresorbable, biocompatible, osteoinductive material which supports cellular attachment, proliferation, migration and differentiation (18, 38, 44-46). It should also have appropriate mechanical strength and timely degradation for successful healing. The macro- and microstructure of the scaffolding also influences the outcome considerably. The scaffolding should have an outer shape appropriate for the size and geometry of

the defect and an inner architecture ensuring an interconnected, open porous system which allows capillary ingrowth and sufficient nutrient and oxygen supply to the cells (18). The degradation characteristics, bioresorbability and integration into the host tissue greatly impact the clinical outcome.

Bone tissue engineering is most commonly represented by the concept of the implantable construct, comprising a scaffold/matrix in combination with progenitor/stem cells and/or osteoconductive growth factors. The function of the scaffold is to support cell colonization, migration, growth and differentiation, and to guide the development of the new tissue and/or to act as a drug delivery device. The optimal scaffold should support new bone formation and early mineralization, while allowing for its own biodegradation once it has served its purpose.

Among the different biomaterials being used as scaffolds, hydroxyapatite (HA) and other calcium phosphate-based ceramics show particular promise because of their osteoconductivity, biocompatibility, and ability to integrate with the host bone (38–41). HA provides the best strength but can remain, unresorbed, within the defect for years. On the other hand, beta-tricalcium phosphate (βTCP) is more soluble than HA and degrades more rapidly; but used alone it is too fragile to sustain physiological loads. Combinations of HA and TCP, biphasic calcium phosphate (BCP) ceramics have been evaluated as controlled biodegradable osteoconductive material, providing improved bone formation and bone bonding (19).

Several clinical trials showed that CaP (calcium phosphate) scaffolds in combination with precultured MSC have substantial capacity to heal bone defects (47-49). Molecular screening of cell/CaP biomaterial interaction effects shows that Ca2+ might be responsible for inducing osteogenic differentiation in MSC, as demonstrated by increased expression of ALP and BMP2 (50).

1.3.2 Cells

MSC were first described in a series of studies by Friedenstein in the 1960's (51). The cell was identified in bone marrow as able to adhere to the surface of a tissue culture plate *in vitro* and generate skeletal tissues, including bone and cartilage, following

heterotopic implantation *in vivo* (52-56). Cell populations with comparable properties were also found in many other tissues, such as adipose tissue (57, 58) and umbilical cord blood (59, 60). It has been reported that MSC from different sources exhibit functional differences, such as surface phenotype and differentiation potential (61, 62). MSC are multipotent and with appropriate induction signaling, MSC in culture can differentiate into bone, cartilage, adipose, and muscle lineage cells (63-65).

In 2006, Yamanaka et al. showed that somatic cells such as fibroblasts could be reprogrammed to become pluripotent by the transfer of four genes via viral vectors, and called these cells induced pluripotent stem cells (iPSC) (66). Their potential is similar to that of embryonic stem cells, but circumvents the ethical concerns associated with embryonic stem cells (67). However, generation of iPSC has a low reprogramming efficiency and as it requires the introduction of exogenous transcription factors via viral vectors, there is a risk that the cells can form teratomas which are difficult to control. Thus, the safety of patient-derived iPSC is not firmly established (67, 68).

In 2006, the International Society for Cellular Therapy (ISCT) proposed minimal criteria to define human MSC (69): (i) MSC are plastic-adherent when maintained in standard culture conditions, (ii) MSC express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79 or CD19, and HLA-DR surface molecules, (iii) MSC can differentiate into osteoblasts, adipocytes, and chondrocytes *in vitro*.

Some researchers have proposed that *in vivo* criteria should constitute the 'gold standard' for definition of MSC (70). It has been shown that bone marrow MSC, inserted onto a hydroxyapatite or related scaffold and subcutaneously implanted in rats, can form mineralized bone containing hematopoietic marrow (70). For many academic laboratories, this *in vivo* assay of bone differentiation remains the definitive standard (71, 72).

In a recent systematic review and meta-analysis, it was reported that most of the available clinical data supporting the efficacy of cell therapy in treating jaw bone defects are from studies using and transplanting the whole tissue fraction (e.g bone

marrow) (73). This has the advantage of minimum manipulation of cells and perhaps the cost. However, bone marrow fraction aspirates may contain other cells besides MSC, such as endothelial cells and hematopoietic stem cells (74). Although MSC are present in multiple tissues, the overall quantity in the body is small, accounting for only approximately 0.001-0.01% of mononuclear cells in the bone marrow (75, 76). Cell therapy protocols generally require hundreds of millions of MSC per treatment; therefore, cell expansion *in vitro* is necessary to obtain a sufficient number of cells for a clinical procedure (18, 77). The need for cell expansion has significant disadvantages. It takes weeks to expand the cells; there is a risk of infection/contamination during the process and only limited evidence for cell culture efficacy (73).

1.3.3 Cell signals/growth factors

In 1965, Urist discovered substances in the extracellular matrix of bone which stimulate the formation of new osseous tissue (78). A multitude of these factors regulate bone metabolism as well as cellular differentiation and proliferation and expression of extracellular matrix proteins. These growth factors (GF), e.g. transforming growth factor β (TGF- β), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (β FGF) and the bone morphogenetic proteins (BMP) are stored within the matrix and osteoid of the skeleton. During the constantly ongoing physiological process of bone turnover, osteoclast activity results in the release of these factors in their biologically active form. These GF then act upon progenitor/stem cells, osteoblasts and cells of other lineages such as endothelial and vascular cells to induce the regeneration of lost tissue *in situ*. Regeneration and degeneration of the tissue are closely interlinked, remain in balanced proportion to each other and are associated with a specific location (18, 37). Growth factors have been shown to play a key role in bone and cartilage formation, fracture healing, and the repair of other musculoskeletal tissues (34, 37).

Because of their therapeutic potential, various proteins/growth factors are being explored in bone regeneration research. Although many studies show that such molecules can have a direct and crucial role, their exact molecular mechanisms have not been fully explained (79). Improved understanding of cross-activation and complex

signaling of these molecules will hopefully lead to the design of advanced bone-substitute materials. More detailed knowledge about regulation of signaling mechanisms in different cell types and the molecular consequences of cell–biomaterial interactions will help to control the regeneration of bone defects (79).

In most studies to date, MSC have been cultured and expanded in media containing fetal bovine serum (FBS), which provides a source of bioactive molecules and growth factors required for MSC attachment and proliferation (80). Although FBS is a well-known supplement for MSC expansion, the major drawback is the possibility of triggering immunological responses in the recipient against xenogeneic antigens (81), the risk that animal-derived products may cause immune-reactions towards foreign factors, as well as cross-species pathogen infections (73, 82-84). For these reasons, FBS should be replaced as a growth supplement in cell culture media. Recent research has focused on substitution of FBS with human platelet-derived products, e.g., platelet lysates, which can be produced from standard platelet transfusion units by lysis (73, 80, 85, 86). Based on these studies, platelet-derived products have been proposed as a viable alternative for the *ex vivo* culture of cells for human therapy (73, 80).

1.4 Clinical bone regeneration trials

1.4.1 Review of the state of the art

The clinical studies discussed below used different approaches, including bone marrow, MSC and scaffolds, and osteoinductive factors (i.e. BMP) in treating a variety of conditions, including complex tibial fractures, tumors, osteonecrosis and bone regeneration. Most were limited, observational phase I-type studies, with no control groups and only short-term follow up (73). Despite their shortcomings, these studies provide valuable information about the clinical application of autologous bone marrow and MSC: the procedure is relatively safe and in the event of failure it does not preclude the use of other techniques (19).

In the bone tissue engineering field, three strategies currently use the patient's own bone marrow cells to engineer autologous osteogenic grafts. The first approach consists of aspirating the whole bone marrow fraction, followed by centrifugation (87) to

concentrate mononuclear cells, and then immediate implantation into the bone defect, with or without a synthetic bone substitute. In the second approach, the harvested cells are cultured for 2–3 weeks in a cell therapy facility in order to isolate and expand the MSC fraction. Several tens of millions of uncommitted MSC are then injected alone into a bone defect, or seeded onto a suitable scaffold. These hybrid MSC/biomaterial grafts or "constructs" have shown bone-inducing ability in preclinical and clinical models (48, 75). In the third strategy, bone marrow is harvested, the osteoprogenitor cells are isolated and expanded for several weeks, then seeded onto a scaffold and cultured for a further few weeks in the presence of osteogenic supplements, to promote the formation of a bone-like layer of tissue on the implant (88, 89). This hybrid construct is finally transplanted into an orthotopic site to regenerate a bone defect. Thus, the latter two strategies require several weeks of culturing under strict aseptic conditions in a GMP-compliant facility. From a regulatory perspective, clinical application for bone tissue engineering is therefore very complicated (90).

The use of osteoprogenitor cells from bone marrow expanded *ex vivo* was first reported in 2001 (91). Cell-based tissue-engineering was used to treat large bone defects in 3 patients, with very good results. The same group later treated one more patient, and in 2007 reported 6-7-year follow-up of these 4 patients (92). The patients had suffered comminuted and complicated fractures of the extremities, all of which were originally managed in a traditional manner, without success. They used 20 ml bone marrow aspirated from the iliac crest, from which nucleated cells were extracted and expanded for 3 weeks. Porous HA was customized to fit the defect and seeded with cells at a density of 2.0×10^7. The HA cylinders were positioned in the bone defects and external fixation was used in all cases. Consolidation between the implant and host bone was completed 5-7 months after surgery. In all patients at last follow-up, up to 7 years post-surgery, there was good integration of the graft and full function of the affected limb.

Lendeckel et al. were the next to report the use of cells to treat a bone defect. The case was a 7-year-old girl with bilateral calvarial defects after a fall and subsequent failed reconstruction (93). 15 mL cancellous bone from the ilium was milled, two resorbable microporous sheets molded to fit the defects were fitted over the defects filled with the

bone chips, and to enhance regeneration they excised 42.3 grams adipose tissue from the left gluteal area and processed it according to Zuk et al. (58). They applied 295 × 10^6 cells to the defect and assumed that 2-3 % of the cells were stem cells. The patient healed uneventfully, CT scans 3 months postoperatively showed marked ossification in the defect areas, and the girl was able to stop wearing a protective helmet. There has been no report on long term follow up.

In the same year, Schimming and Schmelzeisen presented a clinical study in 27 patients, using bone matrix derived from periosteal cells to augment the posterior maxilla (94), using a sinus lift procedure with simultaneous dental implant installation in 12 patients and a 2-stage procedure in 15 patients. The periosteal cells were treated with collagenase CLSII, and suspended in DMEM/Ham`s medium supplemented with 10 % autologous serum; after 4 passages the cells were absorbed into Ethisorb fleece (Ethicon, Norderstedt, Germany) and cultured in medium supplemented with dexamethasone. One patient had an early infection and two implants and the grafted material were removed during the first postoperative week. In 8 cases, all treated by the two-step procedure, almost no bone formation occurred. The remaining 18 patients had excellent results after 3 months, and 9 of these were followed for more than 6 months.

In 2011, the 5-year follow-up was reported for the 10 patients who had undergone the one step procedure in the previous study (95). For the first year after augmentation there was a slight decrease in the height of the augmented bone, but the level remained stable for the next 4 years. No adverse events were reported, and the clinical results were good.

In 2004, Warnke et al. published a frequently referenced report (96) of a case describing use of a titanium mesh scaffold, formed to model the mandibular defect in a 56 year-old male who had undergone mandibulectomy for cancer 8 years previously. The mesh was filled with BioOss blocks (Geistlich Pharma AG, Wolhusen, Switzerland) coated with 7 mg recombinant human BMP7 embedded in 1g bovine collagen type 1, and 20 mL of unmanipulated bone marrow aspirated from the recipient´s right iliac crest. The mesh and its contents were implanted into the recipent´s latissimus dorsi rectus muscle,

and 7 weeks later the graft was transplanted, along with part of the muscle, artery and vein into the defect site via an extraoral approach. Four weeks post-transplantation the recipient had his first solid meal in 9 years. Some exposure of the titanium mesh occurred over time after implantation. Unfortunately, the patient died of cardiac arrest 15 months postoperatively. The family refused permission for postmortem examination of the graft (97). Although not stem cell treatment per se, this approach is included here as it is often cited and can be considered a step on the way to stem cell therapy.

In 2004, Kitoh et al. reported preliminary results using marrow-derived MSC and platelet rich plasma (PRP) during distraction osteogenesis (98). The cells were extracted from bone marrow aspirates, cultured with osteogenic supplements, and injected together with autologous PRP into the distracted callus, first at the beginning of the lengthening period and then at start of the consolidation period. The results were promising. In 2007 they reported on the results from 20 patients with limb lengthening procedures: 11 patients had BM MSC and PRP injected as reported earlier, and 9 served as a control group. An average of 3.2×10^7 cells was transplanted. The average healing index of the test group was significantly lower than that of the control group. It was concluded that transplantation of BM MSC and PRP shortened the treatment period and accelerated new bone regeneration (99).

In 2005, Hernigou et al. (87) reported on 60 patients with tibial non-union. Bone marrow aspirated from both iliac crests was concentrated in a cell separator and injected into the area of non-union. Bone union was achieved in 53 patients. In the seven patients where union was not achieved, both the concentration and total number of progenitors (number obtained later, after injection) were significantly lower than those in the patients in whom bone union was achieved. It was concluded that efficacy of treatment was related to the number of progenitors in the patient's bone marrow.

In the same year, Ueda and Yamada in Japan presented a clinical study in 6 patients, using MSC, PRP and βTCP as grafting material in 3 sinuses and as 3 maxillary onlays (100). They extracted 10 ml of bone marrow from the iliac crest, expanded the cells for 4 weeks in Dulbeccos Modified Eagles Medium (DMEM) enriched with dexamethasone, sodium beta-glycerophosphate and L-ascorbic acid 2-phosphate. For

the patients, they used 1.0×10^7 cells/ml, 10% calcium chloride and PRP mixed with βTCP. Dental implants were installed simultaneously. All 20 installed dental implants were stable 12 months post-loading, and the ridge had an average increase in height of 7.3 mm. Their next report in 2006 was their first using their "injectable tissue engineered bone"(ITEB) on a 9 year old girl with a unilateral cleft palate (101). They extracted 10 mL bone marrow, isolated the MSC and expanded the cells *in vitro* for 4 weeks. They did not report on characterization on the cells. The cells then underwent induction for 1 week with dexamethasone, and together with PRP, 5.0×10^7 MSC and 0.3 ml of 10 % calcium chloride, forming a gel which was injected into the exposed alveolar cleft. The graft was covered with a titanium mesh and closed. The radiopacity increased over the next months, and after 9 months the canine and lateral incisor erupted. In 2008 the group reported on 14 patients treated with their ITEB, with the only change in material being a reduction to 1.0×10^7 cells/ml (102). This study comprised 6 sinus lifts and 8 onlay procedures. The mean age of the patients was 54.6 years, and dental implants were installed simultaneously. They reported 100% success with dental implants, but after 4.8 months of healing, 2 of the 8 onlays did not have complete coverage of the dental implants. In the sinuses, the average increase in height was 8.7 mm, and the mean increase in alveolar ridge height was 5 mm. No adverse events were reported.

This group has several reports using the same type of graft material. In 2008, they reported on 12 patients, mean age 54 years, who underwent maxillary sinus lifts, dental implants and ITEB. They used 5.0×10^6 cells, but the rest of the technique was unchanged (103). Here too they reported 100 % success, with a mean gain in bone height of 8.8 millimeters and no adverse events.

In 2013, they reported the use of ITEB material to augment the mandibular left alveolar ridge in a 58-year-old male (104). The material was applied to the alveolar ridge and covered by a titanium lined membrane. At the second surgery 7 months later, there was enough bone for installation of dental implants. The authors reported a successful outcome at 2-year follow-up. The following year the research group reported the use of ITEB in 104 patients. They used a guided bone regeneration (GBR) technique in 36 of the patients, sinus lift in 39, socket preservation in 12 and also used the material on

17 severe periodontitis cases. The mean patient age was 57.7 years. In this study the cells were characterized. They used 3.21×10^7 cells in GBR, 1.76×10^7 in sinus lifts, 1.19×10^7 in socket preservation and 1.34×10^7 in periodontal patients. No adverse events were recorded, and they reported 100% survival of the dental implants, and in the periodontal cases a mean gain in clinical attachment level of 4.29 mm. They concluded that taken together, regenerative bone therapies using MSC transplantation are highly effective and reduce associated complications by accelerating new bone formation and maintaining good function.

Dental pulp stem cells have also been used to repair defects in the human mandible (105). A paper from 2009 reported 7 patients, in whom dental pulp cells from the maxillary third molars were harvested and expanded for 21 days in α MEM and FBS. Thereafter the mandibular third molars were extracted. A collagen sponge soaked with the cells was placed in the empty socket at one site, and a sponge without cells was used on the control side. After 3 months clinical attachment was higher at the test site than at the control site, and biopsies taken at this time showed more mature bone in the test site. In 2013, the group reported 3-year follow-up biopsies from the same patients (106). The patients did not report any adverse sequelae, and clinical parameters were normal at both test and control sites. The 3D reconstructions of two sub-volumes, showing bone volume density (ratio of bone volume (BV) to total volume (TV)), confirmed that a human mandible treated *in vivo* with stem cells derived from dental pulp was composed of more compact bone than the control human mandible, with a higher BV/TV caused by its conformation (i.e., the presence of more than 20 lamellae) and an absence of marrow-containing lacunae typical of cancellous bone.

In 2009, a research group published a pilot study in 2 cleft palate patients using calcium sulfate incorporating human demineralized bone matrix, mixed with autologous expanded MSC (107). The cells were expanded for 2 weeks in DMEM and autologous serum. The graft material comprised 5×10^5 cells. Evaluation after 4 months showed clinically satisfactory results, but less than 50% bone fill in the defects. The authors speculate that the use of human serum resulted in low bone filling, but it should be noted that the interval between augmentation and follow-up was quite small.

The same group reported on 4 cleft palate patients in 2011, using the same procedure for cell expansion and cell dose, but with the biomaterial changed to a synthetic mixture of 60% HA and 40% βTCP (108). Moreover, at surgery, they used platelet-derived growth factors and platelet-rich fibrin on the graft. Clinically, healing was uneventful, and x-rays showed a mean of 51.3% bone fill 3 months postoperatively.

Mendonca and Juiz-Lopez treated 3 patients using 100 mL of bone marrow aspirate, cultured for 12 days in αDMD supplemented with 10 % FBS and 10 % horse serum (109). The expanded cells were mixed with PRP and βTCP/HA. The average cell dose was 20.5×10^6 cell per mL. One patient had osteoradionecrosis, one had serious sequelae after trauma and one had severe bone loss in the mandible and maxilla. All grafts healed and the patients even recovered some sensation in nerves damaged earlier. No adverse events were reported.

A group from South Korea reported in 2010 on the use of bone marrow stem cells in a young patient suffering from a large central hemangioma (110). During resection of the hemangioma a large part of the mandible was removed. This was freeze dried and stored for use later in reconstruction as a scaffold for the cell construct. Three mL of bone marrow were extracted and expanded with αMEM and FBS, dexamethasone was added and the cells were passaged twice in 6 weeks. The scaffold was seeded with 4.8×10^7 cells for use. One year later the implanted mandible showed evidence of bone regeneration, but with mild asymmetry that was treated with distraction osteogenesis. For the distraction operation 4.8×10^7 cells per mL were injected at the operation site. Activation was initiated after 8 days, to 10 millimeters, and consolidated for 7 months. The distractor was then removed, and GBR with titanium mesh, autologous, differentiated MSC were applied and a dental implant was installed. The result was good, but with a very high burden of care for the patient. Furthermore, in this study, FBS, which is an animal derivative supplement was used during expansion of the cells.

In 2010, Rickert et al. published the results of a prospective randomized clinical trial, of maxillary sinus grafts, with bovine bone mineral (BBM) combined with either autologous bone or autologous concentrated bone marrow in a split mouth design in 11 patients (111). Second stage surgery with biopsies were taken at a mean of 15 weeks

after the augmentation procedure. The results showed that concentrated bone marrow was equivalent to autologous bone.

The same group used BBM and bone marrow aspirate concentrate for sinus augmentation in a controlled, randomized trial where the control was BBM mixed with autologous bone (112). The results confirmed that concentrated bone marrow achieved bone formation equivalent to that achieved by autologous bone.

A Danish group in 2012 published a randomized clinical study comparing cultured, autologous bone cells to deproteinized bovine bone mineral (DBBM), and autologous bone enhanced bone formation compared to DBBM and autologous bone alone (113). No futher benefit was shown for the use of bone-derived cells expanded *in vitro*.

In a randomized, controlled trial for treating extraction sockets in 24 patients, Kaigler et al investigated whether bone growth in response to approximately 1.5×10^7 cells, derived from bone marrow aspirate cultured for 12 days *in vitro* suspended on an absorbable gelatin sponge, was superior to that of the sponge alone in fresh extraction sockets (114). There were no adverse study-related events. Half the patients underwent biopsy after 6 weeks, and the rest after 12 weeks. At 6 weeks there was slightly more new bone in the group receiving cells, but after 12 weeks the differences between treatment and control groups were not significant. Clinical analyses of treatment sites demonstrated that the cell therapy accelerated the regenerative response. Further, there was a significantly reduced need for secondary bone grafting procedures in the group which had received the cellular therapy.

The following year the same group reported treatment of a large defect of the alveolar ridge in a trauma patient (115). βTCP served as the cell carrier and the same culture process was used as in the previous series, with a cell dose of 14.1×10^6, and a barrier membrane was placed over the graft. The site was reentered 4 months later, when CBCT showed an approximately 25% reduction of the graft over time. Two dental implants were installed, and biopsies showed highly vascularized, mineralized tissue, indicative of bone formation.

In 2013, a group from Italy published two articles reporting on 8 patients with pseudarthrosis in the upper limb which had not healed using traditional therapy (116,

117). They harvested 60 mL of bone marrow from the patients' bilateral iliac crests and cultured the cells in autologous serum for 10-18 days, supplemented with osteogenic medium for the last 4 days before harvest. The bone defect was entered, revised and a construct made of cells and biomaterial, with 4 different materials used in the 8 patients, and a fibrin clot from autologous serum was placed in the defect. All cases healed, and at 76-month follow-up no episodes of fracture, ectopic neoformation, infection or overgrowth had occurred.

In 2017, Hernigou et al. published a study on allografts supercharged with bone-marrow-derived MSC (118). They examined 20 patients who had received an acetabular graft 8-13 years earlier and were now to undergo femoral hip revision for reasons other than graft failure. Three types of graft were used: allografts initially loaded with bone marrow-derived MSC (BM MSC); dead, irradiated allografts; and autografts. They reported that the concentration of MSC in allografts previously loaded with BM MSC was greater than that in autografts. Few or no MSC were found in allografts without cells. New bone-formation analysis showed that allografts loaded with BM MSC produced more new bone (mean 35%; range 20-50%) than either uncharged allografts (9%; range 2-15%) or autografts (24%; range 12-32%). It was concluded that the results support the long-term benefit of supercharging bone allografts with autologous BM MSC.

More recently, in 2018 the orthopedic group in our EU funded project (Reborne FP7 EU project) published results for treatment of non-union, using autologous expanded bone marrow MSC combined with bone substitute biomaterials made of bicalcium phosphate. Of the 28 participants in the study, 26 healed. There were no adverse events related to the BM MSC (48).

1.4.2 FBS vs PL as a source of growth factors for MSC expansion

FBS is the most commonly used supplement for *ex vivo* expansion of MSC for bone tissue engineering applications. However, from a clinical standpoint, it is important to use animal or human-derived products derived according to current laboratory guidelines for good manufacturing practice (GMP) (73). The previously described strategies for use of MSC in regenerative therapy have involved the clinical use of

tissue fractions containing these cells (along with other supporting cells), for example, whole or concentrated bone marrow, usually acquired through a ‘‘chair-side’’ procedure outside of GMP, resulting in a wide variation in cell type and number used.

The *ex vivo* expansion of MSC from harvested tissues, for example bone marrow or adipose tissues, under GMP-grade conditions before clinical application, will provide a relevant number of cells and allow their characterization. Traditionally, *ex vivo* expansion of MSC has been performed using basal culture media plus supplements to provide GF, proteins and enzymes to support cell growth (119). The reason that FBS is often used in MSC culture is that the fetal environment is rich in GF and poor in antibodies (80). However, for clinical use it is important that animal-derived products are replaced with human products. MSC can internalize xenogeneic proteins, and thus carry the risk of infection (through viral, prion or other agents) and immunoreaction. It has been reported that a single injection of 100 million MSC expanded in 20% FBS-supplemented media is associated with 7-30 mg of bovine serum proteins (13). Moreover, there are concerns about FBS sample-to-sample consistency, and animal welfare concerns in terms of the ‘‘3 R’s’’ principle (replacement, reduction, refinement) (120, 121).

Up to 2013, proposals submitted to the FDA for MSC-based products were increasing rapidly and were characterized by increased variability in donor and tissue sources, manufacturing processes, proposed functional mechanisms, and characterization methods. Although use of clinical-grade FBS may be permitted by regulatory health agencies in phase 1 clinical studies, with >80% of proposals submitted to the FDA for MSC-based products reporting expansion in FBS (122), according to GMP guidelines later phase trials involving larger patient groups require the use of culture conditions free of animal-derived products, (120). Such ‘‘xeno-free’’ or humanized alternatives to FBS broadly include three categories of products: (1) autologous or allogeneic human serum (HS), (2) pooled human platelet derivatives (HPD), and (3) chemically defined media (serum-free) (CDM) (19). Of particular interest are HPD, as platelets release a wide range of physiological GF and cytokines, which can significantly enhance cell growth and function (123).

1.4.3 Variability in clinical trial protocols is problematic

The literature shows pronounced variations in clinical trials of MSC in bone augmentation. Many are based on small patient numbers. The multitude of protocols, the range of parameters and data in the current literature preclude any clear conclusion as to the most reliable model. It does, however, indicate the need for additional collaborative studies using consistent protocols and data analysis in advancing the science of bone reconstruction using MSC.

1.5 Rationale

The protocols for isolation and expansion of donor MSC vary widely between clinical trials, which may affect the efficacy of the therapy. This is perhaps the reason for the gap between preclinical and clinical findings (73). It is therefore important to develop international standards for MSC production which are evidence-based, regulatory authority-compliant, of good medical practice grade, cost effective, and clinically practical (80). Only once these standards are developed can this innovative approach become an established, reproducible and widely adopted treatment. The EU has acknowledged this need, and has funded several projects, among them VascuBone, FP7-EU project no. 242175 and Reborne, FP7-EU project no. 241879, in tissue engineering. The goal of Study II, was to establish a standardized protocol, and then to test the safety and efficacy of the cell product according to this protocol (Study III). Finally, Study I comprised a retrospective assessment of patients who had undergone advanced autologous alveolar bone augmentation from the anterior iliac crest, with reference to graft and implant survival and to patient-reported outcome measures (PROM), i.e. satisfaction and oral health-related quality of life (OHRQoL).

2. AIMS

The application of stem cell therapy and tissue engineering strategies could provide a feasible alternative to autologous bone grafting, reducing the need for, and thereby the morbidity of, invasive bone harvesting. Therefore, the overall goal of this thesis is to introduce an alternative approach for regenerating human alveolar ridge bone by using autologous bone marrow-derived MSC and biomaterials.

Specific Aims

Study I: The aim of this retrospective study was to evaluate patient-reported outcome measures (PROMs), including overall satisfaction and oral health-related quality of life (OHRQoL); and to evaluate clinical outcomes, graft and implant survival, after advanced autologous alveolar ridge bone augmentation using bone harvested from the anterior iliac crest.

Study II: The primary aim was to implement a previously established protocol for good manufacturing practice (GMP) compliant large-scale expansion of bone marrow derived MSC and determine the feasibility of producing clinical doses of 50 – 100 million autologous MSC for alveolar ridge bone reconstruction prior to dental implant surgery, for patients in a clinical trial (Study III) using platelet lysate from human donors instead of FBS. The osteogenic potential of MSC expanded according to this specific protocol has previously been tested *in vivo* in preclinical models. A second aim of this study was to evaluate the feasibility of interaction between clinic and distant manufacturing center.

Study III: The primary aim was to introduce and validate the protocol described in Study II, using autologous bone marrow-derived MSC and synthetic microporous biphasic calcium phosphate, in a standardized, minimally invasive surgical procedure; and to assess the clinical feasibility, safety and efficacy of this procedure. A second aim was to evaluate the outcome of prosthetic rehabilitation, i.e. installation of dental implants in the augmented alveolar bone and screw-retention of a fixed partial denture on the implants.

3. MATERIALS AND METHODS

3.1 Study population and registration (study I)

The 2002 – 2012 records of the Oral Maxillofacial Department of Haukeland University Hospital were reviewed, in order to identify all patients with a treatment code indicating a bone graft. Those who had undergone autologous bone grafts from the iliac crest to the alveolar ridge were then identified. Each patient record was studied, and identical key features were noted. In all, 69 patients were identified as having undergone bone augmentation of a large area prior to restoration of the dentition with dental implants. All patients had undergone iliac crest grafting of the alveolar ridge at Haukeland University Hospital. At the time of the survey, seven patients had passed away, two had relocated (current addresses unknown), and one was in a psychiatric institution and unavailable for study. Thus, the final study sample comprised 59 patients (29 females, 30 male).

3.2 Medical records (study I)

The records of the 69 patients were analysed with reference to: (a) site of graft; (b) survival of grafts, determined by success or otherwise in installing implants in the grafted site(s); (c) 'implant survival' as determined by the presence of functional implant-supported prostheses at the most recent follow-up. When available, the reasons for implant failure were also recorded.

Questionnaire

A self-administered questionnaire was mailed to all 59 available patients, together with an information leaflet about the survey, a return envelope with prepaid postage and an informed consent form. Reminder letters were sent after two and four weeks if necessary.

The questionnaire contained 36 previously validated questions, which were categorized and related to: (1) demographic and lifestyle, (2) perceived general and oral health, (3) donor site and hospitalization, (4) implant and prosthesis and (5) OHRQoL (OHIP-14)

(see Table 1, Study 1). Responses to questions in categories 1–2 were recorded as 'yes/no' or graded on a 3- to 5-point Likert scale (124). Category 3 included information on duration of hospitalization and sick leave. Category 4 included information on 'graft survival', i.e., whether implants (and prostheses) had been installed in the augmented site(s), and 'implant survival', i.e., post-surgical "loss/loosening" of any implants. OHRQoL was assessed using a Norwegian version of the OHIP-14 (125). These 14 questions addressed seven domains of OHRQoL and the responses were graded on a 5-point Likert scale ranging from "at no time" (0) to "all of the time" (4) (seeTable 1, study 1).

Table 1, study I: Summary of questions

Category Question	**Response**
1) Perceived health-status	
General health	"very good" to "bad"
Oral health	"very good" to "bad"
Overall quality of life	"excellent" to "bad"
2) Lifestyle-related	
Smoking	"yes"," no" or "sometimes"
Appetite	"good" to "bad"
3) Donor-site-related	
Pain	"yes" and "no"
Infection	"yes" and "no"
Presence of a scar	"yes" and "no"
Reduced sensitivity	"no" to "total loss of sensitivity"
Problems walking	"no" to "a lot"
Satisfaction	"very satisfied" to "dissatisfied"
4) Implant-related	
Intraoral pain	"no" to "strong pain"
Installation of implants and prosthetic	"yes", "no" or "just implants"
Loss of implants	"yes" and "no"
Satisfaction with prosthesis	"very satisfied" to "dissatisfied"
5) OHIP-14	"at no time" to "all of the time"

3.3 Bone marrow harvesting and shipping (studies II and III)

Bone marrow (BM) aspirates were collected from 21 healthy volunteer donors recruited at the Cell Production Centre, The Institute for Clinical Transfusion medicine and Immunogenetics (IKT), University of Ulm, Germany to validate the expansion protocol and assess the influence of shipping BM and cells between the clinical and manufacturing centres when they are a considerable distance apart.

For the clinical trial (Study III), the inclusion criteria are described in detail in Study III: the main criterion was an alveolar ridge width of 4.5 mm or less. The patients were recruited at The Department of Clinical Dentistry, University of Bergen. All needed replacement of one or more mandibular posterior teeth, but required bone augmentation prior to dental implant installation. BM aspirates were harvested from 13 patients included in the study at The Section of Haematology, Haukeland University Hospital, Bergen, Norway.

In an operating room, under local anaesthesia, 25 mL BM (target goal) was aspirated from the posterior iliac crest of each patient. The posterior iliac crest was aspirated 2-3 times via a trocar introduced through cutaneous puncture. BM was harvested in 2-4 mL aliquots into 20 mL syringes prefilled with heparin (Ratiopharm, Leopharma, Denmark). The harvest, in its primary packaging, was placed in an isothermal box labelled according to Directive 2004/23/EC and 2006/17/EC. The transport temperature was between 18°C +/- 3°C, with temperature traceability. Delivery to the manufacturing site in Ulm, Germany was ensured within 24 h by couriered transportation, using a qualified transport company.

3.4 Isolation and expansion of MSC (studies II and III)

MSC from the BM aspirates were isolated and expanded as previously described, using a two-step protocol, option 1 by Fekete et al. (126). In brief, BM aspirates were directly plated, without any further manipulation, in Eagle´s Minimal Essential Medium, alpha formulation (αMEM medium) supplemented with 5% platelet lysate (PL, IKT Ulm) and 1 IU heparin/mL at day 0 and incubated at 5% CO2 atmosphere, 95% relative

humidity at 37°C. After staining with 7-amino-actinomycin D, the viability of the cells was evaluated by flow cytometry (FC500 flow cytometer; Beckman Coulter, USA). Samples were considered inadequate for further processing if the total white blood cell (WBC) count was less than 127.2×10^6 cells. After 2-4 days, the supernatants were discarded and replaced by fresh αMEM (Lonza, Basel, Switzerland) supplemented with 5% PL and 1 IU heparin/mL. Clonogenicity was assessed by counting colonies of more than 50 cells/colony and the medium was changed twice a week. The cells were processed and expanded under laminar hood flow, in grade A clean room conditions, for 14 days. The cells were then detached using trypsin (TrypZEAN; Lonza). The harvested passage 0 cells (MSC-P0) were counted and reseeded at a density of 4000 MSC-P0/cm^2 in αMEM supplemented with 8% human PL and 1 IU/ml heparin (Ratiopharm, Ulm, Germany) for 7 days. The cells were then detached and passage 1 MSC were washed with phosphate buffered saline without Ca^{2+}/Mg^{2+} (Lonza), and resuspended in a concentration of 20×10^6 MSCs/ml in clinical-grade physiological saline supplemented with 4–5% human serum albumin (CSL Behring, Munich, Germany).

Five ml of cell suspension were drawn into one or two sterile syringes sealed with a Luer-Lock stopper and shipped by a certified shipping company (World Courier, Stuttgart, Germany) to the clinical unit at the Section of Oral and Maxillofacial Surgery, Department of Clinical Dentistry, University of Bergen, within 24 h of production. Appropriate quality controls of the advanced therapy medicinal product (ATMP) were conducted after each step of the culture procedure. Viability was tested using Trypan blue viability tests and and the number of cells was counted in an Automated Cell Counter (Countess™; Invitrogen, Life Technologies, USA). Cells were characterized for their stemness using a flow cytometer and performing multiple assays for adipogenic, chondrogenic and osteogenic differentiation. Details on manufacturing the MSC, including quality controls, are presented in Study II.

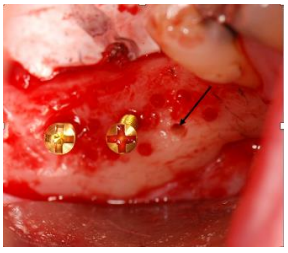
3.5 Patients and study design (Study III)

Thirteen patients were recruited for the clinical trial. All gave written informed consent to participation. The patients were to be aged between 18 and 80 years and had to be healthy non-smokers, with no evidence of infectious diseases. They presented with one or more missing mandibular posterior teeth and an alveolar ridge width in the edentulous area of less than 4.5 mm. For patients who met the inclusion criteria, cone beam computer tomography (CBCT) (Morita 3D Accuitomo F17, Japan) and dental X-ray scans were taken before and 4–6 months after grafting, to evaluate bone volume. The study design is described in detail in Study III.

3.6 Clinical procedures (Study III)

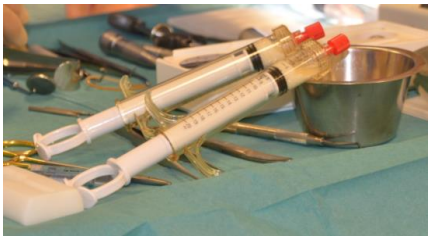
One hour before surgery, the surgical site in each patient was prepared and flaps were raised. The cortical bones were then perforated with a small round burr, to enhance blood flow and facilitate vascular ingrowth into the biomaterials (Fig. 1).

Figure 1; The alveolar rigde was perforated, and tenting screws were plased to support the biomaterial and membrane.



In parallel, the MSC delivered from the cell production center were mixed with particles of bicalcium phosphate (MBCP+™; Biomatlante, France), comprising 20% hydroxyapatite (HA) and 80% beta-tricalcium phosphate (β-TCP). Cells were allowed to attach to the biomaterial for 1 h in closed syringes (Fig.2).

Figure 2; The syringes containing the MSC and biomaterial.



The mixture of MSC and granules in a dose of 20×10^6 cells/cm^3 (75) were placed on the alveolar ridges under a titanium-reinforced PTFE membrane and muco-periosteal flaps (Fig. 3). Nonabsorbable sutures (4/0 Supramide; B. Braun Surgical SA, Spain) were used to close the wounds.

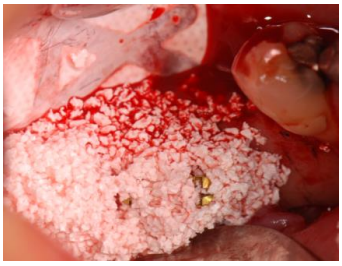


Figure 3; MSC and biomaterial placed on the alveolar ridge, membrane secured ligually.

Part of the mixture was used for bacteriological tests and cell attachment to BCP. Twelve days after surgery, the operation sites were clinically inspected and the sutures were removed.

The patients were recalled after 1, 2, and 4 months, in accordance with the protocol. CBCT scans were taken 4–6 months post-operatively, to determine whether the sites were ready for implant installation. At the time of implant installation, the augmented area was re-entered, if the width was 7 mm or more. Prior to implant installation, bone biopsies were taken and new bone formation was assessed by histology and micro-computed tomography (μ-CT) (Skyscan 1172; Bruker). Dental implants (Bone Level, Roxolid®, SLActive®; Institut Straumann AG, Basel, Switzerland) with a diameter of 4.1 mm and a length of 8–10 mm were then installed according to the manufacturer's recommendations (Fig. 4).

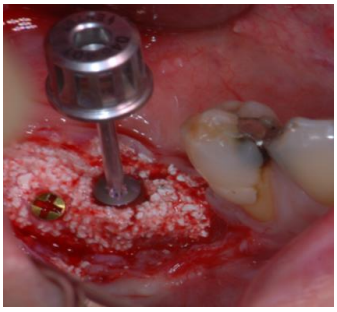


Figure 4; Dental implant placed after core biopsy.

Two months after implant installation, abutment surgery was done and a screw-retained crown was mounted 2–4 weeks later (Fig. 5). The implant stability quotient (ISQ) was measured at each of these procedures using an Osstell® device (Osstell AB, Gothenburg, Sweden).

a b c

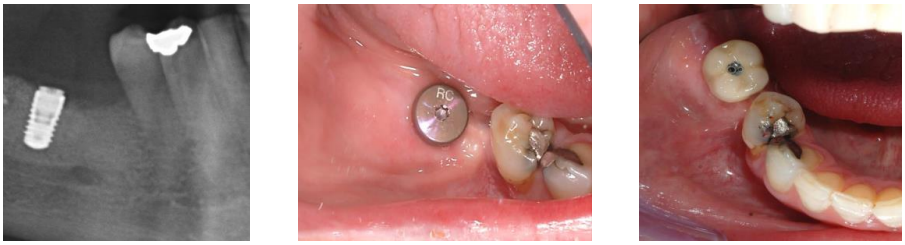


Figure 5; (a) X-ray before abutment surgery, (b) Clinical picture after abutment installation, (c) Clinical picture after crown installation.

3.7 Processing bone biopsies (Study III)

Bone biopsy specimens were collected before implant installation, maintained in 10% buffered formalin and scanned with the high-resolution μ-CT SkyScan1172® (SkyScan, Kontich, Belgium). Images from the scanning of biopsies were reconstructed by the software NRecon® (SkyScan) to obtain 2D and 3D images. CTvox (version 3.2; SkyScan) was used to create 3D images for the biopsies. The histomorphometric parameters analyzed have been described previously (127).

Fixed samples were decalcified in a solution (pH 7.4) containing 4.13% EDTA/0.2% PFA in PBS for 96 h at 50 °C, using an automated microwave decalcifying apparatus (KOS Histostation; Milestone Med. Corp., USA). Samples were dehydrated in an ascending series of ethanol, followed by butanol, in an automated dehydration station (MicromMicrotech, Lyon, France). The samples were embedded in paraffin (Histowax; Histolab, Gothenburg, Sweden). Thin histological sections (3 μm thick) were made using a standard microtome (Leica RM2255; Leica Biosystems, Nanterre, France). The sections were stained by the Masson trichrome technique. Slides were scanned (NanoZoomer; Hamamatsu, Photonics, Hamamatsu City, Shizuoka, Japan)

and observed virtually (NDP view; Hamamatsu). Histomorphometry of images was performed using ImageJ and the percentages of bone and bone marrow were calculated per area of explant. Four sections through each biopsy were analyzed and quantified.

3.8 Ethics

Study I

The Norwegian Committee for Medical Research Ethics ('REK', Health Region West), acknowledged this study as a treatment quality control study. Written consent was obtained from all participants.

Studies II and III

For the validation study, BM aspirates were collected from healthy volunteer donors after obtaining written informed consent according to the Declaration of Helsinki and approval by the Ethics Committee of Ulm University (ethical approval numbers 21/10 and 24/11).

The clinical trial MAXILLO-1 (EudraCT, 2012-003139-50, ClinicalTrials.gov, NCT 02751125, entitled "Jaw bone reconstruction using a combination of autologous MSC and biomaterial prior to dental implant placement") was approved by the Norwegian Ethical Committee (2013/1284/REKvest) and by the Norwegian Medicines Agency (13/12062-15). The trial followed the European guidelines for Advanced Therapy Medicinal Products (ATMP). The participants received oral and written information and were recruited after signing written informed consent according to the Declaration of Helsinki and to REKvest guidelines and approval. The cell manufacturing centre at the Institute for Clinical Transfusion Medicine and Immunogenetics, University of Ulm (Ulm, Germany) is authorised to expand and produce BM MSC for clinical trials (authorization number DE_BW_01_MIA_2013_0040/DE_BW:91_IKT Ulm).

3.9 Statistical analysis

Study I

Data were anonymized and analysed using SPSS v 24 (SPSS Inc, Chicago, IL, USA). Descriptive analyses were applied. Statistical significance was set at 5%.

Study II

GraphPad Prism 7.01 Software for Windows was used for statistical analysis. Each dataset was tested for normal distribution of data using the D'Agostino & Pearson normality test. In the case of normal distribution, data were compared using the unpaired t test with Welch's correction. For datasets which did not pass the D'Agostino & Pearson normality test, data were compared using the Mann-Whitney U test. The Kruskal-Wallis test was used for multiple-parameter analyses. Because of multiple testing of the datasets, differences were considered to be significant at $p < 0.01$.

The correlation of multiple parameters was assessed, computing Spearman correlation (r values) for every pair of the following datasets: time between end of aspiration and end of seeding (h), age (y), aspiration volume (mL), aspiration volume without heparin (mL), white blood cell count ([WBC]/mL), mononuclear cell count ([MNC]/mL), % MNC of WBC, % CD34+ cells in BM aspirate, harvest density of MSCP0 (cells/cm^2), harvest density of MSCP1 (cells/ cm^2), doubling time of MSCP0 (h), doubling time of MSCP1 (h), population doublings in P0, population doublings in P1, cumulative population doublings, CFU-F/10^6 BM-WBC, CFU-F/10^6 MSCP0, CFUF/ 10^6 MSCP1, MSCP0 harvested/mL BM aspirate seeded and MSCP1 harvested/mL BM aspirate seeded. Correlations with r 0.5 and $p < 0.05$ were considered to be significant with 5% PL and 1 IU heparin/mL.

Study III

Bone width and volume are shown as means and confidence intervals. Confidence intervals were based on formulae assuming normally distributed data. The p value was calculated from a one-sample t test, with 0 as the hypothesized difference. A $p < 0.05$ was considered statistically significant.

4. SUMMARY OF MAIN RESULTS

4.1 Results Study I

The final sample consisted of 44 patients (24 women, 20 men), with a mean age of 61.2 years ± 13.1, (range: 27–82 years) who responded and completed the questionnaire, giving a response rate of 74.6%. The mean interval between augmentation surgery and completion of the questionnaire was 7.8 years ± 2.65, (range 1.9–12 years).

4.1.1 Health-related PROM

General

The majority of patients reported "good" or "very good" levels of general health (81.4%), oral health (83.7%) and overall quality of life (90.7%).

Fewer than 5% reported "bad" levels for either of these variables. Most patients reported better general (86%) and oral health (78%) after treatment. Only two patients (4.7%) reported their oral health to be worse after treatment.

Donor site and hospitalization related PROMs

Most patients (85.4%) were satisfied with the hip surgery procedure. Pain at the donor site was reported by 38% of patients, lasting for an average of 18.1 ± 16.1 days and measuring 43.6 ± 27 on the VAS (0-100) scale.

Only 2 patients (4.7%) reported post-operative infection at the donor site. Scar formation on skin (hip) was reported in 49 % of patients, with the majority esthetically acceptable (90.4%). Four (9.5%) and two (4.7%) patients respectively reported "a little" or "a lot" of reduced sensitivity at the donor site. Three patients (7.3%) reported problems walking (Table 2, Study I).

The average period of hospitalization was 4.3 ± 3.5 days and sick leave 20.2 ± 18.5 days.

Table 2, Study I: Patient reported outcomes

Question	Response	Frequency
Oral health	Very good/good	81.8 %
Quality of Life	Very good/good	90.9 %
General health	Very good/good	81.8 %
Pain after hip operation	Excessive	35.0 %
Satisfaction hip operation	Very	85.7 %
Post op infection in hip site	No	95.3 %
Visible scar on hip	Yes	48.8 %
Acceptable scar	Yes	20 of 21*
Reduced sensibility on hip site	No	86.0 %
Problem walking	No	92.9 %
Augmented bone block still present	No	6.8 %
New augmentation	Yes	1 of 4*
Oral pain after augmentation	No/some	83.3 %
Implant/teeth in augmented bone	Yes	90.9 %
Lost implants	Yes	28.6 %
Time lost after installation	0-3 months	42.9 %
	7-12 months	28.6 %
New implants installed	Yes	8 of 11*
Satisfaction with implant-retained teeth	Very satisfied/satisfied	90.5 %

*Incomplete or missing data

4.1.2 Implant/prosthesis-related PROM

Most patients (n=40: 90.9%) reported proceeding with implants and prostheses at the augmentation site(s). This indicates a graft survival rate of 90.9% at patient level. Two patients had implants installed, but did not proceed with prosthetic rehabilitation. Implants could not be installed in 2 patients. However, 28.6 % of patients reported "loosening or loss" of implants in the post-operative period (one year), indicating an implant survival rate at patient level of 71.3%, and most patients (8 out of 11) received new implants.

No pain was reported in 39 patients following implant surgery (82.9 %) and a majority of patients (90.2%) were satisfied/very satisfied with the implant therapy overall, and in terms of overall satisfaction with their teeth (90.5%).

The correlation analyses did not show significant correlation between the complications at the donor site and implant loss.

4.1.3 OHRQoL

The mean OHIP-14 score was 8.4 ± 9.7 (range 0 - 56) in 44 patients, 35 of whom scored 14 or less. Nine patients scored a total sum of one (1) i.e. “hardly ever” impact on any single item and “at no time” on the remaining 13 items. The highest score (2.34) was for the functional limitation domain and the the lowest (0.61) was for the social disability domain.

4.2 Results Study II

4.2.1 Validation of the expansion protocol, table 3, study II

	Volunteer patients	**Maxillo 1 patients**
Cell density cells/cm^2	49 961 ± 264	49 955 ± 65
First culture step days	**13.8 ± 0.1**	**14.0 ± 0.0**
Cell density at passage 0 (MSC P0)	25.7 x 10^3/cm^2 ± 15.7 x 10^3/cm^2	13.4 x 10^3/cm^2 ± 7.0 x 10^3/cm^2
Population doubling time	25.4 ± 1.6 h	25.3 ± 2.5 h
Second culture step	**6.9 ± 0.2 days**	**7.0 ± 0.0 days**
Cell density at passage 1 (MSC P1)	49.1 x 10^3/cm^2 ± 18.0 x 10^3/cm^2	42.7 x 10^3/cm^2 ± 9.4 x 10^3/cm^2
Population doubling time	51.7± 24.1 h	49.3 ± 4.4 h
Cumulative number of population doublings	16.6 ± 1	16.8± 1.5
Overall harvest of the final product	283.2 x 10^6 ± 187.3 x 10^6	273.7 x 10^6 ± 104.5 x 10^6

The robust protocol used in this study demonstrated a stable performance characteristic of expanded MSC, although there was variation in the starting materials. Manufacturing of the autologous clinical grade MSC was possible, requiring 21 days for each product. Transport of BM aspirates and MSC within 24 h was possible and did not affect the viability and quality of the cells. MSC fulfilled the quality criteria requested by the National Competent Authority. In one case, the cells developed a mosaic in chromosomal finding, showing no abnormality in differentiation capacity, growth behavior or surface marker expression during long-term culture. The proportion of cells with the mosaic decreased in long-term culture and cells stopped growth after 38.4 population doublings.

4.2.2 Donor characteristics

Twenty-one BM aspirations were performed from volunteer normal donors for validation, and 13 aspirations from patients enrolled in the clinical trial.

One BM from the validation was split and two BM aspirates from patients in the clinical trial (MAXILLO-1) had to be discarded due to lack of CFU-F in the aspirate and growth of the culture during the passage 0 growth phase.

Although the group of volunteer healthy donors and the MAXILLO-1 patients differed significantly in age ($P < 0.0001$), aspirates did not differ significantly with respect to:

- Aspiration volume ($P = 0.2414$).
- Percentage of CD34+ cells in the aspirate ($P= 0.0946$).

4.2.3 Quality controls

All quality controls were carried out according to the Ph Eur for the corresponding method and all matrices have been validated for the tests applied.

4.2.4 Cell viability, table 4, study II

	Volunteer patients	**Maxillo 1 patients**
BM aspirate (BM MNC)	94.6% ± 3.1%	92.5% ± 3.5%
MSC P0	97.0% ± 3.2%	97.7% ± 1.7%
MSC P1	94.5% ± 4.2%	97.9% ± 1.1%

The was no significant difference in viability of cells from BM (P= 0.0767), of MSCP0 cells BM (P= 0.8995) or MSCP1 BM (P= 0.0104) cells from volunteer healthy donors and MAXILLO-1 patients. The Kruskal-Wallis test revealed no significant difference in viability of MSCP0 and MSCP1 between the two groups of donors.

4.2.5 Analysis of Starting Material

Using flow cytometry, the content of the starting material (i.e, leukocytes vs hematopoietic progenitor/stem cells) was determined by expression of CD3, CD34, CD45 and MHC class II on MSCP0) and MSCP1 cells.

In summary, parameters for identity and impurity were fulfilled for all expansions from both volunteer healthy donors and MAXILLO-1 patients with one exception: MSC P1 expanded from volunteer healthy donor 7575 showed deviations for the parameters CD3 and CD105. The percentage of CD3+ cells was 23.5% (with an allowed threshold of ≤5%), and the expression of CD105+ cells was 88.97% (with an allowed threshold of ≥90%).

Thus, only one preparation of 33 (i.e. 3%) did not fulfill the quality control release criteria for identity and impurity.

4.2.6 Clonogenicity (CFU-F), table 5, study II

	Volunteer patients	**Maxillo 1 patients**
/10^6 seeded MSC P0	192 x 10^3 ± 72 x 10^3 colonies	171 x 10^3 ± 86 x 10^3 colonies
/10^6 seeded MSC P1	210 x 10^3 ± 79 x 10^3 colonies	91 x 10^3 ± 40 x 10^3 colonies

BM aspirates from volunteer healthy donors differed significantly in their CFU-F content (P= 0.0060) and MSCP1 showed significant difference in clonogenicity (P= 0.0003).

Interestingly, there was no difference in the clonogenic potential of MSCP0 from volunteer healthy donors and MAXILLO-1 patients (P= 0.3551).

4.2.7 Differentiation capacity

Adipogenic, chondrogenic and osteogenic differentiation capacity was shown for all expansions performed for validation runs and in the context of MAXILLO-1. All batches of MSC exhibited a multipotent capacity in the three lineages.

4.2.8 Microbial, endotoxin and mycoplasma testing

Microbial testing of the starting material (BM), of the cell culture supernatant at day 7, of MSCP0 and of MSCP1 was negative for all expansions. Endotoxin testing was performed for all expansions and mycoplasma testing was performed for expansions in the context of the clinical trial MAXILLO-1 and for 8 of the 22 cell expansions from volunteer healthy donors. For all tested product samples, anaerobic and aerobic cultures showed negative test results. No mycoplasma DNA was detectable and endotoxin levels were ≤1 IU/mL in all cases.

4.3 Results Study III

4.3.1 Donor and Product characteristics

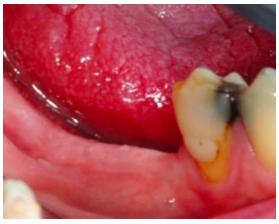
13 patients aged 52–75 years (mean 65 years) were enrolled. For 11 of the 13 patients the expansions fulfilled the release criteria and cells could be delivered to the clinical unit in Bergen. Two expansions were stopped at passage 0 because there were insufficient bone marrow cells in the starting material to attempt expansion.

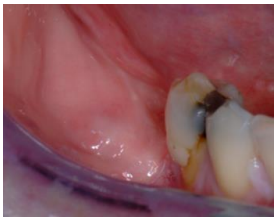
The final cell product consisted of fresh autologous cells (MSC) expanded *in vitro* expressing the markers CD90, CD73, and CD105 and negative for CD14 and CD45, with a 90% viability rate. The product also showed strong expression of the markers CD49d, CD73, CD90, and CD105; moderate expression of CD14 and CD106; and low expression of CD19, CD34, and CD45.

The viability of the cells on arrival was 87–90%, as demonstrated by a Trypan blue assay and cell counting. Mixing of cells and BCP granules was done under aseptic surgical conditions. The cells were mixed with BCP granules for 60 min.

4.3.2 Patient outcomes

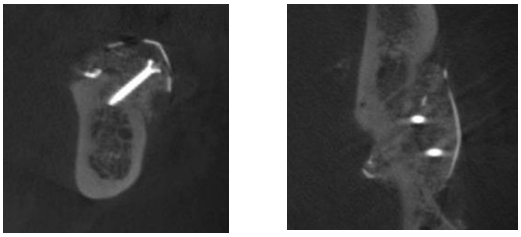
Healing of the augmented area was uneventful in all 11 patients, without any local infection. No adverse events occurred during the trial period. New bone formation was restricted to the granules under the PTFE membrane, with no new bone observed on those granules outside the membrane. All patients had successful ridge augmentation and an adequate amount of bone for implant installation. In five patients the PTFE membrane became exposed and was removed uneventfully 7–8 weeks post augmentation. Casts of the alveolar ridge in each patient, X-ray scans, and clinical examinations (Fig. 6) demonstrated a significant increase of the total bone volume in all 11 patients after treatment.





Linear measurements of the width and height were performed from all CBCT scans in iView software (version 2.2.0.3. J; Morita MFG Corporation). Grafted bone could be distinguished from residual bone by density and structure on the scans taken immediately after the grafting procedure. (Fig.7).

Figure 7; CBCT scans to distinguish grafted from residual bone.



All patients had sufficient increase in alveolar width to accommodate dental implants. The average volume of bone increased by 887.23 ± 365.01 mm^3 (Table 6)

Table 6, Study III. **Clinical outcomes:** Demonstrates bone healing, increased bone width and volume. All patients received implants and prosthesis**.**

Patient no	***Age***	***Sex***	***Healing time weeks***	***Increase in width mm***	***Increase in volume mm^3***	***Implant placement***	***Crown delivered***	***Patient satisfied***
01	75	F	27	4.5	902.92	Y	Y	Y
02	67	M	25	3.7	1047.15	Y	Y	Y
03	55	F	26	3.9	1382.54	Y	Y	Y
04	62	F	18	1.1	440.93	Y	Y	Y
06	52	M	21	4.9	1469.53	Y	Y	Y
07 left	69	M	31	4.6	432.7	Y	Y	Y
07 right	69	M	31	4.9	1187.21	Y	Y	Y
08	69	M	22	1.4	753.52	Y	Y	Y
09	61	F	22	1.4	546.33	Y	Y	Y
11	62	F	21	9.7	1188.47	Y	Y	Y
12 left	65	F	20	2.7	954.98	Y	Y	Y
12 right	65	F	20	3.4	418.36	Y	Y	Y
13 left	69	F	22	3.7	553.56	Y	Y	Y
13 right	69	F	22	6.8	1142.96	Y	Y	Y

The increases in both width and volume of the alveolar ridge were statistically significant. The mean increase in bone width (n = 14) was 4.05 mm (95% CI 2.74, 5.36; $P < 0.001$) and the mean increase in volume (n = 14) was 887.23 mm^3 (95% CI 676, 1097.98; $P < 0.001$).

Formation of mineralized tissues was evaluated by μ-CT and by histological analysis of the biopsies taken during implant installation. From the μ-CT scan datasets, 3D models were constructed for visualization. It was possible to identify accurately the newly formed bone from the BCP granules (based on histogram calculations) when the raw data-reconstructed cross-sections were turned into images. Histological analysis revealed that the BCP granules were well integrated, with deposition of newly formed bone tissue on the surface of the particles, with osteoblast lining cells and subsequent deposition of lamellar bone tissue. The BCP granules demonstrated continuous degradation and dissolution, with the presence of multinucleated cells, probably osteoclasts, as well as macrophage CD68+ cells on the surface of the particles.
Table 6 shows the mean values for each analyzed variable, obtained by μ-CT analyses, in relation to the microstructural properties of the biopsies.

4.3.3 Patient satisfaction

All patients reported satisfaction with the esthetic and functional outcomes of the procedure. No adverse events were reported or observed. There were no postoperative infections in any of the transplants or at the donor site. One patient reported moderate levels of pain after augmentation and it became necessary to remove the exposed membrane. The other patients reported only minor postoperative pain. All patients were satisfied with the clinical outcome of the augmentation procedure and with their new teeth and said they would recommend this procedure to others with a similar clinical condition (unpublished data). Osstell values increased for all patients during the first 12 months after installation of the dental implants.

5. DISCUSSION

The human body has the capacity to regenerate certain tissues, such as the liver, which can regain function after significant loss. Unfortunately, this process of regeneration does not occur when bone is lost or resorbed. Therefore, regeneration requires bone grafting. With more than 2.2 million bone grafts performed worldwide each year (128), this places a large economic burden on the health care system. Other drawbacks to autologous bone grafts include their unpredictable resorption and the finite amount which can be removed for transplantation. Major goals for tissue engineering include decreasing the invasiveness of the procedures by eliminating the need for donor tissue harvest, improving graft outcomes, and making the outcome more predictable, with less alteration in size/dimension of the graft over time.

Even a decade ago, there was evidence that MSC held great promise for bone tissue engineering and bone regeneration (129, 130). However, although there are clinical studies of MSC dating from the early 2000's, there is a notable lack of conformity of studies. Cell preparations vary, from minimally manipulated whole tissue fractions (96) to *ex vivo* (88) conditioned cells (73). The origin of the cells also varies, e.g. bone marrow (88), adipose tissue (89, 131, 132) and other sources (106). The state of the cells has ranged from progenitor (88) to pre-osteoblasts (98).

Because of these variations, no direct comparison is made of the present results with those of other studies. However, it is of interest to consider the present research in the context of reports from other research groups also using cell-based tissue engineering to treat orofacial defects. It should be noted that most such reports comprise primarily clinical cases rather than trials.

In 2008, Meijer et al., reported a clinical study of cell-based tissue engineering in the maxilla (88). In 6 patients with insufficient bone for dental implant installation, 9-15 mL of bone marrow were extracted, and expanded in culture containing α-MEM, FBS, dexamethasone and antibiotics. Thus, although bone marrow cells were extracted from the patients, in contrast to the protocol described in Study II, osteogenic supplements were used for expansion, hence the cells after expansion should be considered pre-osteoblasts rather than stem cells. In contrast, the cells produced in Study II maintained

their stemness with minimum manipulation during culture. The issue of using animal-derived products such as FBS has been discussed previously. The cells were seeded on HA particles after replating 3 times, then grown together for 7 days supplemented with dexamethasone. The scaffold with cells was placed on the alveolar ridge and left to heal for 4 months. A biopsy was taken from the site of planned dental implantation, and the implant was installed. Of the 11 implants installed in the 6 patients, 10 healed uneventfully, and 1 failed. In 3 patients the biopsies showed bone formation: in 2 such cases bone formed on the scaffold material near the pre-existing bone of the defect. In only one biopsy was there an area of 'de novo' bone formation further than 7 mm from the pre-existing bone tissue. This was regarded as a strong indication of osteogenic bone formation by the implanted cells. It is difficult to draw any broad conclusions from the results, because of the pronounced inter-patient variation in the size and site of the bone defects. In contrast, the defect site in Study III, the mandibular ridge distal to the canine, was well-defined and standardized.

Adipose tissue has also been used as a source of stem cells for bone-tissue engineering in the orofacial region (133). Adipose tissue offers the advantage of easy access and a plentiful source for harvesting cells. The first report of human trials using these cells was published in 2009 by a research group in Finland. This was a case report of maxillary reconstruction using 13×10^6 cells expanded from the patient's adipose tissue (131). The cells were expanded for 14 days in DMEM and autologous serum. Prior to combining the cells with βTCP, they were incubated for 48 hours before surgery in basal media containing 12 mg of rhBMP-2. A preformed titanium case was filled with the cells and βTCP and inserted into the patient's left rectus abdominus muscle and left for 9 months. The construct, together with part of the muscle and supplying blood vessels was then placed in the maxillary defect, and the vessels connected. After uneventful healing, dental implants were installed into the construct after 8 months, and progress was monitored for another 12 months. In 2013, the same group reported on 3 patients with mandibular defects, which were treated with adipose stem cells (ASC) and βTCP (89, 132). The cell dosage varied from 4.7×10^6 to 16×10^6. Healing was uneventful and the large defects, ranging from 6-10 cm, were successfully bridged. Histological analysis of the recovered bone cores revealed signs

of bone formation and remodeling. The βTCP granules were interconnected by bridges of vital newly formed bone.

In 2014, the same group also reported treatment of 13 cases of hard tissue cranio-maxillofacial defects: 3 frontal sinuses, 5 cranium defects, 2 nasal septum defects and the 3 mandibular defects mentioned earlier (133). The cell dose varied from 2.8×10^5 to 4.3×10^6 per ml. In the sinus defects the cells were combined with a bioactive glass scaffold. βTCP was used in the remainder. Only the mandibular defects were treated with rhBMP2. The frontal sinus patients were asymptomatic during a follow-up period of up to 37 months. In the cranial defects group one patient had a recurrence of the original meningioma, one showed evidence of graft resorption after one year and was re-operated, and in a third patient the reconstruction resorbed almost completely. The mandibular cases have been discussed previously. In the nasal septum group (two patients) one was successful, but the other failed and this was attributed to the patient`s habitual nose picking.

In 2017, the six-year follow-up results for the cranioplasty group were published. The outcomes were unsatisfactory: only 1 patient had not been re-operated and in this case, radiographs showed hypodensity at the borders of the graft (134). However, the cell culture procedures for stem cells derived from adipose tissue may require the addition of BMP-2 in order to direct the cells toward osteogenesis. In contrast, bone marrow cells have inherent osteogenic potential. Thus, although adipose tissue cells are good candidates for bone regeneration, their osteogenic potential is not as great as that of bone marrow cells (61, 62).

In the context of such widely-ranging approaches to stem-cell based bone tissue regeneration of defects in the craniofacial region, Studies II and III clearly highlight the advantages of a standardized cell expansion protocol, which meets the regulatory requirements for Phase II or later trials, and a well-planned clinical design.

In order to establish a control group for future clinical trials with MSC, our first requirement was to identify a group of patients who had been treated with the current gold standard for alveolar bone restoration, using autologous bone grafts, and to acquire data about patient satisfaction and quality of life after the procedure (Study I).

Data were acquired from 59 patients who had undergone reconstructive surgery and been followed for up to 12 years postoperatively. Although the majority of patients expressed satisfaction with the long-term outcome, in that after augmentation dental implants could be installed, this satisfaction was tempered somewhat because they experienced significant postoperative pain (38% of patients) and morbidity (an average of 4.3 days' hospitalization and 20.2 days' sick leave). As this was a retrospective study, the subjects included many who would have been ineligible for inclusion in Study III, because of poor overall oral health. Afterwards many admitted to being smokers.

Different experimental biomaterials were tested in preparation for the planned clinical trial. However, in order to eliminate another unknown and also to facilitate the process of approval for the human trial it was realized that an FDA approved and commercially available product was needed as substrate for the cells. The adherence of MSC and commercially available biomaterials, and the ideal mixing time for best adherence of the MSC to the biomaterial were therefore evaluated in preclinical trials (75). The bone-inducing potential of this combination was assessed; first ectopically under the skin of nude mice, and later as an implant in mice skulls (75). After this initial validation of the method, the surgical procedure and its efficacy were tested in a split mouth model in a minipig (135). The alveolar rigde of the animal were implanted with the BCP to be used in our planned clinical trial, or with MSC seeded onto BCP granules. Bone regeneration was clearly demonstrated when MSC were combined with the biomaterial (Fig. 8).

a b

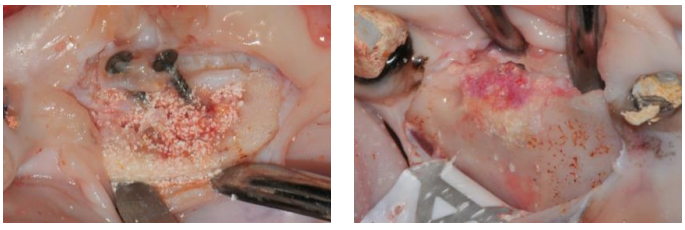


Figure 8; (a) Control (BCP granules) and (b) test (BCP granules + MSC)

Shipping of cells and viability were then validated in animals before transportation of human MSC for 24 hours was validated.

After these preliminary investigations, it was then possible to proceed with Study II: developing a GMP protocol for harvest, isolation and expansion of autologous MSC. Although regulatory agencies permit the use of qualified lots of FBS in phase I clinical cell therapy clinical trials, for phase II or later trials this must be replaced with a non-animal source of growth factors. EU regulations also require a reliable xeno-free product, both for human safety and animal welfare. Therefore, rather than switch sources after the phase I trial, we chose to validate a protocol in which human platelet lysate (PL) was the source of the growth factors that would be used for the phase I/II human trials. As the marrow aspirates were harvested in Bergen, Norway, shipped by qualified courier to Ulm, Germany for expansion *in vitro*, and then the expanded cells were returned to Bergen for clinical use, we also validated the viability and other important characteristics of the expanded MSC after transport.

PL as a supplement for the culture medium was compared with FBS in a recently published systematic review and shown to be at least equally effective for expansion of MSC and osteogenic differentiation (80). The PL used in the trial was made from pooled validated platelet concentrates (PC) from qualified donors, past the mandated blood bank PC shelf life.

It was reported that for isolation and expansion (single step protocol) or for culture with pre-expanded MSC (in our case comparable to the culture of passage 0 cells), there was no benefit in using a PL concentration >10% (126). For the pre-expanded cells in particular, 5% PL was not as supportive as 10%. Experiments extended the investigation to the whole expansion process (126) and involved testing several different protocols. The first tested was a single-step protocol which used 10% PL and cells were not passaged. However, a two-step protocol resulted in more reliable expansion data: seeding a defined number of passage 0 MSC helped to eliminate effects due to variation in expansion capability of BM from different donors. A two-step protocol was therefore chosen for use in the clinical trial.

Study III presents the procedures and outcomes for the 11 patients in the phase I/II trial of expanded autologous MSC for alveolar bone regeneration and placement of dental implants. Bone biopsies were taken at the time of dental implant installation. Patients were evaluated at each post-surgical visit with a clinical examination, VAS scales for postoperative pain and complaints. The patients all reported satisfaction with the outcome, but unlike Study I, these patients reported minimal pain and morbidity.

Methodological considerations may potentially confound interpretation of the results. The trial in Study III included biopsies, which were examined by both μCT and histology. The bone cells were seen clearly dispersed in between the biomaterial. However, it is impossible to tell whether the new bone tissue is derived from the transplanted cells, from homing of new MSC to the area, or via osteogenesis. While labelling the cells might allow this distinction, it might alter their behavior *in vivo*.

An important aspect of Study III is that it has a close-to-standardized defect, located in the posterior mandible, distal to the canine, and has a width less than 4.5 mm. Most trials involving bone transplants and cell therapy in the orofacial area do not utilize a standardized defect and this complicates comparison of treatment results. There are few randomized trials, and more reviews of the procedure than trials (136-138).

In the Maxillo-1 patients, the membrane enclosing the cells and granules determined the area of regenerated bone, and there has been minimal resorption of the reconstructed ridge in patient follow-up over the last 3 years (unpublished data, manuscript in preparation). The non-resorbable membrane used in the study is microporous, and impervious to bacteria while still allowing diffusion of gas and small molecules, but may inhibit vascularization from the periosteum (128, 139). However, the importance of the membrane to bone regeneration is highlighted by the fact that new bone formation was seen only on the granules enclosed by the membrane, and not on those that fell outside.

Although the maxillary sinus has been used to test a graft material, compared to the jaws it is not a very challenging test environment: the sinuses are mostly sterile, the graft is not exposed to the forces of mastication and there is good blood supply. It has even been shown that by lifting Schneiderian membrane over a dental implant, new

bone will develop along the long axis of the implant (140, 141), as would happen with a sinus lift procedure.

The results of the comprehensive clinical trial in human subjects in Study III confirm that MSC can successfully induce significant formation of new bone in a challenging environment, with no adverse events. Hence, this novel augmentation procedure warrants further investigation. It has the potential to form the basis of a valid treatment protocol, which may challenge the current gold standard.

6. CONCLUSIONS

Study I: severely compromised patients who had undergone advanced reconstruction of alveolar ridges with iliac crest-derived grafts and implants reported favorable OHRQoL and satisfaction with the outcome. However, this treatment requires substantial resources including hospitalization and sick leave, and is associated with significant pain. Further, the findings of this study highlight the need for stringent patient selection criteria in order to minimize the risk of implant loss after regenerative bone procedures.

Study II: Clinical use of freshly prepared MSC, manufactured according to a standardized and validated protocol, is feasible for bone regeneration, even with considerable distances requiring transport of material between the manufacturing center and clinical site. Individual variations in several BM parameters, such as CFU-F, % CD34+ cells, MNC and WBC content may serve as a predictive tool for the yield of MSC and may help to avoid unnecessary costs for MSC manufacturing in cases of insufficient cell expansion. Replacing FBS, platelet lysate served as a reliable human-derived source of growth factors.

Study III: The results of this trial in human subjects confirm that MSC can successfully promote formation of new bone, with no untoward sequelae and minimal pain. Hence, this novel augmentation procedure warrants further investigation. It has the potential to form the basis of a new therapeutic approach which may challenge the current gold standard.

7. FUTURE PERSPECTIVES

Future academic clinical perspectives

The next clinical trial (Maxibone, H2020 EU project number 779322) will focus on improving the costs and decreasing the turnaround time for MSC expansion, in order to accommodate an increased number of patients. Culture time has been shown to influence the cell doubling time (126), cells that are cultured for long time may become tightly packed and their doubling time is then slowed, possibly attributable to contact inhibition. It will therefore be possible to shorten culture time for this trial to 15 days compared to 21 days in Study III, which will decrease the costs associated with cell culture (a protocol validated in the ongoing Maxibone project).

In Study III, all surgical procedures were performed by the same surgeon to avoid interoperator variability. The surgical technique is challenging, using a PTFE membrane to expand the alveolar ridge up to 1 cm with enclosed cells and granules and allow tension-free wound closure. Calibration of other surgeons to do the validated procedure is therefore planned, both to increase the number of patients treated and to demonstrate that the procedure can be done by other surgeons. Calibration is always required to ensure uniformity of surgical approach so that surgical technique does not become a significant variable in the multicenter randomized controlled trial (142). Further, it will also be important to train other personnel involved in further care of these patients, e.g. dental technicians who fabricate the prostheses, dental hygienists, and prosthodontists, so that the implants are well-maintained.

We chose the area distal to the mandibular canine, a challenging area to reconstruct, especially with granules and not a block. Having succeeded in this challenging environment, a future goal is to apply the method in an orthopedic trial involving mal-unions in the tibia. We have done this once already in a compassionate setting, to treat a 3 cm defect in the left tibia. The patient was able to place 100% load on the leg at 2 months postoperatively (manuscript in preparation).

Future perspectives for academic research

Improved understanding of cross-activation and complex signaling of growth factors affecting bone and MSC growth and expansion will hopefully lead to the design of advanced bone-substitute materials. The development of biomaterials with easier handling is a future goal of our group. 3D printed scaffolds, with or without cells printed into the scaffold, as well as MSC-containing gels are already available but lack standardized, validated GMP protocols for clinical application. The ability to plan the reconstruction in 3D and print the graft to exact fit is also a future research goal for our group.

Alternatives to autologous MSC will eventually need to be explored, such as the commercially available GMP-compliant allogeneic MSC or expanded MSC from banked cord blood.

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9. ORIGINAL PAPERS

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Patient experience following iliac crest-derived alveolar bone grafting and implant placement

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Abstract

Background: The objective of this study was to assess patient-reported outcomes such as satisfaction and quality of life after advanced alveolar bone augmentation with anterior iliac crest grafting and implant treatment in orally compromised patients.

Methods: This cross-sectional retrospective cohort study included 59 patients (29 women and 30 men) with major functional problems, who underwent advanced alveolar augmentation with autologous iliac bone grafts during a 10-year period (2002–2012).
The self-administered questionnaire included 36 validated questions related to (1) demographics, (2) perceived general and oral health, (3) donor site and hospitalization, (4) status of implants and/or prosthesis, and (5) oral health-related quality of life (OHRQoL).

Results: Questionnaires were completed by 44 patients: 24 women and 20 men (response rate, 74.6%). Most patients reported good tolerance of the operative iliac bone harvesting (85%) and implant (90%) procedures. Post-operative pain at the donor site was reported by 38%, lasting 18.1 ± 16.1 days. An average of 4.3 ± 3.5 days of hospitalization and 20.2 ± 18.5 days of sick leave was reported. The overall satisfaction with prosthetic reconstruction was 90.5%. OHRQoL was reported with a mean Oral Health Impact Profile-14 (OHIP-14) score of 8.4.

Conclusion: Favorable OHRQoL and satisfaction were reported after advanced reconstruction of alveolar ridges with iliac crest-derived grafting and implants in severely compromised patients. However, this treatment requires substantial resources including hospitalization and sick leave.

Keywords: Dental implants, Reconstruction, Quality of life, Bone graft, Iliac crest, Donor site morbidity, PROMs

Background

Insufficient alveolar bone volume, as a result of periodontal disease, trauma, congenital anomalies and/or resorption atrophy, often presents a clinical challenge for optimal placement of dental implants for prosthetic rehabilitation. In such cases, augmentation of alveolar bone, with either autologous bone, allogeneic, xenogeneic, or alloplastic biomaterials, is a prerequisite for placing implants in restoratively and esthetically acceptable positions.

Limited alveolar ridge defects are solved by local grafting. In cases of larger defects and extreme resorption, larger grafts are necessary. The most common donor site for large autologous bone grafts is the iliac crest, due to its accessibility, comparatively abundant bone volume, and high bone quality [1].

Autologous bone is still considered as a "gold standard" for alveolar reconstruction, according to systematic reviews [2–5]. Intra-oral donor sites, like mandibular ramus and symphysis, allow harvesting of limited volumes of autologous bone. The anterior iliac crest is the preferred extra-oral donor site for alveolar augmentation for larger bone volumes [1, 6, 7]. However, complications are reported, including pain, gait disturbance, hematomas, paranesthesia, and infections [8–15].

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Traditionally, objective clinical variables, like the amount of bone gain (in millimeters) after augmentation, are reported as outcome measures after surgical procedures in clinical studies [16]. Patients' experiences like patient-reported outcome measures (PROMs) have been increasingly used as a measure of treatment effect after medical and dental therapies [17, 18]. Importantly, these measures reflect the patients' perceptions of the treatment outcome in addition to conventional clinical measures. Nowadays, Norwegian authorities address clinicians to include patients' perspective in decisions regarding different treatment modalities [19]. It has been suggested that PROMs such as treatment satisfaction, perceived cost-effectiveness, and quality of life (QoL) may be more important and relevant to patients' daily lives than objective clinical measures [16, 20]. Patient satisfaction is an important outcome measure, related to, although not synonymous with QoL, as satisfaction tends to reflect the process, rather than the outcome, of care [21]. Thus, an increase in the use of PROMs has been highlighted in dental implant research [22].

Health-related QoL (HRQoL) is a dynamic concept referring to an individual's subjective assessment and perspective of current general health condition as well as functional, social, and emotional well-being [23, 24]. Most people regard oral health as important for QoL, and this is mediated through the concept of oral health-related QoL (OHRQoL) [25]. In this regard, OHRQoL is an important PROM in dental research, as oral health is an integral part of general health and well-being [26].

Different instruments to assess OHRQoL may be utilized to detect changes in physical, functional, and psychosocial impacts of oral disorders and have been validated for use in clinical studies [27–29]. The Oral Health Impact Profile-14 (OHIP-14) questionnaire is a widely used OHRQoL instrument [27]. It includes 14 questions covering seven domains of oral health and attempts to assess their impact on patients' OHRQoL [30, 31]. OHIP-14 has previously been translated into Norwegian and used in a large study ($n = 3538$) with a calculated Norwegian national norm value [32]. Although previous studies have reported PROMs in relation to bone grafting [9, 33–42], to our knowledge, only one previous study has systematically assessed impact of donor site harvesting on OHRQoL, where (a) a postoperative lowering of OHRQoL was observed following bone grafting from both intra-oral and extra-oral sites and (b) iliac crest grafts compared to intraoral donor sites had a negative impact on postoperative QoL [37]. Moreover, to our knowledge, only one study has previously assessed the cost-effectiveness of autologous iliac crest grafting [43].

The aim of this study was to assess PROMs such as satisfaction and OHRQoL after advanced reconstruction of alveolar bone by anterior iliac crest-derived grafting and implant treatment.

Methods

Study population

This cross-sectional retrospective cohort study was based on records from all patients ($n = 69$) who underwent advanced alveolar augmentation with autologous iliac bone grafts at the Department of Oral and Maxillofacial Surgery, Haukeland University Hospital, Bergen, Norway, over 10 years (2002–2012). These patients were orally compromised with severe chewing problems as well as speech difficulties and had previously undergone several unsuccessful rehabilitation methods, prior to referral. At the time of this survey, seven patients had passed away, two had moved to unknown addresses, and one was hospitalized in a psychiatric institution. Thus, the study sample included 59 patients: 29 women and 30 men.

The Norwegian Committee for Medical Research Ethics ("REK," Health Region West), acknowledged this study as a treatment quality control study.

Treatment protocol—operative procedure

Bone graft surgeries were performed under general anesthesia and sterile conditions. Cortico-cancellous bone blocks were harvested from the anterior superior iliac crest. Reconstructions in the maxilla ($N = 57$) or mandible ($N = 2$) were performed in one operation by two teams using an onlay bone graft fixated with titanium micro-screws (1.5 mm Ø). The surgical procedure was performed according to the protocol commonly used at Haukeland University Hospital. In brief, the harvesting of autogenous bones from the anterior iliac crests started with a skin incision following the skin lines in a posterolateral direction starting from 3 to 4 cm medial to the iliac crests. The superior surfaces of the iliac crests are exposed after a sharp dissection through the periosteum following the crests. The dissections are performed with great attention to avoid laceration of the fascia lata. Both cortical and spongious bone are harvested. The donor sites are closed in layers with special attention to the first layer—the fascia lata. This layer is sutured close to avoid marrowbone bleeding. Activated vacuum drainages are positioned between the fascia lata and the muscles until the patients are mobilized. The skin incisions are closed with continuous intracutaneous resorbable sutures. All patients included in the study were hospitalized 2–3 days postoperatively. Patients received phenoxymethylpenicillin (1 g × 3) or clindamycin (300 mg × 3) for 5 days following the operation. Vacuum drainage at the donor site was used until the patient was mobilized the morning after surgery. Analgesics (paracetamol or non-steroid anti-inflammatory drugs) were prescribed 7–10 days postoperatively.

Implants were placed 4–6 months after the grafting procedure. The implant installations were performed by different oral surgeons (not in the hospital) and different implant systems were used. The implants installed into the augmented bone were allowed to heal for an additional 4–6 months before loading.

Data collection

Medical records

The records of the original 69 patients were examined with regard to (1) grafting site (2), "graft-survival" determined by the ability to place implants in the grafted site(s) and (3) "implant survival" determined by the presence of functional implant-supported prostheses at the most recent follow-up. Reasons for implant failure were recorded when available.

Questionnaire

A self-administered questionnaire (Additional file 1) was sent by post to all 59 patients, together with an information leaflet about the survey, a return envelope with prepaid postage and an informed consent form. Reminder letters were sent after 2 and 4 weeks if no response was received.

The questionnaire contained 36 previously validated questions, which were categorized and related to (1) demographic and lifestyle, (2) perceived general and oral health, (3) donor site and hospitalization, (4) implant and prosthesis, and (5) OHRQoL (OHIP-14) (Table 1). Responses to questions in categories 1–2 were recorded as "yes/no" or graded on a 3- to 5-point Likert scale [44]. Category 3 included information on the duration of hospitalization and sick leave. Category 4 included information on "graft survival," i.e., whether implants (and prostheses) were delivered in the augmented site(s), and "implant survival," i.e., the presence or "loss/loosening" of any implants after surgery. OHRQoL was assessed using a Norwegian version of the OHIP-14 [32]. These 14 questions addressed seven domains of OHRQoL and their responses were graded on a 5-point Likert scale ranging from "at no time" (0) to "all of the time" (4) (Table 1).

Table 1 Summary of questions

Category	Response
Question	
(1) Perceived health-status	
General health	"Very good" to "bad"
Oral health	"Very good" to "bad"
Overall quality of life	"Excellent" to "bad"
(2) Lifestyle-related	
Smoking	"Yes," "no," or "sometimes"
Appetite	"Good" to "bad"
(3) Donor site-related	
Pain	"Yes" and "no"
Infection	"Yes" and "no"
Presence of a scar	"Yes" and "no"
Reduced sensitivity	"No" to "total loss of sensitivity"
Problems walking	"No" to "a lot"
Satisfaction	"Very satisfied" to "dissatisfied"
(4) Implant-related	
Intraoral pain	"No" to "strong pain"
Installation of implants and prosthetic	"Yes", "no" or "just implants"
Loss of implants	"Yes" and "no"
Satisfaction with prosthesis	"Very satisfied" to "dissatisfied"
(5) OHIP-14	"At no time" to "all of the time"

Statistical analysis

Data were anonymized and analyzed using SPSS v 24 (SPSS Inc., Chicago, IL, USA). Descriptive analyses were applied. Statistical significance was set at 5% level.

Results

The final sample consisted of 44 patients that responded and completed the questionnaire, giving a response rate of 74.6%: 24 women and 20 men, mean age of 61.2 years ± 13.1 (range 27–82 years). The mean time from augmentation surgery until completing the questionnaire was 7.8 years ± 2.65 (range 1.9–12 years).

Summary of demographic and lifestyle-related data is presented (Table 2).

Descriptive findings

Health-related PROMs

Most patients reported "good" or "very good" levels of general health (81.4%), oral health (83.7%), and overall quality of life (90.7%). Less than 5% reported "bad" levels for either of these variables. Most patients reported better general (86%) and oral health (78%) after treatment. Only two patients (4.7%) reported their oral health to be worse after treatment.

Donor site- and hospitalization-related PROMs

Most patients (85.4%) were satisfied with the hip surgery procedure. Pain at the donor site was reported by 38% of patients, lasting for an average of 18.1 ± 16.1 days and measuring 43.6 ± 27 on the VAS (0–100) scale. Only two patients (4.7%) reported post-operative infection at the donor site. Scar formation on skin (hip) was reported in 49% of patients, by majority esthetically acceptable (90.4%). Four (9.5%) and two (4.7%) patients reported "a little" or "a lot" of reduced sensitivity at the donor site, respectively. Three patients (7.3%) reported problems in

Table 2 Patients' demographic and lifestyle-related data

Variable	Frequency	
	N or Mean ± SD	%
Patients		
Female	24	54.5
Male	20	45.5
Age (years)	61.16 ± 13.10	
Age at operation	53.73 ± 13.07	
Time from augmentation to completing questionnaire (months)	93.55 ± 31.75	
Civil status		
Married	30	68.2
Single	11	25.0
Widow(er)	3	6.8
Housing		
Alone	12	27.3
With another person	23	52.3
> two persons	9	20.5
Education		
Up to primary	7	11.3
Up to secondary	23	53.5
"Artium"	1	2.3
High school	9	20.9
University	3	7.0
Smoking		
Yes	8	19.0
No	33	78.6
Sometimes	1	2.4
Cigarettes/day	13.65 ± 7.22	
Years of smoking	26.52 ± 11.63	

walking (Table 3). The average time of hospitalization was 4.3 ± 3.5 days and sick leave 20.2 ± 18.5 days.

Implant-/prostheses-related PROMs

Most patients ($n = 40$, 90.9%) reported to have implants placed and received prostheses in the augmentation site(s). This was interpreted as graft survival, indicating a graft survival rate of 90.9% on the patient level. Two patients received implants, although without further prosthetic rehabilitation. Implants could not be installed in two patients. However, 29.3% of patients reported "loosening or loss" of implants in the post-operative period (1 year), indicating an implant survival rate on the patient level of 70.7%, and most patients (8 out of 11) received new implants.

No pain was reported in 39 patients (82.9%) following implant surgery and a majority of patients (90.2%) were satisfied/very satisfied with the implant therapy overall and in terms of overall satisfaction with teeth (90.5%).

The correlation analyses performed did not show a significant correlation between the complications at the donor site and implant loss (Table 4).

OHRQoL

The mean OHIP-14 score (Table 5) was 8.4 ± 9.7 (range 0–56) in 44 patients of whom 35 patients scored 14 or less. Nine patients scored a total sum of 1 [1], i.e. "hardly ever" impact on any single item and "at no time" on the remaining 13 items. The functional limitation domain had the highest score (2.34) and the social disability domain the lowest score (0.61).

Discussion

An important finding in this study is that a majority of patients were very satisfied after iliac crest-derived alveolar bone grafting and implant therapy. Although 90% of the patients in our study had successful bone grafting, only 70.1% reported implant survival together with prosthetic rehabilitation after 1 year. These figures are lower than those reported in previous studies [2, 3, 9]. A review by Chiapasco et al. showed that the mean graft failure in 16 studies was 1.6% and partial loss of graft of 3.3% [45]. The same review showed that the overall survival rate of dental implants in transplanted bone was 87%. However, it must be kept in mind that the patients in our study were orally compromised and very challenging to reconstruct. Moreover, the patients in our study did not report on the number of implants lost, and we do not have reliable records of the exact number of implants each patient had got installed. This could indicate differences in survival on implant or patient levels—a variable of clinical importance as the number of lost implants may be higher.

Another important finding is that patients reported to tolerate the augmentation procedure well; 85% of patients were satisfied with the hip operation (performed under general anesthesia), comparable to a previous report [46]. However, 40% of the patients reported pain for 18 ± 16 days after augmentation, which is in accordance with other studies [37, 46] and which should be considered during the treatment planning of patients scheduled to received iliac crest-derived bone grafts [33]. Two patients reported infection at the donor site. All operations were performed by a strict sterile regime and protocol at the university hospital.

The level of OHRQoL reported by the patients was favorable with an OHIP-14 value of 8.4. In a previous study, Dahl et al. reported an OHIP-14 score of 4.1 in the Norwegian adult population (2441 patients), with 35% of the sample reporting "no oral health problems" [32]. If the study sample in the study of Dahl et al. is considered to be representative of the general population, patients in our study reported poorer OHRQoL than the general population. Thus, even though the participants in this study report good oral health and better than before operation on the single questions, they

Table 3 Patient-reported outcomes

Question	Response	Frequency
Oral health	Very good/good	81.8%
Quality of Life	Very good/good	90.9%
General health	Very good/good	81.8%
Pain after hip operation	Excessive	35.0%
Satisfaction hip operation	Very	85.7%
Post op infection in hip site	No	95.3%
Visible scar on hip	Yes	48.8%
Acceptable scar	Yes	20 of 21[a]
Reduced sensibility on hip site	No	86.0%
Problem walking	No	92.9%
Augmented bone block still present	No	6.8%
New augmentation	Yes	1 of 4[a]
Oral pain after augmentation	No/some	83.3%
Implant/teeth in augmented bone	Yes	90.9%
Lost implants	Yes	28.6%
Time lost after installation	0–3 months	42.9%
	7–12 months	28.6%
New implants installed	Yes	8 of 11[a]
Satisfaction with implant-retained teeth	Very satisfied/satisfied	90.5%

[a]Incomplete or missing data

still report having problems related to their oral condition. This is to be expected as the patients in our study were orally compromised before augmentation with almost no alveolar ridge to retain or support a prosthetic construction. Since the patients had extensive alveolar bone loss rendering them orally handicapped, any improvement in function would be likely to have a positive impact on satisfacation and OHR-QoL. However, it is difficult to relate their reported level of

Table 4 Correlation analyses

Outcome variables	Correlations	Spearman's rho	*P* value
OHRQoL	Oral health compared	0.596	< 0.0001
	General health now	0.369	0.014
	General health compared	0.412	0.005
	Implants placed/teeth installed	0.317	0.036
	Lost implants	− 0.372	0.015
	Smoking	− 0.334	0.005
	Speaking	0.572	< 0.0001
	Chewing	0.375	0.014
Implants placed	General health	− 0.314	0.038
	Oral pain post op	0.334	0.031
	Oral health	0.305	0.044
	General health compared	0.314	0.038
	Satisfaction hip operation	− 0.439	0.004
	OHRQoL	0.317	0.036
Lost implants	General health	− 0.328	0.034
	QoL	− 0.342	0.027
	OHRQoL	− 0.372	0.015
	Satisfied teeth	− 0.328	0.034

Table 5 Summary of OHIP-14 ($N = 44$ and response range 0–8)

OHIP domain	Minimum	Maximum	Mean	SD
Functional limitation	0	7	2.34	1.70
Physical pain	0	7	1.16	1.51
Psychological discomfort	0	8	1.64	2.27
Physical disability	0	8	0.75	1.77
Psychological disability	0	8	1.18	2.11
Social disability	0	8	0.61	1.40
Handicap	0	8	0.70	1.71
Total	0	64	8.4	9.7

OHRQoL to the augmentation and implant installation per se, as this was performed up to 12 years prior to completing the questionnaire (mean 7 years and 10 months). So, patients' present oral situation with fixed teeth could/may alter the "reference" for the patients regarding OHRQoL. However, we cannot reliably ascribe the level of OHRQoL to the treatment performed years ago, since we have no such data either before or soon after the prosthetic rehabilitation, and therefore, cannot estimate the influence the effect of response shift on the study outcomes. Previous reports show a significant influence of implant-retained prosthetic treatment on OHRQoL, but these reports are based on before-and-after registrations [47].

Patients in our study reported satisfaction with the augmentation and implant installation, and as these patients were orally compromised before the operation, their satisfaction with getting fixed teeth most likely improved their perceived oral health condition. This might also, in part, explain why they reported good OHRQoL. Thus, our findings indicate that a majority of patients tolerate the augmentation and implantation procedures very well and with minor long-term sequelae.

The treatment protocol described in this study, i.e., advanced bone reconstructions under general anesthetics, hospitalization, and sick leave, is considered expensive in a public health services. In the present study, an average of 4.3 days of hospitalization and 20.2 days of sick leave was reported, which is costly for the health service and inconvenient for the patient [33, 43]. When comparing iliac bone graft as a treatment to bone substitutes, a previous study clearly demonstrated that iliac bone graft procedure demands more resources and more than three times the costs of bone substitutes [43]. Although the patients reported good satisfaction and OHRQoL after iliac bone grafting, this treatment is demanding for patients as well as health services, indicating the need for alternative treatment modalities [37, 43, 46].

Conclusions

Favorable OHRQoL and satisfaction were reported after advanced reconstruction with iliac crest-derived grafts and implant treatment in orally compromised patients. However, this treatment requires substantial resources including hospitalization and sick leave.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40729-019-0200-8.

Additional file 1. A self-administered questionnaire.

Abbreviations

HRQoL: Health-related quality of life; OHIP-14: Oral Health Impact Profile-14; OHRQoL: Oral health-related quality of life; PROMs: Patient-reported outcome measures; QoL: Quality of life

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Authors' contributions

CG contributed to the design of the trial, acquisition of the data, follow-up of the patient-reported data, and analysis and interpretation of the data and drafted the manuscript. SS contributed to the design of the trial, statistical analysis, and analysis and interpretation of the data and drafted the manuscript. EN contributed to the conception and design and critically revised the manuscript. KM contributed to the conception and design and critically revised the manuscript. HG contributed to the design of the trial, statistical analysis, and analysis and interpretation of the data and drafted the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The Ethics Committee was contacted in 2015, and no ethical approval was needed since this was then considered a quality control study. Written consent was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

Cecilie G Gjerde, Siddharth Shanbhag, Evelyn Neppelberg, Kamal Mustafa, and Harald Gjengedal declare that they have no competing interests.

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Translation of a standardized manufacturing protocol for mesenchymal stromal cells: A systematic comparison of validation and manufacturing data

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Abstract

Background: Many data are available on expansion protocols for mesenchymal stromal cells (MSCs) for both experimental settings and manufacturing for clinical trials. However, there is a lack of information on translation of established protocols for Good Manufacturing Practice (GMP) from validation to manufacturing for clinical application. We present the validation and translation of a standardized pre-clinical protocol for isolation and expansion of MSCs for a clinical trial for reconstitution of alveolar bone. *Methods:* Key parameters of 22 large-scale expansions of MSCs from bone marrow (BM) for validation were compared with 11 expansions manufactured for the clinical trial "Jaw bone reconstruction using a combination of autologous mesenchymal stromal cells and biomaterial prior to dental implant placement (MAXILLO1)" aimed at reconstruction of alveolar bone. *Results:* Despite variations of the starting material, the robust protocol led to stable performance characteristics of expanded MSCs. Manufacturing of the autologous advanced therapy medicinal product MAXILLO-1-MSC was possible, requiring 21 days for each product. Transport of BM aspirates and MSCs within 24 h was guaranteed. MSCs fulfilled quality criteria requested by the national competent authority. In one case, the delivered MSCs developed a mosaic in chromosomal finding, showing no abnormality in differentiation capacity, growth behavior or surface marker expression during long-term culture. The proportion of cells with the mosaic decreased in long-term culture and cells stopped growth after 38.4 population doublings. *Conclusions:* Clinical use of freshly prepared MSCs, manufactured according to a standardized and validated protocol, is feasible for bone regeneration, even if there was a long local distance between manufacturing center and clinical site. Several parameters, such as colony forming units fibroblasts (CFU-F), percentage of CD34+ cells, cell count of mononuclear cells (MNCs) and white blood cells (WBCs), of the BM may serve as a predictive tool for the yield of MSCs and may help to avoid unnecessary costs for MSC manufacturing due to insufficient cell expansion rates.

Key Words: *advanced therapy medicinal products, cell production, Good Manufacturing Practice, karyotyping, mesenchymal stromal cells, quality control, translational medicine*

Background

Mesenchymal stromal cells (MSCs) are well known for their immunomodulatory [1,2] and regenerative potential and have shown their applicability as a promising therapy for tissue regeneration, e.g., liver repair [3], osteoarthritis [4] and bone regeneration [5]. With

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more than one million procedures each year in Europe [6], bone is the most transplanted tissue in humans after blood. Bone losses of traumatic (e.g., non-union fractures) or pathological origin (e.g., tumors or jawbone cysts) are generally filled with an autologous bone graft or autologous bone marrow. Autologous bone transplantation is the gold standard therapy for bone reconstitution in oral and maxofacial surgery [7]. For this treatment, a piece of autologous bone is removed, commonly from the *crista iliaca*, causing a second bone defect in the patient with possible side effects like pain or nerve damage at the site of bone harvesting [8]. In addition, autologous bone therapy may fail, due to preterm transplant resorption [9,10]. MSCs have been shown to be present in almost every tissue [11]. Due to their limited number in tissues, MSCs have to be isolated from the original tissue and expanded *ex vivo* in clean rooms (class A in B) [12–14].

Different protocols for Good Manufacturing Practice (GMP)-compliant isolation and expansion of MSCs have been described previously [15–21], but there is a lack of information on the suitability of pre-clinical protocols for experimental settings and animal models and their translation for GMP-compliant manufacturing of MSCs for clinical trials. We present the validation and translation of a standardized pre-clinical protocol [12] for isolation and expansion of MSCs for a clinical trial for reconstitution of alveolar bone (Jaw bone reconstitution using a combination of autologous mesenchymal stem cells and biomaterial prior to dental implant placement; MAXILLO-1 [EudraCT number 2012-003139-30; ClinicalTrials.gov identifier NCT02751125]) as an example for translation of manufacturing protocols for clinical trials in other indications.

Expanded MSCs for clinical applications are classified as an advanced therapy medicinal product (ATMP) according to the European Medicines Agency (EMA) regulation number 1394/2007 of the European Commission (EC) [22].

In this study, we describe the translation of a previously established protocol for GMP-compliant large-scale expansion of bone marrow (BM)-derived MSCs [12] to produce clinical doses of 50–100 million MSCs for jawbone reconstruction prior to dental implant surgery for 11 patients participating in the clinical trial MAXILLO-1. The MSCs expanded according to the GMP-compliant protocol used in this study have previously been tested for their osteogenic *in vivo* bone formation potential in pre-clinical models [23,24]. In these models, MSCs were immobilized on a macro-microporous biodegradable, resorbable biphasic calcium phosphate.

Minimal criteria for MSCs as defined by the International Society for Cellular Therapy (ISCT) [25,26] or modifications of these criteria [12–14,27,28] are required to define identity and describe impurities of the ATMP. Release parameters for the ATMP may vary and include microbial, endotoxin and mycoplasma testing, tests for viability, clonogenicity, identity and purity and functional tests, depending on the type of clinical trial and the demands from national competent authorities.

For most clinical trials using MSCs, the manufacturing centers and clinical centers are two individual institutions at distinct locations. In this study, a bi-directional transportation lasting 18–24 h was necessary to transport BM aspirate from the clinical site to the manufacturing center and to transport the ATMP back to the clinical site. Part of this study was to analyze feasibility of interaction between a clinical partner and a manufacturing center over a long distance. Freezing ATMP has been shown to be quite inefficient because the recovery rate and clonogenicity [29] are reduced by cryopreservation and the clinical center additionally has to manipulate the MSCs, which may alter the intended clinical dose. It is also not clear if and how quickly MSCs recover their full therapeutic activity after thawing. In principle, cryopreservation of MSCs is possible with loss of viability and clonogenicity, depending on the freezing protocol [30]. Several publications showed maintenance of cell viability, surface marker expression, plasticity [31] and function of MSCs (e.g., in a retinal ischemia/perfusion model) [32]. Viability of (adipose-derived) MSCs after cryopreservation in animal-free formulations may, however, be less than 72% and reduced by more than 20% [29] as compared with pre-freezing viability with a recovery of down to 62%. The viability rate is similar to the one observed for pre-clinical studies at 8°C ± 3°C for transportation of MSCs expanded according to the protocol used in this study [12,24].

Effects of changes on gene expression profiles by freezing and thawing are still unclear [33]. The occurrence of cryopreservation-induced apoptosis [34] and of freeze/thaw and osmotic stress [35] can be avoided when using non-cryopreserved cells. As summarized by Galipeau [36], clinical trials with human MSCs almost always use cryopreserved cells, whereas in the pre-clinical animal models, live, log phase of growth MSCs are used almost universally. It was important for this study to use non-cryopreserved MSCs showing the full potential of bone formation when stored and transported at 4°C within 24 h including transportation. When using freshly produced, unfrozen MSCs for therapy, optimization of transportation conditions is crucial. Therefore, the clinical site often is close to the manufacturing site. In this study we also showed that there is no necessity of a local association of manufacturer and operator when MSCs are shipped at 4°C at conditions previously established in a pre-clinical setting keeping the bone formation potential of the shipped cells [23,24].

Methods

Ethical approval and participating manufacturing and clinical centers

BM (validation runs) was collected from volunteer healthy donors after written informed consent was obtained according to the Declaration of Helsinki and approval by the Ethics Committee of Ulm University (ethical approval numbers 21/10 and 24/11). The clinical trial MAXILLO-1 (Jaw bone reconstruction using a combination of autologous MSCs and biomaterial prior to dental implant placement) was approved by the Norwegian ethical committee (2013/1284/REKvest) and by the Norwegian Medicines Agency (13/12062-15). The clinical trial followed the European guidelines for advanced therapeutic medicinal products. The EudraCT number of the trial was 2012-003139-50 and the trial was incorporated in the database ClinicalTrials.gov with the identifier NCT 02751125. The Institute for Clinical Transfusion medicine and Immunogenetics Ulm (Ulm, Germany, authorization number DE_BW_01_MIA_2013_0040/DE_BW:91_IKT Ulm) received BM aspirates from the Section for Haematology, Department of Clinical Science, University of Bergen at the Department of Medicine, Haukeland University Hospital, Bergen, Norway, and delivered the ATMP to the Institute of Clinical Dentistry, University of Bergen, Norway.

Donor screening

BM donors were screened as described in the biomedical research protocol for the prospective interventional phase 1 clinical trial MAXILLO-1. In summary, donors were between 18 and 80 years (both genders), with lateral (width 5 mm or less) or vertical bone loss (focusing lateral bone loss) of the mandible behind the canine tooth and endented (at least one missing tooth) for more than 6 months in the region requiring reconstitution, and in good general health presenting with normal blood cell counts and renal and hepatic function within normal limits.

Isolation and shipping of BM

Aspiration of 25 mL (target) BM was performed in an operating room from the iliac crest after local anesthesia. By a cutaneous point of puncture, two to three points of puncture of the posterior iliac spine were made with a trocar. BM was harvested by fraction of 2-4 mL in 20-mL syringes, prefilled with heparin (ratiopharm). The harvest, in its primary packaging, was laid out in an isothermal box labeled according to Directive 2004/23/EC [37] and 2006/17/EC [38]. The transport temperature was between 18°C and 24°C, with temperature traceability. Delivery to the manufacturing centers was ensured within 24 h by accompanied transportation using a qualified transportation company.

GMP-compliant isolation and expansion of MSCs

Isolation and expansion of MSCs from the BM aspirates was performed as previously described as two-step protocol, option 1 (TSP1) by Fekete *et al.* [12]. The different steps of the manufacturing process, the corresponding test parameters and the responsibilities are summarized in Supplementary Table 1. Disposables, reagents and excipients are listed in Supplementary Table 2 and Supplementary Table 3. In brief, BM was directly seeded without any further manipulation in Minimal Essential Medium Eagle, alpha formulation (alpha-MEM medium) supplemented with 5% platelet lysate (PL) and 1 IU heparin/mL at a concentration of 50 000 BM white blood cells per cm^2 in one to eight 2-chamber CellStacks (Corning) at day 0 and incubated at 5% CO_2 atmosphere, 95% relative humidity at 37°C. After 2–4 days, the supernatant was discarded and replaced by fresh alpha-MEM (Lonza) supplemented with 5% PL (IKT Ulm) and 1 IU heparin/mL. Twice a week, the supernatant was replaced by alpha-MEM supplemented with 5% PL and 1 IU heparin/mL. At day +14, the cells were rinsed with Dulbeccos Phosphate Buffered Saline (DPBS, Lonza) and detached and MSCs of passage (P) 0 (MSCP0) were harvested using TrypZean (Lonza). Harvested cells were re-seeded at the concentration of 4×10^3 MSCP0 per cm^2 in alpha-MEM medium supplemented with 8% PL and 1 IU heparin/mL in one to seven 2-chamber CellStacks. Twice a week, the supernatant was replaced by alpha-MEM supplemented with 8% PL and 1 IU heparin/mL. At day 21, the cells were rinsed with DPBS and harvested using TrypZean. Cells were resuspended in a 5% albumin solution (CSL Behring) to obtain the final product MAXILLO-1 on which quality controls were applied. Cells were packaged and labeled for the shipment to the clinical center at the University of Bergen. All the materials and reagents used for the production were selected due to their suitability during the validation process to ascertain their compliance to be used in the manufacturing process. Specifications of the final product were as described in Supplementary Table 4.

Clonogenicity (colony forming units fibroblasts; CFU-F)

BM aspirate was seeded in duplicates in T25 flasks (Nunc Thermo Scientific) at the same cell density as the main culture using the same culturing conditions. For MSC of passage 0 (MSCP0), MSC of passage 1 (MSCP1) duplicates of 200 and 400 cells per T25 flask were seeded at the same culturing conditions as the main culture. After 10 days, the medium was discarded and cells were Giemsa-stained (Sigma) on

T25 flasks. Clonogenicity was assessed by counting colonies consisting of more than 50 cells/colony.

Differentiation capacity

Differentiation of MSCP1 was performed as described previously in detail [12,13,39–41], using the commercially available kits for adipogenic (Lonza), chondrogenic (Miltenyi Biotech) and osteogenic (Miltenyi Biotech) differentiation.

Quality controls: microbial testing, mycoplasma screening, endotoxin testing, karyotyping and flow cytometry

Microbial testing was performed after matrix validation according to chapter 2.6.27 of the *European Pharmacopoeia* (*Ph Eur*) 8.0 [42] using the BacT/ALERT iAST aerobic and BacT/ALERT iNST anaerobic culture bottles (Supplementary Table 2) in a BacT/ALERT 3D system (BioMerieux). Samples were shipped to an accredited contract laboratory, the Institute for Transfusion Medicine and Immunology, Mannheim, Germany, for mycoplasma testing using polymerase chain reaction (PCR) as previously described [43] after matrix validation according to chapter 2.6.7/2.6.21 of the *Ph Eur* 8.0 [42] and to Labor L+S AG, Bad Bocklet-Groenbrach, Germany for endotoxin testing by Limulus amebocyte lysate (LAL) test after matrix validation according to chapter 2.6.14 of the *Ph Eur* 8.0 [42].

For karyotyping, 100×10^3–200×10^3 MSCP0 were seeded in 42 mL in a T175 flask (Nunc Thermo Scientific) for 2–4 days until the cells reached approximately 50% confluence. Colchizin (1.63 mL of a 20 mg/mL solution from Eurobio) was added and incubated at 5% CO_2 atmosphere, 95% relative humidity at 37°C for at least 2 h. Cells were rinsed once with 50 mL of DPBS, harvested by TrypZEAN treatment, collected in 10 mL of complete medium and transported within 2 h to the accredited contract laboratory, the Institute for Human Genetics, University Hospital Ulm (Ulm, Germany), for karyotyping according to the national guidelines [44,45] and the guidelines of the European Cytogeneticists Assocoation (E.C.A.) Permanent Working Group for Cytogenetics and Society [46,47].

Flow cytometry was performed as previously described [12,13,28]. Approximately 1×10^6–4×10^6 MSCP0 or MSCP1 were stained per assay. In brief, cells were washed in DPBS and resuspended in 100μL of DPBS. Cells were stained with a combination of either immunoglobulin (Ig)G-fluorescein isothiocyanate (FITC) (20 μL, clone X40), IgG-phycoerythrin (PE) (20 μL, clone X40) and IgG-peridinin chlorophyll protein (PerCP) (20 μL, clone X40), or CD90-FITC (1 μL, clone 5E10), CD34-PE (20 μL, clone 8G12) and CD45-PerCP (20 μL, clone 2D1), or CD105-FITC (10 μL, clone SN6), CD73-PE (20 μL, clone AD2) and CD3-PE (20 μL, clone SK7), or major histocompatibility complex class II (MHC cII) human leucocyte antigen (HLA)-DQ,DP-DR-FITC (20 μL, clone Tü39) and major histocompatibility complex class I (MHC cI) HLA-A,B,C-PE (20 μL, clone G46-2.6), respectively. Antibodies were purchased from BD Bioscience, except CD105 (Bio-Rad AbD Serotec GmbH). After 15 to 20 min of staining at ambient temperature, cells were washed in DPBS and the fluorescence intensity of 50 000 cells was acquired using a FACScan with CellQuest 3.3 software (BD Biosciences).

Shipping of the ATMP

The transportation of freshly detached MSCs at 5°C ± 3°C was performed with temperature traceability. Delivery to the clinical center in Bergen was ensured within 24 h using a qualified transporter (World Courier [Deutschland] GmbH). Stability of the ATMP in 5% saline solution has previously been demonstrated [24].

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.01 Software for Windows. D'Agostino & Pearson normality test was performed for each dataset to test for normal distribution of data. In case of normal distribution, data were compared using the unpaired *t* test with Welch's correction; for datasets not passing the D'Agostino & Pearson normality test, data were compared using the Mann-Whitney *U* test. Kruskal-Wallis test was used for multiple-parameter analyses. Differences were considered as significant for $P < 0.01$ because of multiple testing of the dataset. The correlation between multiple parameters was assessed, computing Spearman correlation (*r* values) for every pair of the following datasets: time between end of aspiration and end of seeding (h), age (y), aspiration volume (mL), aspiration volume without heparin (mL), white blood cell count ([WBC]/μL), mononuclear cell count ([MNC]/μL), % MNC of WBC, % CD34+ in BM aspirate, harvest density of MSCP0 (cells/cm^2), harvest density of MSCP1 (cells/cm^2), doubling time of MSCP0 (h), doubling time of MSCP1 (h), population doublings in P0, population doublings in P1, cumulative population doublings, CFU-F/10^6 BM-WBC, CFU-F/10^6 MSCP0, CFU-F/10^6 MSCP1, MSCP0 harvested/μL BM aspirate seeded and MSCP1 harvested/μL BM aspirate seeded. Correlations with $r \geq 0.5$ and $P < 0.05$ were considered as significant.

Results

Donor characteristics

Overall, 13 aspirations were performed in the context of the clinical trial MAXILLO-1 and 21 aspirations of BM were performed for validation. Production of two of the 13 aspirates for clinical trial MAXILLO-1 was stopped at passage 0 for patients 1-05 and 1-10 because the overall harvest of 0.9×10^6 and 1.2×10^6 cells in passage 0 was not sufficient to start passage 1. In accordance with this, no CFU-F/10^6 BM-WBCs were detected (data not shown). One BM from the validation was split (identification [ID] 7585) and two BM aspirates from patients within MAXILLO-1 had to be discarded (ID 1-05 and 1-10) due to lack of CFU-F in the aspirate and growth of the culture during the passage 0 growth phase (data not shown). Information on shipping and donor characteristics are presented in Supplementary Table 5 and Supplementary Figure 1 and summarized in Table 1.

The group of volunteer healthy donors and MAXILLO-1 patients differed significantly in age ($P < 0.0001$). Significant differences were also observed for clonogenicity of the BM aspirate (CFU-F/10^6 BM-WBC; $P = 0.0060$), time between end of aspiration and beginning of seeding, WBC/mL BM aspirate ($P < 0.0001$) and MNC/mL BM aspirate ($P < 0.0001$), whereas aspiration volume ($P = 0.2414$) and the percentage of CD34 cells in the aspirate ($P = 0.0946$) did not significantly differ.

MSC isolation and expansion for validation

Detailed information on data for the expansions performed is shown in Table 2, Figure 1, Supplementary Table 6 and Supplementary Table 7.

Validation

For validation, BM-WBCs were seeded on 4307 ± 2911 cm^2 culture surface at a cell density of 49 961 ± 264 cells/cm^2. The first culture step was 13.8 ± 0.1 days and resulted in a density of passage 0 MSC (MSCP0) of $25.7 \times 10^3/cm^2 \pm 15.7 \times 10^3/cm^2$. This corresponds with 13.1 ± 0.8 population doublings with a doubling time of 25.4 ± 1.6 h. In the second culture step, 4008 ± 2 MSCP0/cm^2 were seeded on 5753 ± 3481 cm^2 and cultured for an additional 6.9 ± 0.2 days. This culture resulted in a cell density of passage 1 MSC (MSCP1) of $49.1 \times 10^3/cm^2 \pm 18.0 \times 10^3/cm^2$. This corresponds with 3.5 ± 0.7 population doublings with a doubling time of 51.7 ± 24.1 h. The cumulative number of population doublings was 16.6 ± 1.

The overall harvest of the final product was $283.2 \times 10^6 \pm 187.3 \times 10^6$. The calculated yield was $17.5 \times 10^3 \pm 14.8 \times 10^3$ MSCP0/μL BM aspirate and $230.6 \times 10^3 \pm 245.3 \times 10^3$ MSCP1/μL BM aspirate. This theoretically would have allowed a total harvest of $4540.2 \times 10^6 \pm 5227.9 \times 10^9$ MSCP1 from as little as 23.6 ± 8.2 mL of BM aspirate within 20.8 ± 0.3 days, based on the assumption that all BM-WBCs of the BM aspirate were seeded in passage 0 and the total harvest of passage 0 was reseeded for passage 1.

MAXILLO-1 patients

From the MAXILLO-1 patients' BM aspirates, BM WBCs were seeded on 5493 ± 2743 cm^2 culture surface at a density of 49 955 ± 65 cells/cm^2. The first culture step was 14.0 ± 0.0 days and resulted in a density of passage 0 MSC (MSCP0) of $13.4 \times 10^3/cm^2 \pm 7.0 \times 10^3/cm^2$. This corresponds with 13.4 ± 1.4

Table 1. Information on BM aspirates from validation runs (A) and manufacturing for the clinical trial MAXILLO-1 (B): age, volume of BM aspirate, WBC count/mL BM aspirate and number of CFU-F of BM aspirate per million BM WBCs.

(A) Validation runs

N = 22	Donor age	Volume of aspirate (mL)	Cell count WBC x 10^6/mL BM aspirate	CFU-F of BM/10^6 WBCs
Mean	26	23.6	30.7	236
SD	7	8.2	15.7	160
Minimum	21	9.3	9.4	9
Maximum	49	42.5	62.4	453

(B) MAXILLO-1 patients

N = 11	Patient age	Volume of aspirate (mL)	Cell count WBC x 10^6/mL BM aspirate	CFU-F of BM/10^6 WBCs
Mean	63	21.4	16.9	31
SD	6	2.0	8.5	22
Minimum	51	19.0	6.5	2
Maximum	72	26.0	37.2	73

SD, standard deviation.

Table 2. Key information of expansion process for MSCs from validation runs (A) and manufacturing for the clinical trial MAXILLO-1 (B): doubling time in passage 0, passage 1 and number of population doublings in passage 0 and passage 1 and number of cumulative population doublings in passage 0 and passage 1, achieved overall harvest of the final product (MSC of passage 1), and calculated yield (MSC/μL BM aspirate) of MSCs for passage 0 and passage 1.

(A) MSCs from validation runs

	Doubling time		Number of population doublings			Overall harvest (cells x 10^6)	Calculated yield (MSC x 10^3/μL BM aspirate) for		Hypothetical maximum harvest (cells x 10^6)
	P0 (h)	P1 (h)	P0	P1	Cumulative (P0 and P1)		P0	P1	
Mean	25.4	51.7	13.1	3.5	16.6	283.2	17.5	230.6	4540.2
SD	1.6	24.1	0.8	0.7	1.1	187.3	14.8	246.3	5227.9
Minimum	21.8	38.7	11.7	1.2	14.4	11.9	0.5	6.6	13.6
Maximum	28.4	155.4	15.2	4.3	19.1	740.8	54.6	1012.1	20141.3

(B) MAXILLO-1 MSCs

Mean	25.3	49.3	13.4	3.4	16.8	273.7	5.3	63.2	1424.9
SD	2.5	4.4	1.4	0.3	1.5	104.5	5.0	69.1	1653.5
Minimum	20.5	44.1	11.1	2.9	14.5	53.3	0.5	5.3	103.8
Maximum	30.3	57.1	16.4	3.8	20.2	412.0	18.3	243.5	5905.4

The hypothetical maximum harvest indicates the maximum harvest that could have been achieved in case all aspirated BM was used for the MSC isolation and expansion process.
P0, passage 0; P1, passage 1.

population doublings with a doubling time of 25.3 ± 2.5 h. In the second culture step, 3882 ± 374 MSCP0/cm^2 were seeded on 6467 ± 2132 cm^2 and cultured for an additional 7.0 ± 0.0 days. This culture resulted in a density of passage 1 MSC (MSCP1) of $42.7 \times 10^3/cm^2$ ± $9.4 \times 10^3/cm^2$. This corresponds with 3.4 ± 0.3 population doublings with a doubling time of 49.3 ± 4.4 h. The cumulative number of population doublings was 16.8 ± 1.5.

The overall harvest of the final product was $273.7 \times 10^6 \pm 104.5 \times 10^6$. Thus, the clinical dose of 2 x [50×10^6] MSCs could be produced in all cases except for patient 1-07. For this patient, a single dose of 50×10^6 MSC was produced, fulfilling the specifications (Supplementary Table 4).

The calculated yield was $5.3 \times 10^3 \pm 5.0 \times 10^3$ MSCP0/μL BM aspirate and $63.2 \times 10^3 \pm 69.1 \times 10^3$ MSCP1/μL BM aspirate. This theoretically would have allowed a total harvest of $1424.9 \times 10^6 \pm 1653.5 \times 10^6$ MSCP1 and production of a minimum of two to a maximum of 118 doses of 50×10^6 MSCP1 from as little as 21.4 ± 2.0 mL of BM aspirate within 21.0 ± 0.0 days when seeding all BM aspirate for passage 0 and all MSCP0 for generation of the ATMP.

Statistical analysis

When isolating and expanding MSCs, no significant differences of clinical relevant parameters between the group of volunteer healthy donors and MAXILLO-1 were observed for the parameters harvest density (MSCs/cm^2) of MSCP1 ($P = 0.19463$), yield (MSCs harvested/μL BM aspirate seeded) of passage 0 ($P = 0.0153$) and passage 1 ($P = 0.2134$), population doublings in passage 0 ($P = 0.5247$), in passage 1 ($P = 0.7485$) and cumulative population doublings ($P = 0.6553$), doubling time during passage 0 ($P = 0.3551$) and passage 1 ($P = 0.2484$). There was a significant difference in harvest density (MSCs/cm^2) of MSCP0 ($P = 0.0048$). This difference, which disappears during further passaging, is reflected by different CFU-F counts in the BM aspirates and may be caused by differences in the transportation time and age of donors in the group of volunteer healthy donors and MAXILLO-1 patients.

Quality controls

All quality controls were carried out according to the *Ph Eur* (Supplementary Table 8) for the corresponding method and all matrices have been validated for the tests applied.

Viability

Percentage of viable cells was 94.6% ± 3.1% in the BM aspirate (BM-MNC), 97.0% ± 3.2% for MSCP0 and 94.5% ± 4.2% for MSCP1 for validation runs (Table 3, Figure 2A and Supplementary Table 9).

For MAXILLO-1 patients, the percentage of viable cells was 92.5% ± 3.5% in the starting material (BM-MNC), 97.7% ± 1.7% for harvested MSCP0 and 97.9% ± 1.1%, for the ATMP MAXILLO-1 MSCs.

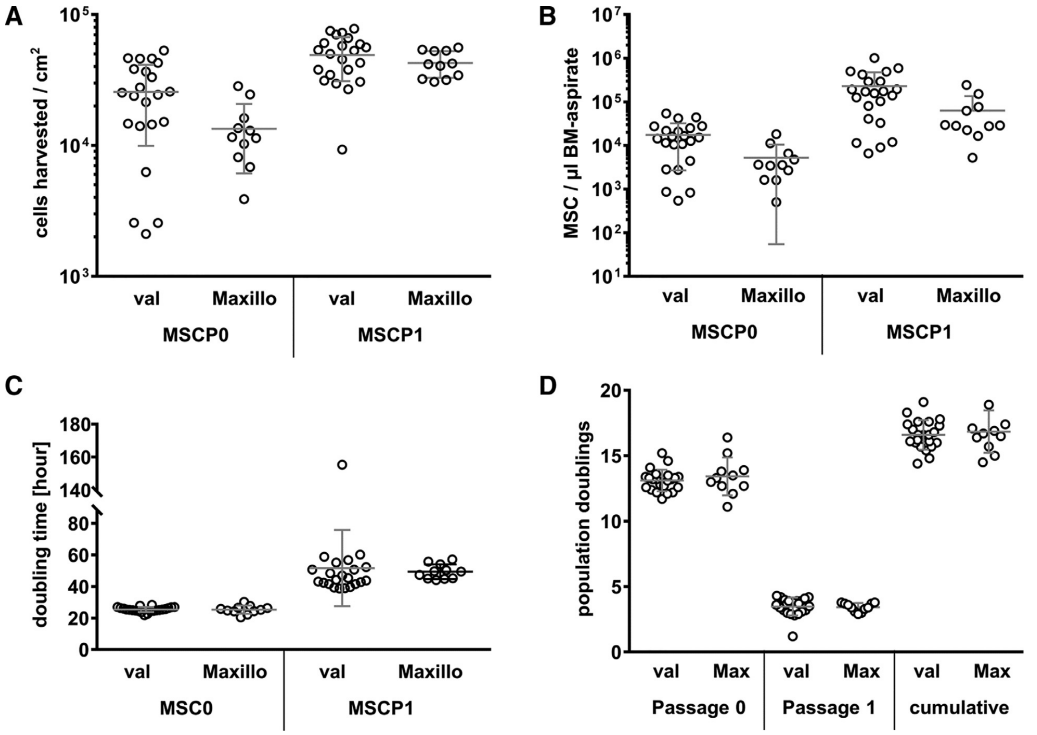

Figure 1. Key parameters of cell expansion. (A) MSC harvesting density (cells harvested/cm^2), (B) yield per µL BM aspirate seeded (MSCs/µL BMaspirate) and (C) doubling times (doubling time [h]), are shown for passage 0 and passage 1 for expansions used for the validation process and for the clinical trial MAXILLO-1. (D) Number of population doublings for MSCP0 and MSCP1 and the cumulative number of populations doublings is shown for expansions used for the validation process and for the clinical trial MacilloCT-1. Grey bars show mean and standard deviation. MSCP0, passage 0 MSC; MSCP1, passage 1 MSC; val, data for validation runs; Maxillo or Max, data for clinical trial MAXILLO-1.

No significant difference in viability of cells from BM ($P = 0.0767$), of MSCP0 cells BM ($P = 0.8995$) or MSCP1 BM ($P = 0.0104$) cells from volunteer healthy donors and MAXILLO-1 patients was seen. The Kruskal-Wallis test failed to reveal significant difference in viability of MSCP0 and MSCP1 between the two groups of donors.

Impurities and identity

The content of impurities of the starting material (i.e,. leukocytes or hematopoietic stem cells was determined by expression of CD3, CD34, CD45 and MHC cII on MSCP0; Table 4, Figure 2B and Supplementary Table 10) and MSCP1 (Table 4, Figure 2B and Supplementary Table 11) cells using flow cytometry. In summary, parameters for identity and impurity were fulfilled for all expansions from both volunteer healthy donors and MAXILLO-1 patients with one exception: MSCP1 expanded from volunteer healthy donor 7575 showed deviations for the parameters CD3 and CD105. The percentage of CD3+ cells was 23.5% (with an allowed threshold of $\leq$5%), and the expression of CD105+ cells was 88.97% (with an allowed threshold of $\geq$90%). Thus, only one preparation of 33 (i.e., 3%) did not fulfill the release quality control criteria for identity and impurity.

Clonogenicity (CFU-F)

Expanded MSCs showed a clonogenicity of $192 \times 10^3 \pm 72 \times 10^3$ colonies/10^6 seeded MSCP0 and of $210 \times 10^3 \pm 79 \times 10^3$ colonies/10^6 seeded MSCP1 for cells from volunteer healthy donors and of $171 \times 10^3 \pm 86 \times 10^3$ colonies/10^6 seeded MSCP0 and of $91 \times 10^3 \pm 40 \times 10^3$ colonies/10^6 seeded MSCP1 for cells from MAXILLO-1 patients. BM aspirates from volunteer healthy donors differed significantly in their CFU-F content ($P = 0.0060$) and MSCP1 showed significant difference in clonogenicity ($P = 0.0003$;

Table 3. Percentage of viable cells in the starting material, for harvested passage 0 MSCs (% viable cells after harvest of P0) harvested passage 1 MSCs (% viable cells after harvest of P1) from validation runs (A) and manufacturing for the clinical trial MAXILLO-1 (B).

(A) MSCs from validation runs			
% of viable cells	In BM aspirate	After harvest of P0	After harvest of P1
Mean	94.6	97.0	94.5
SD	3.1	3.2	4.2
Minimum	87.0	86.2	84.7
Maximum	98.2	100.0	99.6
Threshold for release	ND	≥80	≥80
(B) MAXILLO-1 MSCs			
Mean	92.5	97.7	97.9
SD	3.5	1.7	1.1
Minimum	86.9	94.4	93.3
Maximum	97.7	99.8	99.4
Threshold for release	ND	≥80	≥80

Thresholds for release of the ATMP MAXILLO-1 are indicated at the bottom of the table.
ND, not defined (declaration parameter only).

Figure 2C). Interestingly, MSCP0 from volunteer healthy donors and MAXILLO-1 patients did not differ in their clonogenic potential ($P = 0.3551$).

Differentiation capacity

Adipogenic, chondrogenic and osteogenic differentiation capacity was shown for all expansions performed for validation runs and in the context of MAXILLO-1. Representative photographs are shown in Supplementary Figure 2. All batches of MSCs exhibited a multipotent capacity in the three lineages.

Microbial, endotoxin and mycoplasma testing

Microbial testing of the starting material (BM), of the cell culture supernatant at day 7, of MSCP0 and of MSCP1 was negative for all expansions (Supplementary Table 12). Endotoxin testing was performed for all expansion and mycoplasma testing was performed for expansions in the context of the clinical trial MAXILLO-1 and for 8 of the 22 cell expansions from volunteer healthy donors. For all tested products samples, anaerobic and aerobic cultures showed a negative test result. No mycoplasma DNA was detectable and endotoxin levels were ≤1 IU/mL in all cases.

Karyotyping

Karyotyping was set up for all 33 expansions. In one case (ID 1-07), only an insufficient number of metaphases could be achieved (Supplementary Table 13). A chromosomal change in only one metaphase could be detected for validation run ID 7537, 7543, 7562 and 7574. From the occurrence of such abnormalities no conclusion can be made on the culture.

In one case (ID 1-04), four different chromosomal changes of the active substance were observed after release of the ATMP. The karyotype was as follows: 46,XX[29]; 46,XX,t(3;5;13)(p1?3;q33;p1)[6]; 46,X,?inv(X)(p22q1?1[3]; and 46,X,+8[1]; 47,XX,+21[1]. Two of the four changes occurred in more than one metaphase. To exclude chromosomal instability, immortalization and any effect on growth regulation, long-term cultures were set up.

Long-term culture of MSCs from patient 1-04

Cells were cultured in accordance with the expansion protocol for 5–19 days and passaged as indicated in Supplementary Table 14A. This process was continued until cells stopped growth. At each passage, the number of population doublings, doubling time and viability were determined. Karyotping of cells from passage 1, 2, 4, 7 and 9 was performed. In addition, identity of the cells by flow cytometry (Supplementary Table 14B) and adipogenic, chondrogenic and osteogenic differentiation capacity (Supplementary Figure 3) was assessed for cells of passage 4 and 7.

During long-term culture, cells with the chromosomal finding 46,X,?inv(X)(p22q1?1[3], 46,X,+8[1] and 47,XX,+21[1] disappeared after one additional passage, whereas cells with the karyotype 46,XX,t(3;5;13)(p1?3;q33;p1) persisted until passage 7 (i.e., 34.9 cumulative population doublings of the CFU-F from BM) but the relative proportion of cells with this marker decreased from 15% (passage 1) to 3% (passage 7). Cells seeded after nine passages stopped growth. The culture was maintained for 98 days. During this time, 38.4 population doublings occurred and the doubling time increased from 26.4 h (passage 0) to 340.9 h (passage 9). Viability was always >80% and flow cytometry analysis showed that <5% of cells were positive for CD3, CD45, CD34 and MHC cI and >90% of cells were positive for CD73, CD90 and CD105 (Supplementary Table 14B). All these were release criteria for clinically applicable MSCs in MAXILLO-1. Interestingly, expression of MHC cI decreased to 75.75% for passage 7 cells. Patient 1-04 was screened a second time about half a year later with patient ID 1-11 [48] and cells were produced for transplantation of MSCs to the opposite mandibular site before dental implantation. For this second expansion, karyotyping was without findings. Overall, there was no evidence for expansion of the clone with the cytogenetic marker and no evidence for autonomous proliferation with appearance of phenotypically abnormal cells.

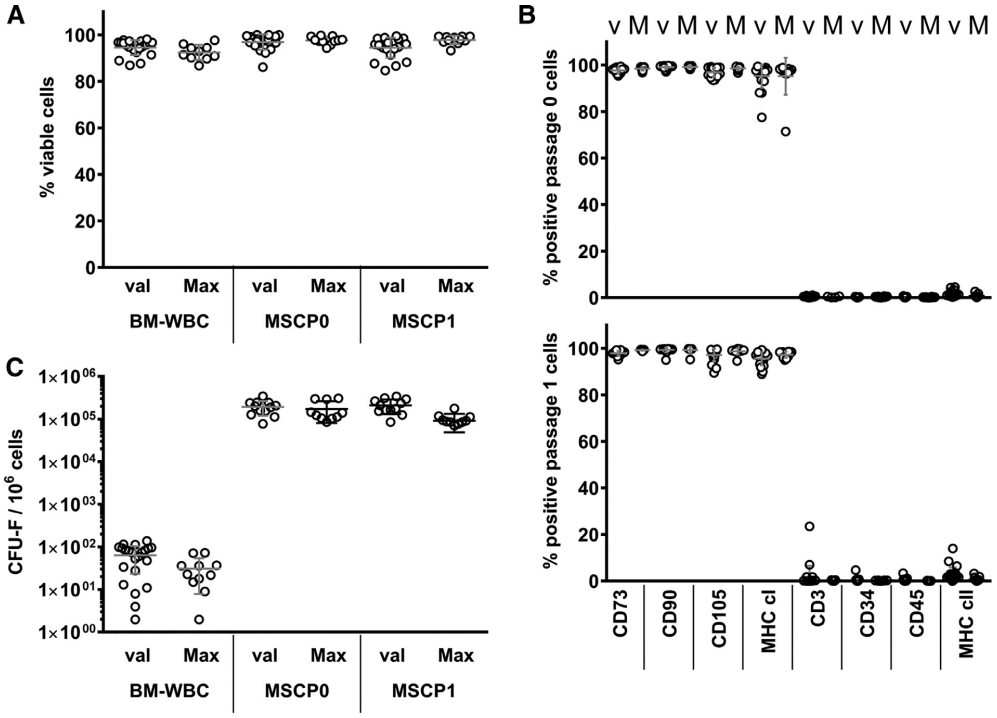

Figure 2. Key parameters of quality controls. (A) Percentage of viable WBCs in the BM aspirate and of viable MSCP0 and MSCP1, (B) results of flow cytometry analysis for identity (CD73, CD90, CD105 and MHC cI) and impurities (CD3, CD34, CD45 and MHC cII) of MSCP0 and MSCP1 and (C) number of colony-forming units fibroblasts per 10^6 cells (CFU-F/10^6 cells) for MNCs from BM aspirates, MSCP0 and MSCP1 are shown for expansions used for the validation process and for the clinical trial, respectively. Grey bars show mean and standard deviation.

Statistical correlations

Statistical correlations were calculated based on all 33 expansions (both from volunteer healthy donors and MAXILLO-1 patients). Spearman correlation matrix for multiple-parameter analysis is shown in Supplementary Table S15A, and the corresponding P value matrix is shown in Supplementary Table 15B.

As expected, a high correlation between BM-WBC count and BM-MNC count ($r = 0.8436$; $P < 0.0001$) and between the percentage of CD34+ cells and BM-WBC ($r = 0.5938$; $P = 0.0005$) count and BM-MNC count ($r = 0.6351$; $P = 0.0002$) was observed because one of the inclusion criteria for treatment of MAXILLO-1 patients was the absence of hematopoietic disorders.

Effects of the parameters "age" and "transportation time" cannot be deciphered because transportation time for BM aspirates from elder donors (mainly MAXILLO-1 patients) was significantly higher (see donor characteristics) than transportation time for aspirates from younger patients (volunteer healthy donors). According to this, the correlation of age and transportation time was $r = 0.7719$ ($P < 0.0001$). Both age and transportation time show negative correlation with WBC count, MNC count, percentage of CD34+ cells in the BM aspirate, harvest density of MSCP0 (but not MSCP1), CFU-F from BM aspirate and MSCP1 (but not from MSCP0) and the yield of both MSCP0 and MSCP1 harvested per microliter BM aspirate seeded (for r and P values refer to Supplementary Table 15).

The percentage of CD34+ cells in BM aspirates correlated with the number of CFU-F from the BM aspirate ($r = 0.6288$; $P < 0.0002$) and also with the harvesting density of MSCP0 ($r = 0.6586$; $P = 0.0001$). In accordance with this, the number of CFU-F from the BM aspirate correlated positively with the harvesting density of MSCP0 ($r = 0.8016$; $P < 0.0001$).

A positive correlation between WBC count in the BM aspirates and MSCP0 cells ($r = 0.8522$; $P < 0.0001$) and MSCP1 cells ($r = 0.7721$; $P < 0.0001$) harvested per microliter BM aspirate as well as between MNC count in the BM aspirates and MSCP0 cells ($r = 0.7865$; $P < 0.0001$) and MSCP1 cells ($r = 0.7064$;

Table 4. Flow cytometry in process quality control of passage 0 MSC and control of validation runs (A) and the ATMP MAXILLO-1 MSC (B).

(A) MSCs from validation runs

	% Positive MSCs of passage 0 for indicated marker								% Positive MSC of passage 1 for indicated marker							
	CD3	CD34	CD45	MHC cII	CD73	CD90	CD105	MHC cI	CD3	CD34	CD45	MHC cII	CD73	CD90	CD105	MHC cI
Mean	0.07	-0.12	0.08	1.47	97.71	99.09	96.93	95.32	1.43	0.12	0.23	2.67	97.91	99.39	97.21	95.65
SD	0.44	0.38	0.28	1.21	1.18	0.73	1.95	4.88	5.07	1.11	0.77	3.14	1.07	0.98	2.67	2.78
Minimum	-0.60	-1.34	-0.41	0.30	95.41	97.20	93.56	77.58	-0.90	-1.38	-0.76	-0.06	95.29	95.08	89.54	88.97
Maximum	1.06	0.53	0.80	4.60	99.49	99.80	99.36	99.45	23.51	4.74	3.39	14.02	99.43	99.89	99.72	99.41
	Thresholds for in process controls of passage 0 cells								Thresholds for controls of the ATMP MAXILLO-1 MSCs							
	≤20	≤20	≤20	ND	≥80	≥80	≥80	ND	≤5	≤5	≤5	ND	≥90	≥90	≥90	ND

(B) MAXILLO-1 MSCs

	% Positive MSC of passage 0 for indicated marker								% Positive MSC of passage 1 for indicated marker							
	CD3	CD34	CD45	MHC cII	CD73	CD90	CD105	MHC cI	CD3	CD34	CD45	MHC cII	CD73	CD90	CD105	MHC cI
Mean	-0.05	0.31	0.18	0.78	98.49	99.30	98.63	95.25	0.00	0.11	0.01	0,58	99.34	99.36	98.79	97.34
SD	0.42	0.23	0.16	0.82	0.72	0.52	0.79	8.60	0.23	0.13	0.18	1.07	0.10	0.07	0.37	1.26
Minimum	-0.74	-0.14	-0.01	0.19	96.85	98.23	96.67	71.53	-0.39	-0.09	-0.25	-3.03	98.79	95.31	94.66	95.07
Maximum	0.64	0.71	0.52	2.68	99.25	99.69	99.47	99.02	0.44	0.29	0.18	3.27	99.56	99.87	99.98	98.72
	Thresholds for in process controls of passage 0 cells								Thresholds for controls of the ATMP MAXILLO-1 MSCs							
	≤20	≤20	≤20	ND	≥80	≥80	≥80	ND	≤5	≤5	≤5	ND	≥90	≥90	≥90	ND

Thresholds for release are indicated at the bottom of the table. Negative figures mean that the proportion of positive cells after staining with the respective specific antibody was lower than percentage of positive cells in the isotype control.

$P < 0.0001$) harvested per microliter BM aspirate and between the percentage of CD34+ cells in the BM aspirates and MSCP0 cells ($r = 0.7195$; $P < 0.0001$) and MSCP1 cells ($r = 0.6163$; $P < 0.0001$) harvested per microliter BM aspirate was observed. The harvesting density of MSCP0 correlated positively with the clonogenicity of MSCP1 ($r = 0.5035$; $P = 0.0143$). For the passage 1 culture step, there was a positive correlation between harvesting density and number of population doublings ($r = 0.9771$; $P < 0.0001$) and, in accordance with this, a negative correlation between harvesting density and doubling time ($r = -0.9721$; $P < 0.0001$). For further correlations refer to Supplementary Table 15.

Discussion

Production of cells

In this study, we have presented validation data of a GMP-compliant protocol for MSC isolation and expansion and have proven the feasibility of this protocol to manufacture MSCs for a clinical trial. Pre-clinical studies (e.g., in the context of bone formation [15,49] or osteoarthritis [4]) using non-cryopreserved MSCs from BM or adipose tissue have been performed previously, but to our knowledge a systematic comparison of data on growth behavior, yield and quality controls relevant for release of the cell product (i.e., the manufactured ATMP) obtained in the validation process and in manufacturing of the clinical product has not been performed before.

In this study we also have shown that the production of clinical doses of MSCs for the clinical trial MAXILLO-1 was possible from a remote production site. Transport of BM aspirate from Bergen (Norway) to Ulm (Germany) was possible within 24 h by using a conventional courier service. The shelf life of 24 h for freshly produced, non-cryopreserved, clinical-grade MSCs was sufficient for release of the product and transportation from the manufacturing site in Ulm, Germany to the clinical site in Bergen, Norway, where the product was implanted the day after. Before starting the production of 11 clinical doses for jaw augmentation, the production process was validated using 22 expansions from BM aspirates of 21 volunteer healthy donors. The availability of volunteer healthy BM donors is limited and, because the age of patients treated within MAXILLO-1 was not predictable prior to the recruitment of patients for the clinical trial, the group of volunteer healthy donors and MAXILLO-1 patients could not be matched for parameters like gender, age, body mass index or smoker status. Volunteer healthy donors had an average age of 26 years, and MAXILLO-1 patients had an average age of 62 years. In addition, mean time between end of aspiration and beginning of seeding was 6.7 h for volunteer healthy donors and 22.3 h for MAXILLO-1 patients. The high correlation for the parameters "donor age" and "time between end of aspiration and beginning of seeding" is caused by the experimental setting of this study, because transportation time for BM aspirates from elder donors (mainly MAXILLO-1 patients) was significantly higher than transportation time for aspirates from younger patients (volunteer healthy donors). Therefore, it is not possible to decipher the reason for significant differences of the two groups in clonogenicity of the BM aspirate (CFU-F/10^6 BM-WBC), in WBC/mL BM and in MNC/mL BM. Interestingly, the percentage of CD34+ cells in the aspirates did not significantly differ. The percentage of CD34+ cells in BM aspirates correlated with the number of CFU-F from the BM aspirate. However, Kurt Yuksel *et al.* [50] showed no correlation between the clonogenic potential of stromal cells (CFU-F) and hematopoietic cells (colony foming units granulocyte-macrophage; CFU-GM) for patients with hematologic malignancies, patients with a diagnosis of BM failure and patients without hematologic disease. Both, CD34+ cells in BM aspirates as well as CFU-F from the BM aspirate correlated with the harvesting density of MSCP0. Obviously, a high percentage of CD34-positive cells and/or high CFU-F count and/or high cell count of MNCs and/or WBCs in the BM aspirate also positively correlate with the yield of not only MSCP0, but also MSCP1. Because BM cell count and also the percentage of CD34+ cells can easily be assessed at the day of BM harvest, it may be possible to predict whether a determined cell target of MSCP1 can be achieved from each individual aspirate. Analysis of a higher number than 33 large-scale expansions and analysis of an independent set of expansions is necessary to calculate the positive predictive value and the validity of the above-mentioned assumption. Starting from only 23 mL of BM aspirate, a total harvest of 13.6×10^6 – 20 141.3×10^6 to MSCP1 within 21 days would have been possible when using the whole BM aspirate for cell expansion. This range of the hypothetical overall yield shows the necessity to screen for early available, reliable prediction parameters for calculation of the expectable yield. In the setting of large-scale manufacturing for clinical use, an appropriate cell number for seeding passage 0 has to be used, sufficient to guarantee the target dose of the respective clinical protocol, but not causing an "overproduction," taking account of economic issues.

Because published expansion protocols show a high variability, we compared the doubling times in this study with the doubling times from publications using a similar expansion protocol [51,52]. We obtained mean doubling times of about 25 h for cells in passage 0 and of about 39 and 44 h for passage 1 (Table 2). These doubling times are comparable to the ones

published by Schallmoser *et al.* [52] (doubling time: 25 to 34 h, and calculated from available data: 10.5 population doublings in 11–15 days) and by Schallmoser *et al.* [51] (doubling time: 36 to 89 h, and calculated from available data: 2.7–6.7 population doublings in 10 days; doubling time: 36 for comparable seeding density to the protocol in this study).

It is also important to mention that the harvesting density of MSCP0 correlated positively with the clonogenicity of MSCP1, indicating that a higher harvesting density in passage 0 might have a beneficial effect on clonogenicity of cells applied to the patient. For long-term cultures for up to 39 population doublings, replicative aging, induction of alkaline phosphatase, bone sialoprotein, osteocalcin and collagen 1 have been described [53]. It has been shown that donor age and the number of cumulative population doublings impact the quality of MSCs in long-term cultures [54,55]. Beane *et al.* [54] showed lower cell yields and impaired adipogenesis with age in rabbits. Long-term cultured BM-derived MSCs exhibited slower population doublings, increased senescence and inferior chondrogenic differentiation potential. It has previously been shown that CFU-F content [29] and bone morphogenetic protein 7 (BMP7) [32] secretion increased after cryopreservation. Comparisons of the CFU-F content after different handling or harvest procedures of the tissue [56,57], from different tissue sites [56] or from donors with different malignancies [50] have been published, but to our knowledge nothing is known so far about the impact of seeding density of MSCs on their clonogenicity or capacity to form CFU-F in the straight following passages. We did not observe any significant correlation of harvesting density for passage 0 and the clonogenicity of the harvested cells from this passage, whereas Bartmann *et al.* [51] used a similar xenogenic-free expansion system for MSCs and observed an inverse correlation of seeding density to MSC proliferation and CFU-F frequency of the same passage.

Quality controls and release of cells

All cell productions performed for the clinical trial MAXILLO-1 fulfilled the release criteria accepted by the Norwegian Medicines Agency (NoMA) for this ATMP (Supplementary Table 4). During the validation process, cell marker surface expression of MSCP1 expanded from volunteer healthy donor 7575 showed deviations for the parameters CD3 and CD105. Overall, only one single expansion of 33 (i.e., 3%) failed to pass quality controls for identity and impurities.

Bone augmentation capacity of manufactured cells

MSCs isolated and expanded to the described protocol showed *in vivo* bone formation potential. Seven MSC preparations manufactured in the context of validation were successfully used in combination with a biphasic calcium phosphate biomaterial (BCP+) to induce ectopic bone formation and bone regeneration of induced critical size defects of the calvaria in immunocompromised mice [23]. Bone formation was observed and human cells were detected in the freshly formed bone. MSCs for the clinical trial MAXILLO-1 were implanted into patients with severely atrophied mandibular bone and successful augmentation of alveolar bone was observed in all study participants and shown by histology and X-ray microtomography (μCT) images [48].

Karyotyping

We observed chromosomal abnormalities in 5 expansions (Supplementary Table 13). These findings occurred in 4 of 22 (i.e., 18%) and in 1 of 11 (i.e., 9%) MSCP1 obtained from volunteer healthy donors and MAXILLO-1 patients, respectively. However, because only one metaphase was affected, this observation was considered as irrelevant; from the occurrence of such abnormalities no conclusion can be made [44–47,58,59]. Only in two cases (donor 7574 and patient 1-04), a specific finding occurred with higher frequency. Long-term cultures were set up for MSCP1 from patient 1-04 with a total culture time of 105 days to exclude chromosomal instability, immortalization and any effect on growth regulation. Cells with the specific chromosomal finding 46,XX,t(3;5;13)(p1?3;q33;p1) persisted until the culture stopped growth. The percentage of affected cells decreased from 15% to 3%. The release criteria for identity, impurity and viability were fulfilled and adipogenic, chondrogenic and osteogenic differentiation potential of cells were fulfilled also for cells harvested from long-term culture passage 4 and passage 7. During the long-term culture, doubling time constantly increased until cells showed progressive growth arrest after 38.4 population doublings.

The MSCP1 with a positive finding in karyotyping were applied to the patient because results from karyotyping were available only after implantation. Noteworthy, the same patient was screened and included a second time for MAXILLO-1. Different individual patient IDs (1-04 and 1-11) were given for the two independent treatments on the left and right mandibular side. MSCP1 cells obtained in the second production process showed a normal female karyotype. No phenotypical abnormalities or changes in differentiation capacity were seen at any time point analyzed, neither for ID 1-04 nor for ID 1-11. In long-term culture, cells rather underwent senescent. This is in line with the clinical observations on patients who received the MAXILLO-1 MSCs and had an uneventful clinical course up to now (last follow-up May 2017) [48]. Overall,

laboratory analysis and clinical observations of patient 1-04 did not reveal evidence for unlimited proliferation of the clonal population with a cytogenetic marker.

The presence of cells with atypical findings in karyotyping has previously been described for MSCs in the context of clinical trials to prevent acute graft-versus-host disease or to treat irradiation-induced lesions [60]. In five of 20 (i.e., 25%) cases, chromosomal abnormalities occurred and 7–75% of the cells were affected by aneuploidies, independent from the culture protocol. These frequencies are in accordance with our observations. In all cases with chromosomal changes reported by Tarte *et al.* [60], human telomerase reverse transcriptase (hTERT) activity was not detectable using quantitative PCR. A recent publication reports an increase of hTERT activity and a high spontaneous malignant transformation of BM-derived MSCs in long-term cultures [61]. Transformation of MSCs occurred in 45.8% of long-term cultures and MSCs showed loss of expression of typical MSC markers like CD73 and CD90, down-regulated expression of CD105 and limited differentiation potential. We did not observe these alterations in surface marker expression and differentiation capacity, even in the long-term cultures of MSCs from patient 1-04. This difference may also be caused by different culture conditions. Røsland *et al.* [61] cultured cells in medium supplemented with fetal calf serum. In our study, cells were grown in a xenogenic free system using human platelet lysate as a source for growth factors. This difference may contribute to suppression of transformation and favor senescence. Nevertheless, the significance of karyotyping as quality control parameter thus remains questionable. Quantitative analysis of hTERT may be the quality parameter of choice.

Conclusions

In this study, we have shown that there is no need of close proximity between manufacturing facility for MSCs and the clinical center where the cells are applied, even if cryopreservation has to be avoided and freshly produced cells have to be shipped on wet ice. We demonstrated that several parameters, like CFU-F, percentage of CD34+ cells, cell count of MNCs and WBCs of the BM, may serve as predictive tools for the yield of MSCs and thus may help to develop strategies to avoid unnecessary costs for production of MSCs due to insufficient cell expansion rates. Further investigations may be necessary to interpret the physiological and clinical impacts of the positive correlation between harvesting density of MSCs from early passages with low numbers of population doublings and the clonogenicity in the straight following passages.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2019.03.001.

III

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Cell therapy induced regeneration of severely atrophied mandibular bone in a clinical trial

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Abstract

Background: Autologous grafting, despite some disadvantages, is still considered the gold standard for reconstruction of maxillofacial bone defects. The aim of this study was to evaluate bone regeneration using bone marrow-derived mesenchymal stromal cells (MSCs) in a clinical trial, a less invasive approach than autologous bone grafting. This comprehensive clinical trial included subjects with severe mandibular ridge resorption.

Methods: The study included 11 subjects aged 52–79 years with severe mandibular ridge resorption. Bone marrow cells were aspirated from the posterior iliac crest and plastic adherent cells were expanded in culture medium containing human platelet lysate. The MSCs and biphasic calcium phosphate granules as scaffolds were inserted subperiosteally onto the resorbed alveolar ridge. After 4–6 months of healing, new bone formation was assessed clinically and radiographically, as were safety and feasibility. Bone at the implant site was biopsied for micro-computed topography and histological analyses and dental implants were placed in the newly regenerated bone. Functional outcomes and patient satisfaction were assessed after 12 months.

Results: The bone marrow cells, expanded in vitro and inserted into the defect together with biphasic calcium phosphate granules, induced significant new bone formation. The regenerated bone volume was adequate for dental implant installation. Healing was uneventful, without adverse events. The patients were satisfied with the esthetic and functional outcomes. No side effects were observed.

Conclusions: The results of this comprehensive clinical trial in human subjects confirm that MSCs can successfully induce significant formation of new bone, with no untoward sequelae. Hence, this novel augmentation procedure warrants further investigation and may form the basis of a valid treatment protocol, challenging the current gold standard.

Trial registration: EudraCT, 2012-003139-50. Registered on 21 August 2013. ClinicalTrials.gov, NCT 02751125. Registered on 26 April 2016.

Keywords: Bone tissue engineering, Biphasic calcium phosphate, Dental implants, Alveolar ridge augmentation, Mesenchymal stem cells, Bone regeneration

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Background

Bone is among the most frequently transplanted tissues, with about 2.2 million procedures annually worldwide [1]. In bone reconstruction procedures, surgeons harvest autologous bone from the patient and transplant this bone graft to the defect. This is currently regarded as the gold standard in bone regeneration, using the patient's own cells and growth factors and providing scaffolding for bone regeneration [2]. However, the procedure has several major disadvantages: harvesting requires a second surgical site and provides only limited bone stock; the two-stage procedure increases surgery time; and patients often suffer pain and nerve damage at the harvest site. Moreover, autologous bone has an unpredictable resorption rate [3–5]. These factors all increase treatment costs and patient discomfort.

In the maxillofacial region, reconstruction may be necessary to treat congenital malformations, severe facial trauma, or resection of tumors [6, 7]. Bone defects also occur in the maxilla and mandible, often after tooth loss, which results in atrophy of hard and soft alveolar tissue, and reduction of both horizontal and vertical dimensions [2]. In clinical practice, patients often present with severe alveolar ridge resorption, leaving insufficient bone volume for optimal installation of dental implants. Various surgical procedures have been developed to enlarge the alveolar crest [8].

Because of the disadvantages inherent in autologous grafting, alternative methods for bone regeneration have been proposed, including bone substitutes of animal, human, or synthetic origin [9–12]. There are, however, documented cases of infection associated with bone substitute materials. Furthermore, the risks of bacterial contamination and immune rejection of the graft must be considered [9]. While these procedures can be used to reconstruct small bone defects, they are less effective in larger defects [3, 12, 13]. Thus, there is currently an unmet clinical need for effective, safe interventions which do not expose the patient to the risk of donor site morbidity [14–18].

Multipotent stromal cells or mesenchymal stem cells (MSCs) are the cells most extensively investigated and applied [19–30]. These cells are nonhematopoietic and of mesodermal derivation, capable of self-renewal and multilineage differentiation (e.g., into osteoblasts, adipocytes, and chondrocytes). MSCs are found throughout the body and numerous extraction protocols have been established for different tissues (e.g., umbilical cord, adipose tissue, skeletal muscle, deciduous teeth, and other tissue) [20, 21, 23, 24, 31, 32]. For more than 40 years, bone marrow-derived stem cells have been the most frequent sources for cell therapy. These cells can be isolated from bone marrow and from bone chips (cortical or trabecular bone). If seeded onto or cultivated on calcium phosphate ceramic matrices in vitro, these cells can induce bone formation in vivo [14, 33–35]. For many years, biphasic calcium phosphate (BCP) has been used alone or in combination with autologous bone chips to reconstruct the floor of the maxillary sinus and to fill extraction sockets [36, 37].

Recent preclinical studies have shown that BCP ceramics consisting of 20% hydroxyapatite (HA) and 80% beta tricalcium phosphate (β-TCP) are appropriate matrices for MSC culture in vitro and bone formation in vivo [38, 39]. In the present clinical study, the maxillofacial region was selected as an appropriate site for evaluating the safety and feasibility of using MSCs and BCP as a new therapeutic approach to regenerate alveolar bone defects. There were several reasons for this selection. Firstly, repair of facial bone defects is a major clinical challenge [40]. Currently, therapeutic options for repairing large, critical-sized defects are limited to autografts, allografts, or transplanting vascularized bone and soft tissue from autologous secondary sites [40]. Secondly, while a functional dentition is part of the normal facial anatomy, loss of teeth initiates a process of continuous resorption of the alveolar ridge. This is accelerated by denture wear and often results in pronounced loss of bone volume and reduction in the strength of residual bone in the edentulous area. Thirdly, reconstruction of the severely atrophic mandible to restore oral function remains a difficult surgical and prosthetic challenge because of the minimal residual bony volume and the progressive nature of the resorption process [41–43]. Although only a small proportion of edentulous people need bone augmentation for implant installation, for the patients who do, the procedure is essential for restoration of oral function and treatment options are limited [10]. Finally, the implant installation procedure makes it ethically acceptable to biopsy the implant site to inspect the quality of newly formed bone.

The present clinical trial in humans introduced a novel bone augmentation protocol. The primary aim was to introduce and validate the protocol, which uses bone marrow-derived MSCs for the clinical trial and synthetic BCP in a standardized, minimally invasive surgical procedure, and to assess the feasibility, safety, and efficacy of this new procedure. The autologous cells were harvested and cultured for 3 weeks before being implanted into the defect sites. The secondary outcome was to install dental implants in the augmented alveolar bone and screw-retain a fixed partial denture on the implants.

Methods

Ethical approval

This study conforms with the Declaration of Helsinki, and was approved by the Norwegian ethical committee

(2013/1284/REK Vest, University of Bergen) and by the Norwegian Medicines Agency (13/12062-15; EudraCT 2012-003139-50). The clinical trial followed the European guidelines for advanced therapeutic medicinal products (ClinicalTrials.gov, NCT 02751125; https://clinicaltrials.gov/ct2/show/NCT02751125).

Two experienced clinicians informed the patients about the study. After signing the consent form, the patients underwent clinical examination, including clinical photographs and dental impressions, and provided a medical history. If the patient met the inclusion criteria, cone beam computer tomography (CBCT) (Morita 3D Accuitomo F17, Japan) and dental X-ray scans were taken.

Study design and participants

Thirteen patients were recruited for this clinical study at the Section of Oral and Maxillofacial Surgery, Department of Clinical Dentistry, University of Bergen, Norway. To be eligible, the patients had to be healthy nonsmokers, with blood tests showing no evidence of infectious diseases, aged between 18 and 80 years, missing one or more teeth in the mandibular posterior region, and have an alveolar ridge width in the edentulous area less than 4.5 mm. All participants provided written informed consent before any study-related intervention. The study design and time points for each intervention are presented in Table 1.

Inclusion criteria

- Patients presenting with a subjective indication for a fixed implant-retained prosthesis in the mandibular posterior region (i.e., distal to the canine).
- Extensive lateral bone loss of the edentulous alveolar ridge.
- Edentulous alveolar ridge width less than 4.5 mm.
- Edentulous for more than 6 months in the region requiring reconstruction.
- At least one missing tooth to be replaced in the edentulous area.
- Absence of clinical signs of infection in the region requiring reconstruction.
- Absence of any major oral pathology.
- Age 18 years and older.
- In good health.

Exclusion criteria

- Evidence of infection with HIV, or hepatitis B or C, or any contagious disease (specifically, serologically negative 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).
- Smoker.
- Pregnant or breastfeeding.
- Untreated infections.
- History of malignancy.

Table 1 Study design and time schedule for the intervention

Intervention	Day −21 (pre inclusion visit or earlier)	Day 0 (inclusion visit)	Days 12–14	Month 1	Month 6	Month 9	Month 18
Verification of the selection criteria, information given to the patient, patient records and informed consent obtained	X						
Panoramic X-ray scans	X				X	X	X
Loco-regional clinical examination	X	X	X	X	X	X	X
Impression of both dental arches	X					X	
Facial and oral cavity photographs	X						X
Dental radiographs	X				X	X	X
VAS score for pain			X	X	X	X	X
Questionnaire on use of painkillers			X	X	X	X	X
Bone marrow harvest	X						
Grafting procedure		X					
CBCT scan	X		X		X		X
Implant placement, bone biopsy					X		
Resonance frequency Analysis (ISQ RFA)					X	X	X
Implant loading (prosthesis)						X	
Adverse events, clinical examination		X	X	X	X	X	X

VAS visual analog scale, *CBCT* cone beam computer tomography, *ISQ* implant stability quotient, *RFA* resonance frequency analysis

- History of or scheduled cervico-facial radiation therapy.
- Chronic treatment with steroids, immunomodulatory drugs, or bisphosphonates.

Cell production

In 13 participants, bone marrow aspirates were harvested from the posterior iliac crest under local anesthesia at The Adult Clinical Trial Unit at Haukeland University Hospital, Bergen, Norway using a trocar to make two or three cutaneous punctures. Each bone marrow sample was harvested in fractions of 2–4 ml in 20-ml syringes prefilled with 1000 IU of heparin (Leo Pharma A/S, Denmark) and sealed with a Luer lock stopper (Omnifix 20 ml Luer Lock Solo; B. Braun Melsungen AG, Melsungen, Germany). A total of 15–20 ml of bone marrow aspirate from each patient was transported at 21 ± 3 °C with temperature recording and monitoring to provide traceability, and dispatched by a special courier service to the cell manufacturing center at the Institute for Clinical Transfusion Medicine and Immunogenetics (IKT), Ulm, Germany. This center has a production license for MSCs from BM aspirates (production license DE_BW_01_MIA_2013_0040/DE_BW_01_IKT Ulm), using Good Manufacturing Practices (GMP), according to defined standard operating procedures and in compliance with the established quality management system. The advanced therapy medicinal product MSCs were manufactured at IKT Ulm as previously described by Fekete et al. [44].

On arrival in Ulm, BM aspirates from the syringes were pooled and a cell count of the bone marrow was performed using an automated hematology analyzer (Sysmex KX-21 N; Sysmex Deutschland GmbH, Norderstedt, Germany) before any manipulation. Viability was evaluated by flow cytometry following 7-amino-actinomycin D staining (FC500 flow cytometer; Beckman Coulter, USA). If the total white blood cell (WBC) count was less than 127.2×10^6 cells, the sample was considered inadequate for processing. Viability of MSCs (passage 0 and passage 1) was evaluated by Trypan blue staining (Sigma, Taufkirchen, Germany). All manipulations were conducted under laminar hood flow in grade A clean room conditions. The expansion was carried out as previously described [44]. In brief, the cell expansion started with 15–20 ml of bone marrow aspirate; the cells were seeded on one to eight 2-chamber CellSTACKs (Corning/Fisher Scientific, Schwerte, Germany) at a density of 50,000 WBCs/cm^2 in Minimal Essential Medium alpha modification (αMEM) (Lonza, Basel, Switzerland), supplemented with 5% human platelet lysate (PL; IKT Ulm) and 1 IU/ml heparin (Ratiopharm, Ulm, Germany) for 14 days. The cells were then detached using trypsin (TrypZEAN; Lonza). The harvested passage 0 cells (MSC-P0) were counted and reseeded on one to seven 2-chamber CellSTACKs at a density of 4000 MSC-P0/cm^2 in αMEM supplemented with 8% human platelet lysate and 1 IU/ml heparin (Ratiopharm) for 7 days. The cells were detached and passage 1 MSCs were washed with phosphate buffered saline without Ca^{2+}/Mg^{2+} (Lonza), resuspended in a concentration of 20×10^6 MSCs/ml in clinical-grade physiological saline (Kochsalz 0.9% INJ.-FL.(injection fluid), 50 ml; B. Braun Melsungen AG) supplemented with 4–5% human serum albumin (CSL Behring, Munich, Germany). Doses of 5 ml were drawn into one or two sterile syringes sealed with a Luer lock stopper. Transport was undertaken by a certified shipping company (World Courier, Stuttgart, Germany) as an accompanied transport to the clinical unit at the Department of Oral and Maxillofacial Surgery, Institute of Clinical Dentistry, University of Bergen, within 24 h of production. Appropriate quality controls of the cell therapy product were conducted after each step of the culture procedure. Viability and the number of cells were conducted using a Trypan blue viability test and a Countess Automated Cell Counter (Countess™; Invitrogen, Life Technologies, USA) respectively. Details on manufacturing the MSCs including quality controls are presented in a separate manuscript (Rojewski et al., submitted).

Clinical procedures

All procedures were carried out under local anesthesia by an experienced surgeon (CG). CBCT scans were taken for each patient using a CBCT scanner (Morita 3D Accuitomo F17, Japan) to evaluate the bone volume before (T0) and 4–6 months after grafting (T1). One hour preoperatively, the patients received 1 g amoxicillin orally (or 300 mg clindamycin if allergic to penicillin). The site was surgically prepared under local anesthesia (Xylocain/adrenalin 2%; Astra Zenical AS, Sweden). A flap was raised and the cortical bone was then perforated with a small round burr, to enhance blood flow and facilitate vascular ingrowth into the biomaterials (Fig. 2A). Titanium-reinforced, nonresorbable polytetrafluoroethylene (PTFE) (Cytoplast; Osteogenics Biomedical, Lubbock, TX, USA) membranes were then fixed to the underlying bone by micro-screws and mini-screws (Biomet, Jacksonville, FL, USA) to provide a "tenting" effect [45–47].

For each patient, 5 cm^3 of BCP (MBCP+™; Biomatlante, France), comprising 20% HA and 80% β-TCP in the form of granules 0.5–1 mm in size and packed in two syringes, were used and mixed with 100 million MSCs at the time of surgery. During this step, MSCs attached to the BCP granules in the syringes within a contact time of 60 min. The final number of cells mixed with BCP was in a dose of 20×10^6 cells/1 cm^3 [39]. When the graft was ready to be inserted, the BCP granules loaded with MSCs were withdrawn from the syringe and immediately inserted into the implant site (Fig.2B). Part of the mixture was preserved for additional analyses, particularly

bacteriological tests and cell attachment on BCP. For cell attachment, the fluorescent dye DAPI (Sigma-Aldrich), which binds selectively to DNA and forms strongly fluorescent DNA–DAPI complexes, was used. The cell-seeded material was introduced into the pocket formed by the bony ridge and the regenerative membrane and then covered by the membrane and muco-periosteal flaps (Fig. 2C). Finally, the flaps were sutured to the vestibular mucosa using nonabsorbable sutures (4/0 Supramide; B. Braun Surgical SA, Spain).

The patients were instructed to eat only soft food for the next 10–14 days, and to rinse daily with chlorhexidine. The antibiotics were continued for 7 days. If necessary, pain was managed by oral administration of paracetamol (1 g tablets) or codeine phosphate sesquihydrate (30 mg) four times per day.

The operation site was examined clinically and the sutures were removed 12 days after surgery. CBCT scans were taken of the augmented area (T1). The patients were recalled for clinical examination after 1, 2, and 4 months (Fig. 2D). CBCT scans were taken 4–6 months postoperatively to determine whether the sites were ready for implant installation.

At the time of implant installation the augmented area was reentered if the width was 7 mm or more (Fig. 2E). Prior to implant installation, bone biopsies were taken under local anesthesia: new bone formation was assessed by histology and micro-computed tomography (μ-CT) (Skyscan 1172; Bruker) at 40 kV and 2.4-μm voxel size. Dental implants (Bone Level, Roxolid®, SLActive®; Institut Straumann AG, Basel, Switzerland) with a diameter of 4.1 mm and a length of 8–10 mm were then installed according to the manufacturer's recommendations (Fig. 2F). Abutment surgery was done 2 months after implant installation (Fig. 2G) and a screw-retained crown was mounted 2–4 weeks later (Fig. 2H). The implant stability quotient (ISQ) was measured at each of these procedures using an Ostell® device (Ostell AB, Gothenburg, Sweden).

Bone volume measurements and CBCT analyses

CBCT scans (Morita 3D Accuitomo F17, Japan) were taken before grafting (T0) and 6 months after grafting (T1), at 85 kVp, 9.5 mA with a field of view (FOV) of 6 cm × 6 cm (diameter × height), scanning time of 17.5 s, and a voxel size of 0.125 mm.

Reconstruction of 3-dimensional models

The DICOM files of the images were then imported to Mimics program 19.0 (Materialize NV, Leuven, Belgium). The threshold of each case was selected manually, based on subjective evaluation of the apparent display of the residual jaw bone and the graft, this defined the boundary of the region of interest (ROI) of each case. The mask of the ROI at T0 was achieved and visualized in axial, sagittal, and coronal views. The 2D masks were then transformed into 3D models using the so-called "calculate 3D" function. The volume in cubic millimeters of the graft models was acquired automatically with a display of a color-coded 3D model.

The superimposition of the images at T0 and T1 was applied to the Standard Tessellation Language (STL) registration method [48]. Once the models were optimally superimposed, 3D models were reconstructed from the same region in the T0 and T1 images, specifying the augmented bone volumes (ROI).

Processing bone biopsies

Micro-computed topography analyses

The bone biopsy specimens were maintained in 10% buffered formalin. Selected bone biopsies were scanned with the high-resolution μ-CT SkyScan1172® (SkyScan, Kontich, Belgium) with the following technical parameters: 100 mA and 100 kV power intensity, copper–alumina filter and 360° rotation, and pixel size or resolution for acquisition and image reconstruction of 2.7 μm. Images from the scanning of biopsies were reconstructed by the software NRecon® (SkyScan) to obtain 2D and 3D images. CTvox (version 3.2; SkyScan) was employed to create 3D images for the biopsies. The analyzed histomorphometric parameters have been described previously [49]: bone volume (BV); tissue volume (TV); bone volumetric fraction (BV/TV); trabecular thickness (Tb.Th), the mean thickness of the trabeculae in the volume of interest (VOI); trabecular separation (Tb.Sp), the mean separation of the trabeculae in the VOI; structural model index (SMI), which gives information about the preponderance of trabecular morphology; degree of anisotropy (DA), which is the presence or absence of aligned trabeculae in a particular direction (1 is considered isotropic, >1 is considered anisotropic); and fractal dimension (FD), which indicates the complexity of the specimen surface.

Histological analyses

Fixed samples were decalcified in a pH 7.4 solution containing 4.13% EDTA/0.2% PFA in PBS for 96 h at 50 °C, using an automated microwave decalcifying apparatus (KOS Histostation; Milestone Med. Corp., USA). Samples were dehydrated in an ascending series of ethanol followed by butanol in an automated dehydration station (MicromMicrotech, Lyon, France). The samples were embedded in paraffin (Histowax; Histolab, Gothenburg, Sweden). Thin histological sections (3 μm thick) were made using a standard microtome (Leica RM2255; Leica Biosystems, Nanterre, France). The sections were stained by the Masson trichrome technique, which colors cell nuclei blue/black with hematoxylin, colors cytoplasm, muscle, and erythrocytes red using fuchsine, and colors collagen green using light green solution.

Slides were scanned (NanoZoomer; Hamamatsu, Photonics, Hamamatsu City, Shizuoka, Japan) and observed virtually (NDP view; Hamamatsu). Histomorphometry of images was performed using ImageJ and the percentages of bone and bone marrow were calculated per area of explants. Four sections through each biopsy were analyzed and quantified.

Statistical analysis

Bone width and volume are presented as means and confidence intervals. Confidence intervals were based on formulas assuming normal distributed data. The p value was calculated from a one-sample t test, with 0 as the hypothesized difference. $p < 0.05$ was considered statistically significant.

Outcomes

The primary outcomes of the trial were safety and feasibility of the procedure, assessed 12 months after reconstruction. In order to evaluate safety, a system was established for reporting adverse events. With guidance from the European Medicines Agency, these events were further classified into serious adverse events or serious adverse reactions. Adverse events, local (e.g., infection or hematomas) or systemic (e.g., fever or allergic reaction), were to be managed according to the Guidelines for Good Clinical Practice from the International Conference on Harmonization and the German Verordnung über klinische Versuche mit Heilmitteln. The feasibility of the procedure was evaluated on the basis of two factors: surgical manipulation of the graft and the ability to install the implants as planned.

Secondary outcomes were osseointegration of the dental implant and function of the prosthetic restoration.

Results

The final cell product consisted of fresh autologous cells (MSCs) expanded in vitro expressing the markers CD90, CD73, and CD105 and negative for CD14 and CD45, with a 90% viability rate. The product also showed strong expression of markers CD49d, CD73, CD90, and CD105; moderate expression of CD14 and CD106; and low expression of CD19, CD34, and CD45.

The viability of the cells on arrival in the operating theater was 87–90% as demonstrated using Trypan blue assay and cell counting. The mixing was undertaken in theater by the surgeon, under aseptic surgical conditions (Fig. 1A, B). Cells were mixed and attached well to the BCP granules within 60 min (Fig. 1C, D).

Between June 2014 and December 2015, 13 patients aged 52–75 years (mean 65 years) were enrolled. For 11 of the 13 patients the expansions fulfilled the release criteria and cells could be delivered to the Department of Oral and Maxillofacial Surgery in Bergen. Two expansions were stopped at passage 0 because there were insufficient bone marrow cells in the starting material for expansion (Patients 5 and 10, Table 2).

All 11 patients had uneventful healing of the augmented area, without any local infection.

No adverse events occurred during the trial period. Moreover, the soft tissues covering the augmented bone showed an increased area of keratinized gingiva, providing a healthy soft tissue profile (Fig. 2d). Finally, the amount of new bone was strongly influenced by the position of the membrane.

All 11 patients had successful ridge augmentation and an adequate amount of bone for dental implant installation (Table 3). In five patients the PTFE membrane

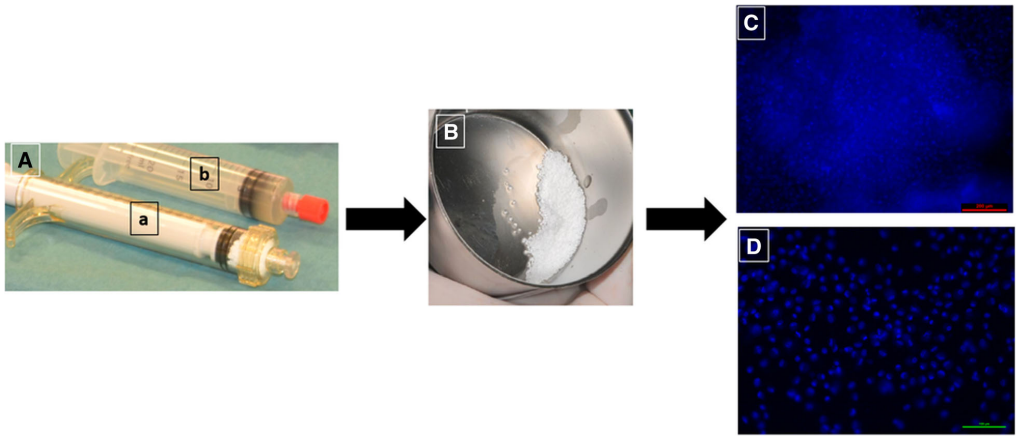

Fig. 1 Cell attachment assay. **A** Syringes containing BCP granules (a) and MSCs (b). **B** Mixture of BCP and MSCs. **C**, **D** Cell attachment to biomaterial determined using DAPI staining after arrival at operating theater

Table 2 Expansion of cells derived from bone marrow of 13 patients

Patient number	BMSCs/μl BM number of MNCs	BMSCs/μl BM aspirate in passage 1	Overall harvest after culture passage 1
1	3.46E + 03	2.98E + 04	3.06E + 08
2	1.13E + 04	1.52E + 05	4.12E + 08
3	3.59E + 03	2.89E + 04	2.46E + 08
4	1.83E + 04	2.44E + 05	4.05E + 08
5	5.74E + 01	-[a]	-[a]
6	4.77E + 03	6.27E + 04	4.02E + 08
7	5.03E + 02	5.27E + 03	5.33E + 07
8	1.61E + 03	2.26E + 04	2.86E + 08
9	1,64E + 03	1.67E + 04	1.55E + 08
10	-[b]	-[a]	-[a]
11	6.54E + 03	7.67E + 04	2.42E + 08
12	2.70E + 03	2.85E + 04	2.69E + 08
13	3.63E + 03	2.79E + 04	2.34E + 08
Mean	4.84E + 03	6.32E + 04	2.74E + 08
SD	4.98E + 03	6.91E + 04	1.04E + 08

BMSC bone marrow-derived mesenchymal stromal cell, *BM* bone marrow, *MNC* mononuclear cell, *SD* standard deviation
[a]No colony-forming unit fibroblast CFU-F growth
[b]Insufficient cell count

became exposed and was removed uneventfully 7–8 weeks post augmentation.

Casts of the alveolar ridge in each patient, X-ray scans, and clinical examinations demonstrated a significant increase of the total bone volume in all 11 patients after treatment (Fig. 3a, b).

Linear measurements of the width and height were performed from all CBCT scans in iView software (version 2.2.0.3. J; Morita MFG Corporation). Grafted bone could easily be distinguished from residual bone by density and structure on the scans taken immediately after the grafting procedure. As these measurements are known to be operator dependent, the measurements were all done by one specialist in oral radiology (SS) [50, 51]. All patients had sufficient increase in alveolar width to have dental implants installed (Fig. 4 and Table 3). The average volume of bone increased by 887.23 ± 365.01 mm^3 (Table 3). Both the increase in width of the alveolar ridge and the increase in volume of the alveolar ridge were statistically significant. The mean increase in bone width ($n = 14$) was 4.05 mm (95% CI 2.74, 5.36; $p < 0.001$) and the mean increase in volume ($n = 14$) was 887.23 mm^3 (95% CI 676, 1097.98; $p < 0.001$).

Formation of mineralized tissues was evaluated by μ-CT and histology from the biopsies taken during implant installation. From the μ-CT scan datasets, 3D models were built for visualization (Fig. 5A). It was possible to identify

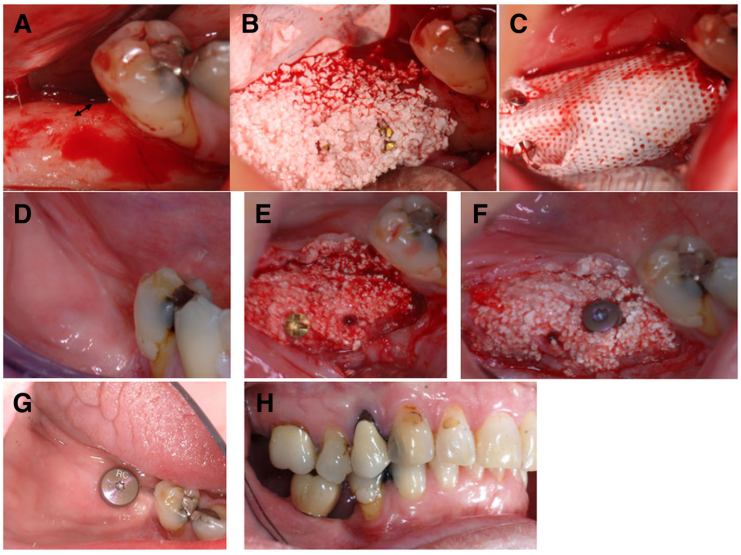

Fig. 2 Clinical procedure. **a** Narrow alveolar ridge before augmentation (arrow). **b** Mixture of BCP and MSCs placed on alveolar ridge. **c** Membrane placed over transplanted graft. **d** Soft tissue healing after 5 months. **e** New alveolar ridge after 5 months of healing. **f** Core biopsy taken and dental implant installed on newly formed bone. **g** Eight months post augmentation and 2 months after implant installation. **h** Implant-supported crown in occlusion

Table 3 Clinical outcomes: demonstrates bone healing, increased bone width and volume

Patient number	Age (years)	Sex	Healing time (weeks)	Increase in width (mm)	Increase in volume (mm^3)	Implant placement	Crown delivered	Patient satisfied
1	75	F	27	4.5	902.92	Yes	Yes	Yes
2	67	M	25	3.7	1047.15	Yes	Yes	Yes
3	55	F	26	3.9	1382.54	Yes	Yes	Yes
4	62	F	18	1.1	440.93	Yes	Yes	Yes
6	52	M	21	4.9	1469.53	Yes	Yes	Yes
7 left	69	M	31	4.6	432.7	Yes	Yes	Yes
7 right	69	M	31	4.9	1187.21	Yes	Yes	Yes
8	69	M	22	1.4	753.52	Yes	Yes	Yes
9	61	F	22	1.4	546.33	Yes	Yes	Yes
11	62	F	21	9.7	1188.47	Yes	Yes	Yes
12 left	65	F	20	2.7	954.98	Yes	Yes	Yes
12 right	65	F	20	3.4	418.36	Yes	Yes	Yes
13 left	69	F	22	3.7	553.56	Yes	Yes	Yes
13 right	69	F	22	6.8	1142.96	Yes	Yes	Yes

All patients received implants and prostheses
F female, *M* male

accurately the newly formed bone from the BCP granules (based on histogram calculations) when the raw data-reconstructed cross-sections were turned into images.

Histological analysis revealed that BCP granules were well integrated with deposition of newly formed bone tissue on the surface of the particles with osteoblast lining cells and subsequent deposition of lamellar bone tissue (Fig. 5B). The BCP granules demonstrated continuous degradation and dissolution, with the presence of multinucleated cells, probably osteoclasts, as well as macrophage $CD68^+$ cells on the surface of the particles.

Table 4 presents the mean values for each analyzed variable obtained by μ-CT analyses in relation to the microstructural properties of the biopsies.

All patients were satisfied with the esthetic and functional outcomes and no adverse events were reported or observed. There were no postoperative infections in any of the transplants or at the donor site. One patient reported moderate levels of pain after augmentation and after the exposed membrane had to be removed. The other patients reported only minor pain postoperatively. All patients were satisfied with the clinical outcome of the augmentation procedure and with their new teeth. All patients said they would recommend this procedure to others with a similar clinical condition. Ostell values increased for all patients during the first 12 months after installation of the dental implants (Fig. 6).

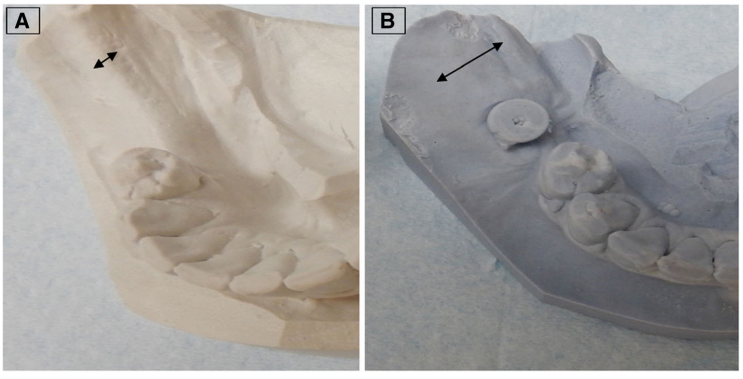

Fig. 3 Cast of alveolar ridge. Before (**a**) and after (**b**) augmentation illustrating amount of bone reconstructed. Arrows indicate the width of the alveolar ridge

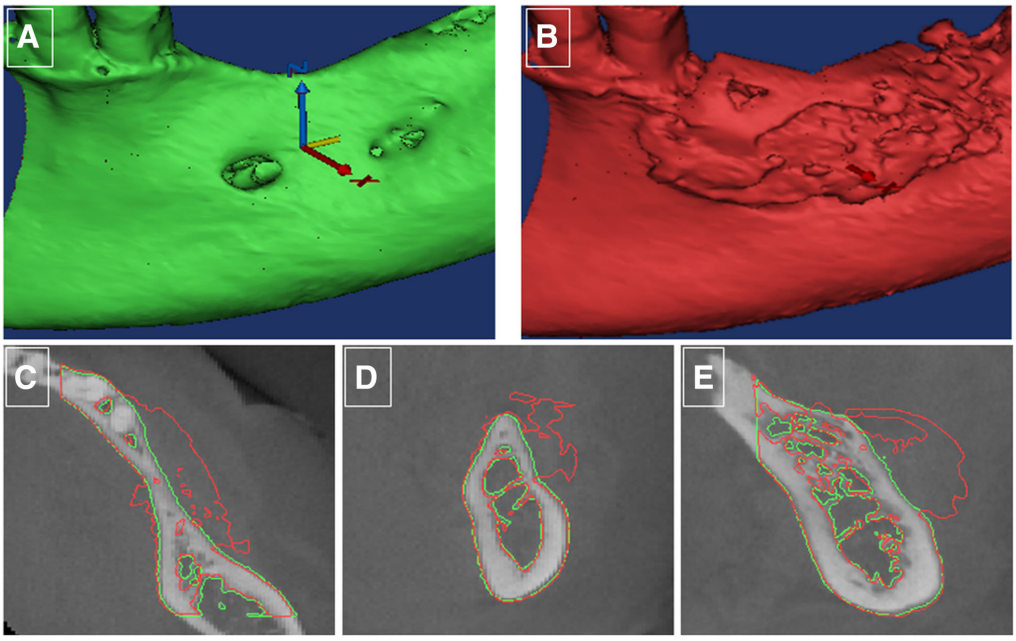

Fig. 4 CBCT measurements. Overlapping of bone outline contours of superimposed models at T0 (before grafting, green) (**a**) and T1 (6 months after grafting, red) (**b**), achieved and viewed in axial (**c**), sagittal (**d**), and coronal (**e**) images of ridge before and after reconstruction

Discussion

Successful augmentation of alveolar bone was observed in all study participants in this clinical trial of a novel protocol using bone marrow-derived MSCs. The site selected for bone augmentation was the posterior mandibular ridge. This is one of the most challenging sites for reconstruction, because of the relatively limited blood supply [52, 53], nonsterile environment [54], and oral functions such as chewing, speaking, and swallowing, which interfere with the stability of the graft. Despite these obstacles and the use of granules as scaffolding, we succeeded in inducing the formation of significant new bone and increasing the volume of the alveolar ridge.

Horizontal bone augmentation of the alveolar ridge is considered to be predictable, whereas vertical augmentation is not [55, 56]. Major drawbacks in relation to the bone graft treatment are donor side morbidity, limited amount of bone to be harvested, and unpredictable resorption of the graft [7, 57–62]. Using the stem cell/biomaterial approach in the present trial promoted both horizontal and vertical augmentation [56]. The donor site morbidity reported by the patients was minimal. The novelty of this approach was related to the development of an appropriate protocol to produce clinical-grade cells that could be used successfully for bone regeneration. The MSCs were expanded using no osteogenic factors, and no osteogenic factors were used in the clinical procedure [63–65], as growth factors may have different effects on different tissue [66] and also increase the cost of producing the cells.

In preclinical studies, MSCs were expanded and produced by the manufacturing center according to the protocol used in this clinical trial. Cells were shipped within 24 h and applied fresh in different animal models to demonstrate the formation of new bone in combination with the BCP biomaterial [39, 67]: the biomaterial alone fails to bridge bone defects in critical size calvarial defects in nude mice while full bridging was achieved with MSC/BCP combinations [39]. However, formation of bone seems to be dependent on a critical number of cells or a critical cell-to-biomaterial ratio. The number of cells and the cell-to-biomaterial BCP ratio used in this clinical study were adapted from the preclinical findings, where 20×10^6 MSCs were mixed with 1 cm^3 BCP [39]. We believe that the intrinsic capacity of MSCs to form bone makes the trial reproducible and safer, because the cells were not manipulated. However, a positive effect on osteogenic "predifferentiation" of MSCs using PL as a supplement during the isolation and expansion phases

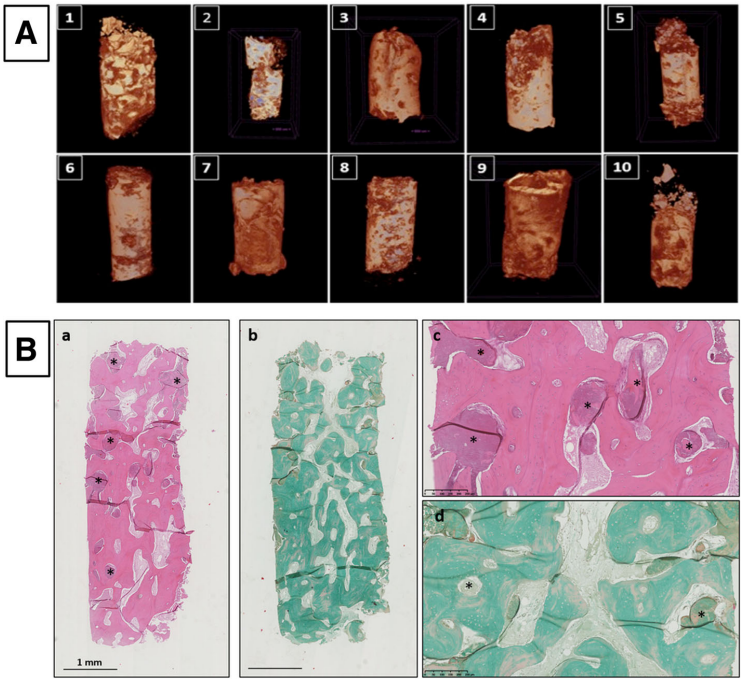

Fig. 5 μ-CT and histological analyses. **A** μ-CT images of biopsies from Patients 1–10. **B** Histology of core biopsies from patients. Note abundant lamellar bone with entrapped osteocytes in extracellular matrix at high magnification around remaining BCP particles (*). a, c Hematoxylin and eosin staining, b, d Masson trichrome staining. Magnification ×1.25 and ×10

cannot be excluded, although this has been a somewhat controversial topic [68, 69]. In this clinical trial, PL was produced from up to 80 individual donors: as shown in a recent study, this minimizes variations in the content of growth factors, chemokines, and cytokines [44] and ensures stable conditions for the ex-vivo expansion of MSCs.

Two of the patients had insufficient cell expansion in vitro, perhaps due to the variable content of MSCs (CFU-F) in bone marrow aspirates from different

Table 4 Mean values for each analyzed variable in relation to microstructural properties of the biopsies

Patient	TV (mm^3)	BV (mm^3)	BV/TV (%)	Th.Tb (mm)	Tb.Sp (mm)	SMI	DA	FD
1	5.187	1.2	23.131	0.023	0.131	0.542	1.153	2.63
2	5.436	0.961	17.677	0.046	0.251	0.277	1.29	2.485
3	4.717	0.495	10.501	0.004	0.359	0.742	1.367	2.256
4	5.333	0.963	18.055	0.039	0.288	0.354	1.256	2.467
5	5.358	0.791	14.762	0.033	0.279	0.529	1.107	2.422
7	4.933	0.741	15.022	0.031	0.239	0.215	1.410	2.46
8	5.546	0.881	15.891	0.045	0.255	0.812	1.549	2.46
9	4.413	0.568	12.867	0.032	0.25	0.437	1.333	2.390
11	5.064	1.106	21.844	0.051	0.180	0.740	1.144	2.542
12	5.488	0.567	10.317	0.037	0.246	0.609	1.333	2.433

In Patient 13, the biopsy disintegrated during transport and could not be measured. However, all dental implants have osseointegrated and are still in successful clinical function

TV tissue volume, *BV* bone volume, *BV/TV* bone volumetric fraction, *Tb.Sp* trabecular separation, *Th.Tb* Trabecular thickness, *SMI* structural model index, *DA* degree of anisotropy, *FD* fractal dimension

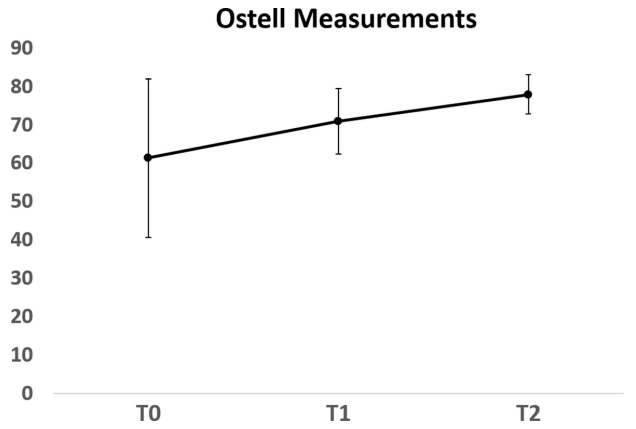

Fig. 6 Ostell measurements. Implant installation (T0), at loading (T1), and at 18 months follow-up (T2). Data presented as mean ± SD showing increased implant stability after loading

individuals [65]. This variability may be a limiting step in the procedure, but may be overcome by increasing the number of cells harvested or by developing methods for identifying the relevant cells prior to initiating culture.

There are few published papers on mandibular and maxillary defect reconstruction using bone marrow or adipose-derived stem cells [33, 70–77], many of which are case reports [70, 74, 75, 77]. The published studies vary in cell source, defect site, scaffold material, cell number, use of growth factors, and membrane or hardware [33, 70, 74–79]. However, the present data generated by treating 11 cases differ from these earlier reports as no growth factor or stimulants were used on the cells prior to implantation. Furthermore, the posterior mandibular region (i.e., distal to the canine) in all patients was selected as an inclusion criterion, as the bone healing is dependent on the location of the defected bone. Although the membrane was the determinant of augmentation volume, it complicated the surgical procedure and postoperative healing procedure. The high-density membrane is microporous, impervious to bacteria while still allowing diffusion of gases and small molecules, but probably inhibits vascularization from the periosteum, limiting the blood supply to the graft. The granules that remained outside the compartment made by the membrane did not induce bone formation, indicating the importance of using an appropriate membrane. Further supporting the importance of the membrane in bone formation, a study by Meijer et al. [76] using no membrane and grafts of bone marrow MSCs grown for 7 days in osteogenic medium and loaded with ceramic bone substitutes did not succeed in inducing bone formation.

In a randomized, controlled trial reported recently, osseous defects generated after tooth extraction were treated successfully with bone marrow-derived cells loaded on gelatin sponge. They showed accelerated healing after 6 weeks, but no significant difference after 12 weeks compared to no cells applied to the defect [33]. However, it is well known that extraction sockets heal without intervention [58, 80].

In the present study, the volumetric measurement on CBCT images was a visual protocol for assessing the outcome of grafting. The volumetric changes to the bone were achieved at T0 and T1. The objective measurement on CBCT images was performed to confirm the clinically observed volumetric changes in the graft [81–83]. This methodology has also been used in follow-up after grafting procedures in alveolar cleft patients [84–86]. Further, the biopsy specimens taken 4–6 months after augmentation showed significant new bone formation, with abundant blood supply and without inflammatory cells. The BCP scaffold was still visible in the histological samples as the reported resorption time is up to 2 years [38]. The scaffold material provides the extracellular microenvironment for support and stimulation of the cells, and also acts as the delivery system for the cells [18]. Although no direct evidence is provided relative to the source of the cells that produced the regenerated tissue (i.e., labeling of the cells), the assumption can be made that the transplanted cells at least partly contributed to bone regeneration, because the bone core specimen was taken from the central region of the defect and graft site.

Normally, there is a gradual resorption of keratinized mucosa simultaneously with bone resorption and this resorbed keratinized mucosa is known to not regenerate [87, 88]. The presence of keratinized mucosa of at least 1–2 mm around an implant is beneficial in decreasing

plaque accumulation, tissue inflammation, and attachment loss [87, 89]. In our patients, an unexpected benefit of the augmentation procedure was an increase in the width of keratinized mucosa (Fig. 2d, g). It therefore appears that the cells used to regenerate bone also have a positive effect on neighboring soft tissues and contribute to wound healing, even when covered by a membrane. MSCs have demonstrated a beneficial effect on wound healing [90, 91]. This observation warrants further investigation. However, MSCs have demonstrated a beneficial effect on wound healing, which appears to be mediated by paracrine signaling [91]. The role of paracrine factors produced by stem cells in tissue regeneration and healing has been investigated and reports showed that angiogenesis and osteogenesis were promoted in response to the paracrine effect of stem cells [65, 90]. This paracrine effect is exerted through cytokines and chemokines such as insulin-like growth factor (IGF)-1, vascular endothelial growth factor (VEGF), and transforming growth factor (TGF)-β1. These growth factors were found to enhance cell proliferation, mobilization, angiogenesis, and expression of osteogenic markers such as alkaline phosphatase, collagen type I, and Runx2 genes [92]. Furthermore, these factors recruit endogenous stem cells to the grafted site [90, 92].

Because of the small cohort and follow-up time (now up to 3 years), the promising results of this study should be interpreted with caution. In order to validate this treatment protocol for application in a standard clinical setting, further study is warranted, with a larger study cohort and a longer follow-up period. Nevertheless, the results of this study are promising and could lead to the development of new strategies for regenerative medicine and therapeutic interventions, and thus have a direct and positive impact on large groups of patients.

Conclusions

The results of this novel clinical study in human subjects show that clinical reconstruction of the alveolar ridge using autologous MSCs and BCP is feasible, safe, and predictable. All sites were successfully augmented; all dental implants osseointegrated and were restored with screw-retained dental crowns as planned. Hence, this novel augmentation procedure warrants further investigation and may form the basis of a valid treatment protocol, challenging the current gold standard.

Abbreviations

BCP: Biphasic calcium phosphate; BM: Bone marrow; BV: Bone volume; BV/TV: Bone volumetric fraction; CBCT: Cone beam computer tomography; μ-CT: Micro-computed tomography; DA: Degree of anisotropy; FD: Fractal dimension; FOV: Field of view; HA: Hydroxyapatite; IGF-1: Insulin-like growth factor; ISQ: Implant stability quotient; αMEM: Minimal Essential Medium alpha modification; MSC: Mesenchymal stromal cell; PL: Platelet lysate; PTFE: Polytetrafluoroethylene; ROI: Region of interest; SMI: Structural model index; STL: Standard Tessellation Language; Tb.Sp: Trabecular separation; Tb.Th: Trabecular thickness; β-TCP: Beta tricalcium phosphate; TGF: Transforming growth factor; TV: Tissue volume; VEGF: Vascular endothelial growth factor; VOI: Mean thickness of the trabeculae in the volume of interest; WBC: White blood cell

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CG contributed to conception and design, data interpretation, patient screening, surgical treatment of the patients, manuscript writing, and final approval and was a principal investigator. KM contributed to conception and design, data interpretation, manuscript writing, final approval, and project technical and strategic management and was a principal investigator. SH contributed to conception and design, manuscript writing, administrative support, and data interpretation and was leader of the maxillo work package. MR contributed to cell production, data generation, and manuscript writing. HG contributed to conception and design, data interpretation, patient screening, and prosthetic treatment of patients. MAY contributed to μ-CT data generation, analysis, and interpretation. XF contributed to CBCT analysis. SS contributed to data interpretation and patient screening. X-QS contributed to CBCT data interpretation. TB and AR contributed to conception and design, and
manuscript writing. ABA and BTG contributed to conception and design, bone marrow acquisition, and administrative support. HS contributed to conception and design, administrative support, and data interpretation and was leader of the cell production work package. PL contributed to conception and design, data interpretation, and final approval and was coordinator of the REBORNE project with technical and strategic management, financial and budgetary management, compilation, elaboration, and communication of the official reports to the European Commission, and internal communication. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study conforms with the Declaration of Helsinki, and was approved by the Norwegian ethical committee (2013/1284/REK Vest, University of Bergen) and by the Norwegian Medicines Agency (13/12062-15; EudraCT 2012-003139-50). The clinical trial followed the European guidelines for advanced therapeutic medicinal products (ClinicalTrials.gov, NCT 02751125, https://clinicaltrials.gov/ct2/show/NCT02751125).
All patients consented to participate in the clinical trial and to publish the data.

Consent for publication

All authors consented to publication of this manuscript.

Competing interests

The authors declare that they have no competing interests.

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