Complementing surgical with biomedical and engineering methods to evolve lip and nose reconstruction

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Medizinischen Fakultät

der Universität Basel

von

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aus Basel (Basel-Stadt), Oftringen (Aargau), Schweiz

Basel, 2013

Genehmigt von der Medizinischen Fakultät

auf Antrag von

Prof. Dr. Dr. Dr.h.c. Hans-Florian Zeilhofer (Fakultätsverantwortlicher und Korreferent)

Prof. Dr. Ivan Martin (Dissertationsleiter)

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Basel, den 16. September 2013

Prof. Dr. Christoph Beglinger

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Contents

Abstract

Facial integrity and self-perception are intimately related to each other. A facial defect therefore affects a patient in his physical and psychosocial health as well. Every reconstructive method exhibit certain imperfections or burden for the patient, which motivates the surgeon's to strive for improvements. While many imperfections can be improved by refinements of the surgical techniques, some aspects might be not solvable by surgical principles alone. At that stage, biomedical and engineering methods should be considered to complement the surgical treatment for further improvements.

We developed and explored a variety of biomedical and engineering methods to overcome shortcomings of current lip-nose reconstruction techniques.

The unknown shape of a missing nose was computed from a morphable face model that comprise the facial shape information of 200 healthy individuals. It led to a more natural shape planning than by hand carving. A biocompatible facial prosthesis was then made out of polyamide using computer assisted design and manufacturing. Alternatively to facial prosthesis large facial defects can be covered by means of tissue transfer from a distant body site performing a microsurgical vascular anastomosis. In this area, the importance to develop and use monitoring devices and pharmaceutical drugs for anastomosis patency remained unclear. We assessed therefore the current practice for microsurgical head and neck tissue transfer in clinics of Germany, Switzerland and Austria. There was a high variability with equal success rate, technical monitoring devices and pharmaceutical drugs seemed to have a negligible effect on the success rate, while the surgical anastomosis having the main effect. To repair small naso-labial defects of inborn cleft lips, the use of the adjoining tissue is sufficient. However, since both lip parts contain a labial artery of normal thickness they could be as well unified by a microsurgical anastomosis, however its biological rational needed exploration. We measured the lip artery blood flow and nose-lip microcirculation in cleft lip patients before and after surgical repair and in normal using laser Doppler flowmetry and white light tissue spectrometry. We found no circulation deficit in cleft patients and therefore no need to strive for a surgical anastomosis. Nonetheless, since blood flow is a precondition for growth and development, visible vessels in the surgical field should be preserved best possible. We therefore studied the intraoperative vascular anatomy for constant vascular findings. A perforating artery of the Musculus transversus nasalis was identified at the nasal ala on the cleft side, which could be constrantly preserved after it became aware.

The aim to refine a surgical treatment should not exclusively focus on the surgical technique but need also consider the burden of the entire treatment plan. More than 95% of the European cleft surgery centers use 2 to 4 surgeries to close the cleft of the lip, alveolus, hard, and soft palate –considering that this optimizes growth of the cleft maxilla. But facing the burden of repetitive surgeries for patient and family, Dr. Honigmann introduced in Basel 1991 the cleft repair in one single operation at "one-stage". We were now able to assess the long-term growth effect of this procedure, which showed the same growth results as compared to multi-stage procedure. But when compared to normal, 20% to 45% of the cleft patients still showed a growth deficit that would require surgery to normalize the dental relation and facial profile. The orthognathic surgery rate in cleft patients from the literature ranges also widely from about 20% to 45%, whatever surgical technique and treatment plan is applied. It is therefore doubtful that by surgical means alone the growth deficit can be

avoided. This prompted us to assess the in-vitro and in-vivo osteogenic capacity of stem cells from the umbilical cord Wharton's jelly (WJMSC) under fully defined conditions allowing for clinical translation. Due to prenatal ultrasound the cleft lip malformation is frequently known before birth, and the umbilical cord could thus serve as an autologous stem cell donor site without any harvesting morbidity. Both, Osteogenically differentiated WJMSCs and WJ tissue biopsies produced a mineralized extracellular matrix. The expression of genes of osteoblastic lineage increased significantly after 3 weeks of osteodifferentiation. Although the WJMSCs formed in-vitro a dense collageneous matrix with signs of osteoblastic differentiation, no mature bone tissue was found after 8 weeks after subcutaneous implantation in immunoincompetent mice. Further in-vivo tests are therefore necessary applying more favorable bone forming conditions by using ostegenic predifferentiated cells and implantation into a bone defect.

In sum, biomedical and engineering methods have been applied to solve surgical problems or to establish new therapeutic strategies where conventional lip and nose reconstruction methods reach their limits. This has been demonstrated at different lip and nose reconstructive levels reaching from prosthetics, over microsurgery, to stem cell tissue engineering.

1. Introduction

When we look into someone's face, our gaze fixes on the eyes, lips and nose. These facial parts strongly define the facial identity of an individual. Therefore any defect in this facial region intimately affects the person's self-perception. The facial defect thus becomes a twofold burden for the patient a physical and psychosocial. This makes it crucial to reconstruct the natural form and function of the lip and nose region best possible, and to constantly strive for improvements in this field of reconstructive surgery.

Surgical clinic goes beyond technical aspects. Innovations for surgical treatments must ad substantial benefit to the entire process of surgical clinic, otherwise they do not successfully translate into clinical routine. The process of surgical clinics can be simplified as interactions between surgical technique, patient's biology and social factors. Ideally an innovation offers a well-balanced benefit between these three aspects. Obvious, that a technical advancement without biological or social benefit for the patient has minor importance. And in turn a specific treatment that appears technically or socially appealing should not compromise the biological outcome.

Surgical improvements rarely appear as radical new techniques. Rather it is an incremental process, in which operational steps are reduced, refined, or replaced. That means, the improvements commonly either reduces the surgical burden, refine the outcome, or replace a current procedure. We herein present surgical and biomedical studies that address specific challenges of lip-nose reconstruction, highlighting how they can reduce, refine or replace current procedures. Achievements and limitation of each study are outlined in the discussion section of the respective study, whereas the thesis discussion outlines how the studies are strategically linked striving for new regenerative surgical strategies.

Shape reconstruction of central facial defects

Facial defects arise secondary to trauma, ablative tumor surgery or inborn malformations. In blunt facial trauma, the facial soft tissue breaks open and can usually be completely reconstructed by meticulous suturing. In contrast, traumas after wide surgical resection, gunshots, burns or animal bites leave defects with a loss of tissue that need to be reconstructed by using the patient's own tissue or a facial prosthesis.

The most common use of facial prostheses is in the field of tumor surgery. Malignant tumors tend to recur. If a few cells are left behind after resection, they can re-grow and lead to a new tumor mass within months. Immediate defect coverage with the patient's own tissue may therefore hide tumor re-growth, especially in a complex anatomical area such as the nasal cavity. While the reconstruction of lateral facial defects can be guided by the healthy counterpart, this is not possible for midline or bilateral facial defects. In such cases the reconstruction of a natural-looking facial part and nose relies on the technician's artistic knowledge and taste.

We describe herein how to compute the three-dimensional shape of a missing facial part that is based on the shape characteristics of the remaining face. Furthermore, we have clinically tested the direct, computer-aided manufacture of the planned prosthesis.

Current standards of microsurgical tissue transfer to the face

Facial defects that are too large to be closed from the neighboring tissue require vascularized free tissue transfer. For this, tissue from a suitable body site is taken including its supplying blood vessels and after tissue transfer to the defect site the supplying vessels are connected to blood vessels at the defect site. The surgical training and techniques vary considerably among different maxillofacial surgical centers to perform such operations as well as the used monitoring techniques and pharmacotherapy. This situation prompted us first, to assess a survey over routinely applied microsurgical techniques in Swiss, German and Austrian maxillofacial centers. Although the survey addressed the topic of microsurgical tumor reconstructive surgery, the knowledge about microsurgical technique has an interest beyond its classical use for tumor defect reconstruction. Microsurgical techniques are optionally to be considered for reconstruction methods including vascularization of large tissue engineering constructs and reconstructing the abnormal vascular anatomy in cleft lip.

Introducing the functional vascular anatomy to cleft lip surgery

The most frequent facial birth defect is the cleft lip and palate malformation, which occurs, with varying degrees of severity, in about 1 in 700 newborn babies. This defect may involve only the lip or the palate, or both. It may separate the tissues completely or in parts, and involve one side of the face or both. However, in all types of cleft malformation, the main surgical challenge is to achieve best esthetics and function with a minimal number of surgeries and minimal surgical trauma.

Current surgical techniques for cleft lip repair differ mainly according to the skin incision design and the extent of muscle dissection. However, current techniques do not specifically address the course of blood vessels and the blood perfusion of the tissue. A striking fact, since blood supply is a precondition for growth and development due to it delivering oxygen and nutrients to the tissue. As such, microsurgical techniques that respect the vascular anatomy during cleft surgery offers an unexplored field to improve cleft lip surgery.

The vascular anatomy in the cleft lip is altered by the cleft. Normally a lip artery runs from both lip sides to the midline where they unify. This normal pattern is interrupted by the cleft. However, it is unknown if this leads to an alteration in the tissues blood supply. So far only structural anatomical studies exist about the blood vessels in clefts, however, no functional conclusions can be deduced from it. There are two principal strategies to influence the blood circulation in cleft lip surgery. Either to maximally preserve the main vessels during lip surgery or to surgically unify main arteries from both lip sides.

These options prompted us first to investigate the vascular blood circulation and tissue oxygenation before and after cleft repair surgery. This will show, whether current techniques lead to a normalization of blood supply or if an attempt to unify the lip arteries should be performed. Second, surgical techniques are identified to maximally preserve the microvascular anatomy during cleft repair.

Combined cleft lip and palate repair to reduce the burden of care

Disturbance of the upper jaw growth is a frequent problem after cleft lip and palate repair. It is commonly considered that repair in two to four separate surgical procedures can minimize this problem. However, multiple hospitalization and surgical event is a stressful time for the family, which they want to avoid if possible. With the objective to reduce this burden, a one-stage surgical technique was introduced in Basel 20 years ago, offering obvious social advantages.

We assess herein the long-term growth after one-stage repair of complete cleft lip and palatal defects. This clarifies whether the reduction of surgical and psychological burden by the one-stage procedure is justified or whether it compromises the biological outcome.

Umbilical cord stem cells to heal inborn bone defects

The alveolar bone is the teeth bearing bone of the upper jaw. This region plays a key role in growth of the upper jaw and its consecutive teeth relation to the lower jaw. Further, form and function of the upper lip is intimately related to the underlying alveolar bone. The cleft in the alveolar bone is commonly closed in a separate surgery in preteen age by autotransplantation of a pelvic bone graft into the cleft.

The pelvic bone graft has multiple shortcomings. First, there is a donor site morbidity. Second, pelvic bone and craniofacial have a different embryological identity and diverging biological behaviors are presumed. The pelvic bone is cartilage derived, whereas craniofacial bone forms directly from embryological connective tissue. Third, the bone graft must be delayed to preteenage, since earlier grafting leads to growth inhibition and graft resorption. Consequently, an autologous graft material, without donor site morbidity, that can be used early in life and which complies with craniofacial bone growth would have multiple benefits for the patient.

Umbilical cord stem cells showed in-vitro osteogenic differentiation. Since the clefts malformation is commonly diagnosed in the prenatal ultrasound, the umbilical cord could potentially serve for a stem cell based strategy to heal the bony cleft. The limitation however, is the growth of the cells under a fully defined and regulated protocol leading to mature bone formation in vivo.

We therefore developed a protocol to isolate, expand and cryopreserve Wharton's jelly mesenchymal stromal cells (WJMSCs) under fully defined conditions, devoid of animal-derived products. The fully defined cultured WJMSCs were included into hydroxyapatite granules-fibrin constructs and the bone formation ability was tested in vivo in immunoincompetent mice.

2. First study:

Missing facial parts computed by a morphable model and transferred directly to a polyamide laser sintered prosthesis: an innovation study

Study design: A. A. Mueller

Financing: NCCR Co-Me (Swiss National Science Foundation; National center of competence in research, Computer aided and image guided medical interventions). Phase 2.
Project N°12 Systems face computer aided treatment of facial disease
Sub Project 12/2 Registration and predictive modeling of human faces

Project Leaders: Prof. H.-F. Zeilhofer / Prof. T. Vetter

Publication: First authorship, British Journal of Oral and Maxillofacial Surgery, Impact Factor 2.717 (2012), Ranking 29/198 (Q1) Surgery

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Abstract

Mirroring of missing facial parts and rapid prototyping of prosthesis templates have become widely used techniques for prosthesis manufacture. However, mirroring is not applicable for central facial defects, and the manufacture of a template still requires labour-intensive transformation into the final facial prosthesis. We explored innovative techniques for these remaining challenges. We used a morphable face model for the reconstruction of non-mirrorable missing facial parts, and skin-coloured polyamide laser sintering for direct prosthesis manufacture. Based on the statistical knowledge gleaned from a data set of 200 coloured, three-dimensional (3D) scans a missing nose was generated that was statistically compatible with the remaining parts of the patient's face. The planned prosthesis was manufactured directly from biocompatible skin-coloured polyamide powder by selective laser sintering. The presented prosthesis-planning system produced a normal-looking shape reconstruction. The polyamide will need adjustable colouring and the ability to combine it with a self-curing resin to fulfil the requirements for realistic permanent use.

Introduction

The engagement and exploration of technology solutions has been identified as a key factor for the future of facial prosthetics.[1, 2] The computer-aided design and computer-aided manufacturing (CAD-CAM) technique is usually used to produce mirror-image templates from the non-affected side of the face for realistic remodeling of ear and orbital prostheses.[3-7] However, in this way CAD-CAM does not realize its full potential to transform prosthesis manufacture from an artistic process into a biomedical manufacturing process.[2] First, the problem remains of what should be regarded as normal or ideal, if the defect area has no healthy mirror image. Second, the labour intensive transfer of the CAD-CAM template into the final prosthesis remains. We therefore explored innovative solutions to these two challenges.

In the present study we used a morphable model as a planning innovation for facial prosthetics, which allows patient-specific shape reconstruction in cases where the mirroring technique is not applicable or undesirable. We also explored automatically defining the margin using distance mapping and the use of a skin-coloured medical-grade polyamide laser-sintering process as a manufacturing innovation for facial prosthetics.

Materials and Methods

The study was approved and found to meet the ethical standards of the declaration of Helsinki. The patient in this study was referred to us with recurrence of a sclerosing basal cell carcinoma of the nose. He underwent total rhinectomy, including resection of the anterior nasal floor, premaxilla and anterior wall of the right maxillary sinus.

To model the missing facial subunit, we developed a morphable model of human faces.[8] The morphable model was computed from a data set of 200 three-dimensional (3D) scans of male and female Caucasian faces. Dense point-to-point correspondences to a reference face were computed for the individual faces. This resulted in a single vector space of faces. Hence, the morphable model can generate continuous transitions between the faces and form linear combinations thereof.

The patient's face was scanned three-dimensionally (ABW, Frickenhausen, Germany). The face scan was automatically matched with the morphable face model using a non-rigid registration algorithm.[9] This produced a complete face that was congruent with the healthy parts of the patient's face.

The prosthesis margins were defined using an automated distance-mapping process. The facial defect borders protruded inward, while the generated nose shape protruded outward. The points of minimal distance form the margin line. The final prosthesis plan was superimposed onto a computed tomography (CT) scan of the patient. This allowed to check the bone quality at the planned implant positions and the leeway for precision attachments at the planned implant positions .

The planned prosthesis was directly manufactured on an EOSINT P machine (EOS, Munich, Germany) using CO_2-laser sintering of PA2200 polyamide powder, with a layer thickness of 0.1–0.15 mm. The prosthesis margins were adapted to the patient's facial movements by milling off areas of interference. Four magnetic precision attachments were polymerized into

recessed boreholes at the implant sites. The surface of the prosthesis was finalized using watercolours and a layer of varnish.

The prosthesis was scanned by a laser (VI-910, Konica Minolta, Munich, Germany) before and after the margin adaptation. Both scans were superimposed on the unchanged nasal shape and a false-colour area map was used to analyse the extent of necessary margin reduction.

Results

The algorithm of the morphable face model generated a nose reconstruction with a natural-looking shape (Fig. 1), which fitted harmoniously to the patient's face.

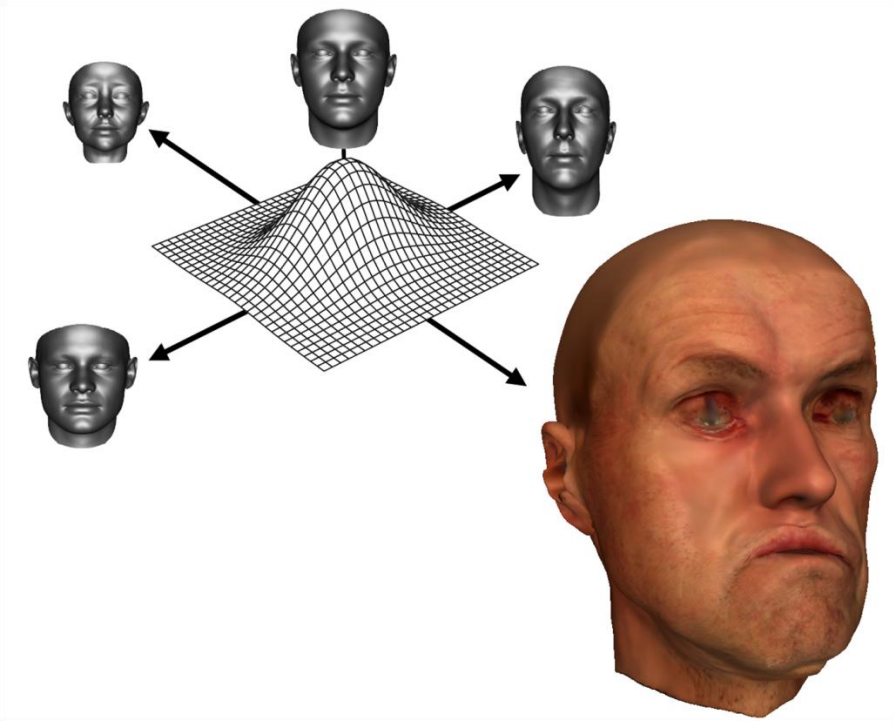

Fig. 1. The morphable model is a statistical model, computed from 200 three-dimensional (3D) face scans of young adults, which can form continuous transitions between the individual faces. The healthy part of the patient's 3D face scan is expressed in terms of this morphable face model, which consecutively provides a reconstruction for the missing facial part. The generated reconstruction is unique, statistically consistent with the patient's face and independent of the artistic skills of a prosthetist.

The polyamide laser sintering resulted in a nearly homogeneous surface which was polishable using standard millers and polishers. The intrinsic stain of the PA2200 polyamide

was darker than the patient's skin colour, which could not be corrected by a coat of paint (Fig. 2).

Fig. 2. Laser sintered prosthesis direct from the plan made of biocompatible, skin-coloured, PA2200 polyamide powder. The prosthesis margin, as defined by distance mapping, resulted in complete defect coverage with circumferential skin contact but it needs fine adjustment with a self-curing material to become inconspicuous. It was not possible to match the extrinsic staining to the patient's skin hue due to the intrinsic staining of the PA2200 polyamide, which was too dark and invariant. Published with the patient's consent.

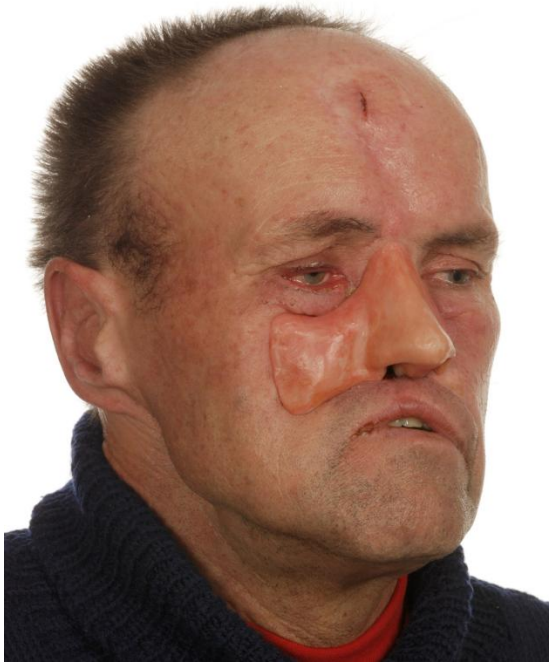

Fig. 3. Polymethylmethacrylate duplication of the laser sintered prosthesis with conventionally adapted margins. The morphable-model-based nasal reconstruction generated a natural-looking shape reconstruction of the nose that fits harmoniously to the patient's face. Published with the patient's consent.

The automatic margin definition achieved by distance mapping led to complete coverage of the defect area. Since polyamide cannot be added with self-curing resin the margin line was planned with a circumferential safety border of 2 mm. Adaptation of the prosthesis margins on the patient resulted in a maximal margin reduction of 3.9 mm. This was necessary at the nasal bridge, below the right lower eyelid and at the nostrils.

The transition between the margins and the skin could not be leveled out completely, since polyamide is not combinable with self-curing resin. Because of the shortcomings, a duplicate polymethylmethacrylate prosthesis was constructed, in which colour and margin adaptation was optimized while keeping the nasal shape unchanged (Fig. 3). The duplicate prosthesis served for permanent use.

Discussion

Morphable modeling

The face is the visible part of a person's unique identity. In central facial defects and after total rhinectomy there is no contralateral side available to act as a shape reference for reconstruction and the method of choice is often a prosthetic reconstruction [10,11] In these cases, the shape of the prosthesis lies in the hands of the maxillofacial prosthetist, whose artistic skill gives the patient back his or her facial identity. Morphable modeling can provide complete 3D shape information of a missing facial part based on the remaining facial characteristics of the patient. In the present case the shape of the generated nose was comparable to that observed in photographs of the patient's original state (Fig. 4).

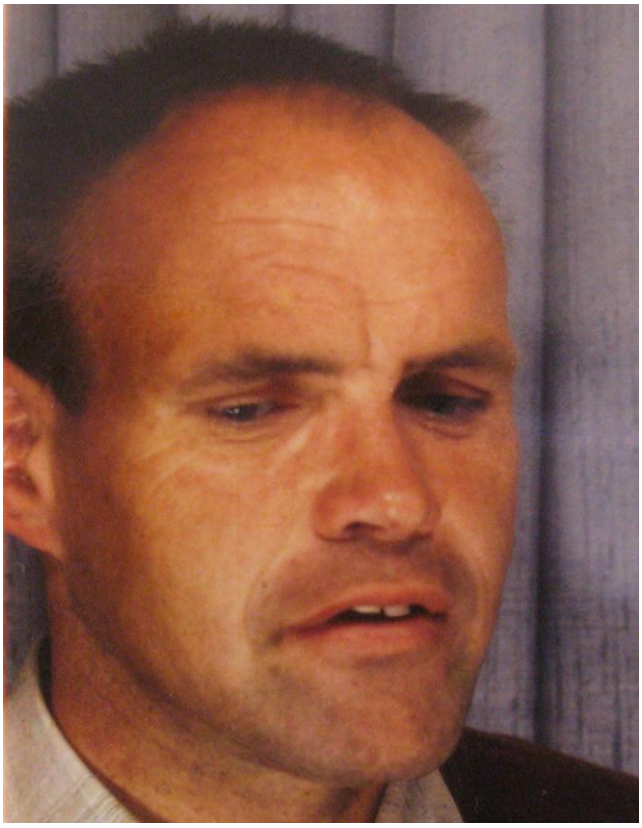

Fig. 4. The patient's original nasal shape, as seen on a old photograph. The automatically generated nose (Fig. 3), was slightly underprominent when compared to the original shape, which can be attributed to the young age of the reference faces upon which the morphable model was based. Published with the patient's consent.

Overall, the generated nose was slightly underprominent. One reason for this is that the morphable model is based on 200 facial scans of young adults, and the nose is known to become more prominent throughout life.

The proposed shape of the morphable model can be adjusted manually with respect to three facial attributes: male–female, bony–fleshy and facial distinctiveness (the degree to which

the face differs from the average face).[8] This allow fine adjustments according to the patient's facial characteristics and wishes. Age- and ethnicity-related facial attributes would also be helpful to complete the medical applicability of the morphable model.

Two-dimensional (2D) photographs of the patient's original state are traditionally used to guide the technician when shaping a wax model. Our morphable face model allows a 3D visualization to be produced from scanned 2D photographs of the patient's original state.[12] Currently such a visualization does not contain sufficient 3D shape information for transformation into a rapid-prototype model. However, it can be used for the fine adjustment of the proposed shape in the morphable model.

The combined data set of CT scan and planned prosthesis allows a simultaneous visualization of bone conditions, prosthesis thickness and soft-tissue thicknesses. This helps to define the optimal implant position and type of implant. If necessary, the marked implant position and axis can be used for intraoperative navigational support.

Simulation systems for craniofacial surgery have become an important tool for the clinician. The trend towards web-based applications, as seen in home computing, might also increasingly affect craniomaxillofacial applications.[13] The algorithm of the morphable model has been published, and open-source implementation is therefore technically feasible.

Laser sintering

A crucial step in the manufacture of a prosthesis is the correct colour and an inconspicuous transition to the skin. The application of CAD-CAM techniques allows both of these requirements to be achieved if manual adjustments are made on a wax template and silicone is used.[14] This might improve the results achieved by the maxillofacial prosthetist, but it does not substantially shorten the manufacturing process of a manual prosthesis.

A rapidly manufacturable material for direct prosthetic use would shorten the manufacturing process, and hence it would open new opportunities for facial prosthetics by reducing the time and effort of manual manufacturing, evolving facial prosthetics from an art into a field of biomedical engineering, attracting industry partners, and making facial prosthetics more widely available including in new applications such as temporary prostheses or prosthesis-dressing combinations.

Polyamide is availabe as a rapidly manufacturable, biocompatible and skin coloured material. PA2200 polyamide is a fine powder based on a polymer of lauryllactam ($C_{12}H_{23}NO$) and is tested for polonged skin contact according to International Organization for Standardization ISO 10993-1. After laser sintering the polyamide is a physically and chemically durable material with low density (0.9–0.95 g/cm^3), which render the material suitable for implant and glued fixation.

The direct manufacture of realistic facial prostheses requires two improvements to PA2200 polyamide: (1) the intrinsic staining must be adjustable to make it better match the skin colour and (2) a self-curing material must be combinable with PA2200 to level out the transition between the prosthesis and skin. The prosthesis margin, as defined by distance mapping, resulted in complete defect coverage with circumferential skin contact, but it needs fine adjustment with a self-curing material to become inconspicuous.

Material scientists could solve these shortcomings. Further, the development of a soft, rapidly manufacturable material is necessary, since a soft material usually fits better at the margins and conforms better with facial movements.

Conclusions

The morphable face model can reconstruct realistic facial parts in cases where mirroring is not applicable. The technique reduces the dependence on the artistic skills of a technician. The direct manufacture of a prosthesis from biocompatible skin-coloured PA2200 polyamide by selective laser sintering currently only fulfils the requirements for temporary use. Adjustable colouring and compatibility with a self-curing resin is necessary to reach the requirements for permanent use.

Acknowledgements

The authors thank the Swiss National Science Foundation for funding the “Systems Face: Computer Aided Treatment of Facial Disease” project, which is part of the National Centre of Competence in Research for Computer Aided and Image Guided Medical Interventions. The sponsor did not influence any step of the study or the publication of this report.

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3. Second study:

Microsurgical reconstruction of the head and neck – Current practice of maxillofacial units in Germany, Austria, and Switzerland

Study design: Dr. Dr. A. Mueller, Dr. T. Mücke, Prof. F. Hölzle

Financing: None

Project Leaders: Prof. F. Hölzle

Publication: First authorship, Journal of Cranio-Maxillo-Facial Surgery, Impact Factor 1.610 (2012), Ranking 80/198 (Q2) Surgery

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Abstract

Refinement in microvascular reconstructive techniques over the last 30 years has enabled an increasing number of patients to be rehabilitated for both functional and aesthetic reasons. The purpose of this study was to evaluate different microsurgical practice, including perioperative management, in Germany, Austria, and Switzerland.

The DÖSAK collaborative group for Microsurgical Reconstruction developed a detailed questionnaire which was circulated to units in the three countries. The current practice of the departments was evaluated.

Thirty-eight questionnaires were completed resulting in a 47.5% response rate. A considerable variation in the number of microsurgical reconstructions per year was noted. In relation to the timing of bony reconstruction, 10 hospitals did reconstructions primarily (26.3%), 19 secondarily (50%) and 9 (23.7%) hospitals used both concepts. In the postoperative course, 15.8% of hospitals use inhibitors of platelet aggregation, most hospitals use low molecular heparin (52.6%) or other heparin products (44.7%).

This survey shows variation in the performance, management, and care of microsurgical reconstructions of patients. This is due in part to the microvascular surgeons available in the unit but it is also due to different types of hospitals where various types of care can be performed in these patients needing special perioperative care

Introduction

Reconstructive surgery that allows free transfer of soft tissue and bone from all over the body following resection for head and cancer or after trauma is considered when there is a functional or aesthetic loss of structures. Depending on the site, size and complexity of the defect, the reconstruction may involve free tissue transfer of soft tissue, bone or a composite of both. The operation is complex and time-consuming and surgeons have to learn both the techniques of raising flaps of different types and microsurgery. In addition to the surgical requirements, other problems include the co-morbidities often seen in these patients, particularly heavy smoking, alcohol abuse and age-related conditions. These can affect intraoperative and postoperative management. High dependency care is usually required postoperatively and varying pharmacotherapeutic and intensive care regimens can influence flap success or failure. Although the mortality of this type of surgery is low, the physical and psychological impact on the patient is significant. These operations involve a large investment of resources, in both time and monetary terms. Despite these issues, there is limited clinical outcome data published relating to the current practice of reconstructive surgery and microsurgical practice. Where these exist it is usually based on departmental and personal practices (Kruse et al., 2010; Marsh et al., 2009; Spiegel and Polat, 2007). The purpose of this study was to evaluate current practice in reconstructive surgery and the different strategies including the perioperative management of microvascular cases in Germany, Austria, and Switzerland.

Material and Methods

Using the German, Austrian and Swiss Association of Oral and Maxillofacial Surgeons database of oral and maxillofacial hospital units, 82 separate units were identified. A questionnaire was developed by the DÖSAK collaborative group for Microsurgical Reconstruction to evaluate the following information: number of surgeons in the department, number of microsurgeons performing anastomoses regularly, number of patients receiving free microsurgical flaps per year, type of free flaps, and type of osteosynthesis for insertion of flaps containing bone. Information about the surgeons' opinion regarding the influence of perioperative treatment was assessed on a scale from 1 to 7 (1 = not important, 7 = most important). A second part of the questionnaire focused on the different perioperative stages of managing microsurgical patients as classified by Chen et al. (2006). The survey was performed by sending the questionnaire to the heads of each unit via Email and via mail in June 2009. Evaluation of questionnaires was performed after 6 months.

Data analysis

Descriptive statistics for quantitative variables are given as the mean standard deviation. If appropriate, medians and ranges were also computed. Statistical analysis was performed using JMP 7.0.1 (SAS Institute Inc., SAS Campus Drive, Cary, NC 27513, USA) Microsoft Office Excel (Microsoft Excel for Windows, release 2007, Microsoft Corporation, Redmond, WA, USA).

Results

In total, 38 questionnaires were completed adequately and 2 returned undelivered (47.5% response rate). Of these departments, 21 were University hospitals (55.3%), and 17 General hospitals (44.7%).

General microsurgical performance

The size of the department in the contributing hospitals varied from hospitals with 3 surgeons to one with 22, a mean of 12.7 ± 5.6 surgeons (median 12, range 3e22 surgeons). The number of surgeons regularly performing microsurgical procedures was 3.4 ± 1.5, range from 1 to 7 surgeons (median 4, range 1e7). Eight hospitals treat less than 50 patients with oral squamous cell carcinoma per year (21%), 20 hospitals see 50e100 patients (52.6%), 10 more than 100 patients per year (26.3%). Numbers are clearly lower for cases undergoing microvascular reconstruction as not every cancer case requires this form of treatment (13 (34.3%) versus 15 (39.5%) versus 10 (26.3%)). There was a considerable variation in the number of microsurgically performed cases per year (Table 1). The microvascular free flaps used for reconstruction of defects are shown in Table 2. In relation to timing of bony reconstruction, 10 hospitals did reconstructions primarily (26.3%), 19 secondarily (50%) and 9 (23.7%) hospitals used both approaches. Frozen sections are performed in the vast majority of participating hospitals to optimize tumour resection intraoperatively (86.8%). For reconstruction of osseous defects, both mini-plates (42.1%) and reconstruction plates (23.7%) are used. One-third of hospitals (31.6%) use both systems.

Table 1. Number of cases by units per year (n= 38).

Number of cases per year	Number of units
0	2
1–9	3
10–19	4
20–49	11
50–99	16
>100	2

Table 2. Different flaps performed by units per year (n = 1300).

Type of microvascular free flap	Average number of cases
Radial forearm free flap	700
Fibular free flap	200
Latissimus dorsi free flap	170
Anterolateral thigh flap	110
Scapular free flap	80
Deep circumflex iliac crest flap	40

Preoperative management

Twenty-eight hospitals (73.7%) perform special preoperative management before a microvascular procedure in cases where bone is necessary for reconstruction. These units perform angiography for a fibular bone graft for vessel evaluation and possible stenoses of the branches or anatomical variations (Hölzle et al., 2010b). The Allen's test is performed if the radial forearm flap is used (Williams and Schenken, 1987).

Intraoperative management

Perioperative anticoagulation is used by 28 hospitals and is mostly heparin, followed by HAES (Hydroxyethylstärke) and acetyl-salicylic acid (ASS) (Fig. 1). Application protocols vary, as it is started both before, at time of and after ischemia. Most units apply pharmacological anticoagulation before (15–30 min) or immediately after completion of the anastamosis and before re-establishing of blood flow (Table 3).

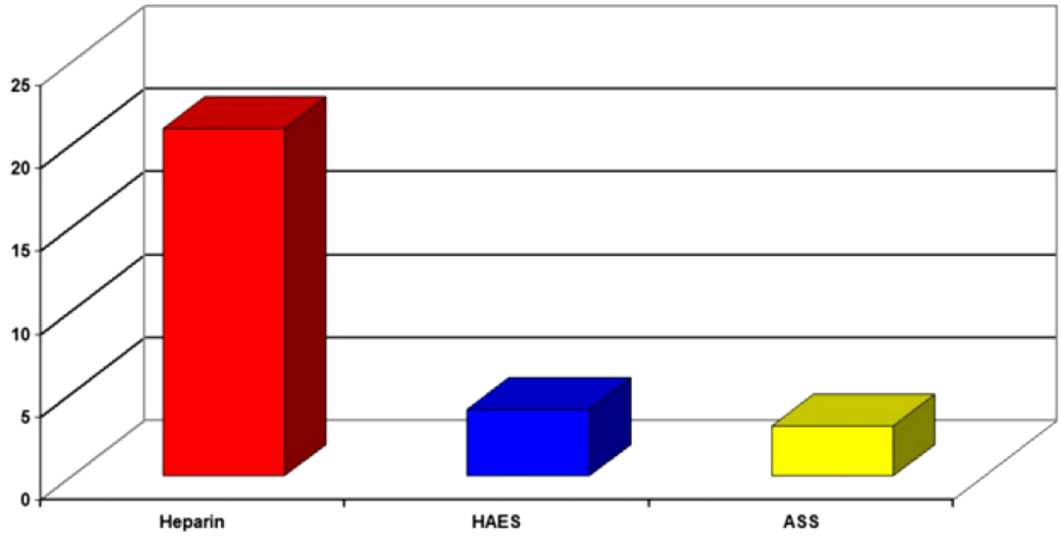

Fig. 1. Anticoagulation used in different units used for perioperative or postoperative treatment.

Table 3. Time point of perioperative anticoagulation performed in different units. Note how many units do not perform "anticoagulation".

Time stage of anticoagulation	Number of units
Starting operation	3
Before (within 15–30 min)	8
Immediately after anastomosis before blood flow is re-established (up to 10 min before anastomosis is opened)	7
After anastomosis and establishment of blood flow	2

44.7% of surgeons performed only an end-to-end technique in arteries and 10.5% in venous vessels. 34.2% of surgeons use end-toside technique in veins. Vessel grafts were never or

rarely used by 15.8% of hospitals and only in 1 hospital (2.6%) were they used regularly. Most hospitals flush recipient vessels (71.7%) intraoperatively, using heparin or saline. Intraoperative infusion therapy for vessel resistance reasons after anastamosis was applied in the minority of cases (26.3%), using HAES. Steroids are given in one third of cases (33.3%).

Postoperative management

15.8% of hospitals use inhibitors of platelet aggregation postoperatively; most hospitals use low molecular heparin (52.6%) or other heparin products (44.7%). HAES was used by 26.3% of hospitals for prevention of systemic venous thromboembolism and specifically prophylactically for the microvascular anastamosis (Mardel et al., 1996). When asked about the impact of supportive measures, substances were rated as presented in Fig. 1. The ratings regarding postoperative monitoring, influence of medication, and experience in microsurgical technique are presented in Fig. 2.

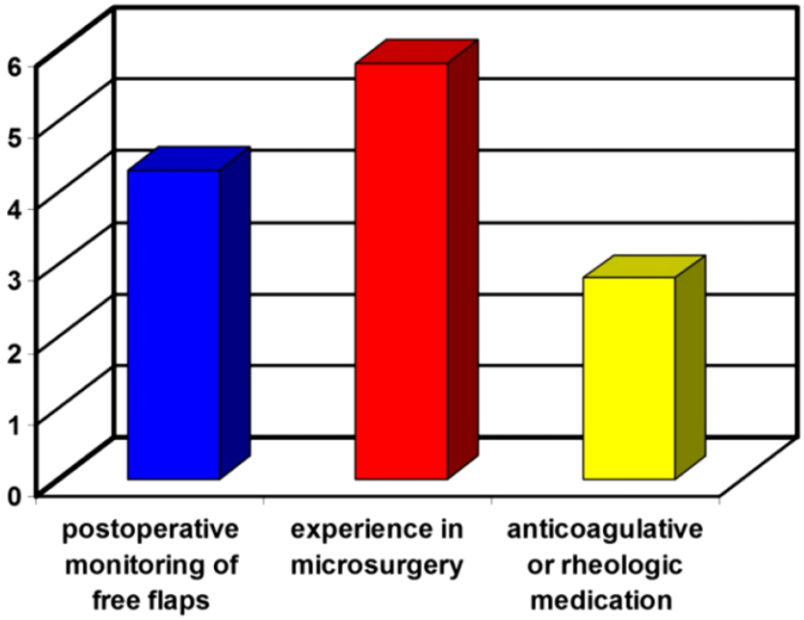

Fig. 2. Ratings regarding the importance of postoperative monitoring (mean 4.3), experience in microsurgery (mean 5.8), and anticoagulative or rheologic medication (mean 2.8) asked on a scale from 1 to 7 (1 = not important, 7 = most important).

Discussion

This study revealed that in most of the units responding to the questionnaire reconstructive microsurgical practice is performed frequently in German, Austrian and Swiss Departments of Oral and Maxillofacial Surgery. Although we had access to all the maxillofacial units in all three countries we expected a lower response rate from those not performing free flaps. This might be an explanation for our 48% response rate overall. Of the replies received, 95% did perform microsurgical free flap reconstruction. We therefore believe that our survey is likely to be representative of the current practice of the participating units. This type of surgery is performed in many units in Germany, Austria, and Switzerland, both in General

and University hospitals, and there was a wide variability in the number of cases performed by each unit per year. Only 2 units perform more than 100 cases, and 18 units perform more than 50 patients per year, representing t half of all units performing microsurgical and reconstructive surgery who had responded to the questionnaire. Only 9 units performed less than 20 cases per year.

In one of the largest surveys performed (Salemark, 1991), microsurgical procedures performed in the year 1989 in Departments of Plastic and Reconstructive Surgery worldwide were evaluated. In 83 units about 6500 operations were performed, but all body sites and reimplantation surgery were included. Whitaker et al. (2007) reported in a monthly-based analysis in Britain, that 75% of participating units had been performing 2e5 cases per month (24e60 per year) which is in the same range as our results, although the numbers presented in this survey are overlapping in this point (Table 1). Similarly in our survey only a few centres had performed more than 100 cases per year. These results show that microsurgical operations are regularly performed in Britain. Although these data are not comparable to each other, they show that the frequency of microsurgical reconstructive procedures has increased over the last decades. This is also related to the development and refinements of postoperative management performed regularly in major oncologic and reconstructive surgery as reported in the literature (Bradley, 2007; Bui et al., 2007; Chen et al., 2006; Marsh et al., 2009).

Postoperative care and monitoring were felt to be important by all the units despite wide variations in practice and the absence of any standard monitoring devices (Fig. 2). This suggests that the success rate does not depend on the postoperative medication regimen, supported by anticoagulation and vessel resistance treatment. Although several studies have been described in the literature, there is still no consensus regarding the optimal treatment regimen for anticoagulation in microvascular free flap surgery (Ashjian et al., 2007; Chien et al., 2005; Davies, 1982). Interestingly, in the literature different monitoring modalities, varying from clinical observation to technical devices for the detection of flap compromise, were not associated with a higher success rate or a lower complication rate (Spiegel and Polat, 2007). This also reflects the importance of experience in microsurgical procedures and individual flap management as rated by the different departments (Hölzle et al., 2010a; Kruse et al., 2010; Salemark, 1991; Smit et al., 2007).

Conclusions

This survey shows variation in the management and care of patients who have undergone microvascular reconstruction. This is due in part to the availability of microvascular surgeons in the different units, but it is also due to different types of hospitals where various levels of care can be provided for these patients needing special perioperative management. The lack of robust data is likely to continue with the presence of multiple small units with different practice performed in oral and maxillofacial surgery. Consolidation of data into a national or an international database would seem logical to inform decision-making and also to allow meaningful analysis of unit performance. This database would be also beneficial for evaluating different applications of microsurgical and reconstructive practice, both from the oncological and surgical points of view.

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4. Third study:

Intraoperative vascular anatomy, arterial blood flow velocity and microcirculation in uni- and bilateral cleft lip repair

Study design: Dr. Dr. A. Mueller

Financing: Prospective Researcher Fellowship Grant to A. A. Mueller (PBBSP3-128279) SNF (Swiss National Science Foundation): CHF 65‘180

Project Leaders: A. A. Mueller

Publication: First authorship, Plastic and Reconstructive Surgery, Impact Factor 3.535 (2012), Ranking 13/198 (Q1) Surgery

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Abstract

Cleft lip repair aims to normalize the disturbed anatomy and function. We determined whether normalization of blood circulation is achieved.

We measured the microcirculatory flow, oxygen saturation, and hemoglobin level in the lip and nose of controls (*n*=22), and in patients with unilateral and bilateral cleft lip and palate (UCLP and BCLP, respectively). We measured these parameters before lip repair (*n*=29 and 11, respectively), at the end of lip repair (*n*=27 and 10, respectively) and in the late post-operative period (*n*=33 and 20, respectively). The arterial flow velocity was measured in UCLP groups at the same time points (*n*=13, 11 and 12, respectively). Statistical differences were determined using analysis of variance.

Before surgery, the arterial flow velocities and microcirculation values were similar on each side of the face and among the groups. The microcirculatory flow was significantly higher in the prolabium of BCLP patients than in the philtrum of controls. All circulation values in UCLP and BCLP patients in the late post-operative period were within the range of controls and of those before surgery. Intraoperatively, we consistently found a perforating artery on the superficial side of the transverse nasalis muscle.

There appears to be no intrinsic circulatory deficit in UCLP and BCLP patients. The increased flow in the prolabium indicates a strong hemodynamic need in this territory, compelling its vascular preservation. Whether the surgical preservation of the nasalis perforator artery is of long-term benefit should be addressed in future studies.

Introduction

Cleft lip repair techniques differ mainly in the design of the skin incisions, how the muscle portions are reconstructed, and how the nasal framework is repositioned.[1] The vascular anatomy has remained largely unaddressed in current surgical techniques and the reasons for this have yet to be explored.

Normal blood supply is a precondition of development and growth. Thus, it would be of clinical interest to determine whether cleft anatomy leads to a change in the blood supply before or after surgery.

Current techniques for cleft-lip repair exclude surgical anastomosis of the lip artery. However, this clinical approach is not based on blood circulation data and so the current standard must be challenged. Vascular damage in cleft surgery interrupts the existent hemodynamics and necessitates further trauma to stop the bleeding, after which the blood circulation may take several months to recover.[2] Gentle surgical soft-tissue handling with blood vessel preservation is perhaps one of the decisive factors**,** why long-term outcome is better if the cleft surgery is performed by experienced surgeons operating with "soft hands."[3,4]

Cadaver cleft lip studies provide timeless information on the vascular network, but they lack in-vivo functional information.[5-9] To improve our understanding of the blood circulation in cleft lip, we assessed both the arterial vascular flow and the microcirculation before, at the end and late after cleft lip repair, as well as in a healthy control group. The results are discussed in relation to the vascular anatomy encountered during cleft lip surgery and in relation to the vascular anatomy of normal cadaver dissections.

Material and Methods

Patients and surgery

Ethical committees at the institutions of two of the authors (A.A.M. and S.G.R.) approved the study protocol. We included patients with unilateral or bilateral complete cleft lip and palate (UCLP and BCLP, respectively). Those in the healthy control group had undergone various surgical procedures, while having a healthy nose-lip anatomy. During the measurements the patients were kept in a supine position and under general anesthesia. All patients were operated on by the same surgeon (R.R.R.) using a previously published surgical technique.[10] Before the skin incision, 1 ml of lidocaine (1%) with epinephrine (1:100,000) was injected submucosally and intramuscularly along the incision markings. Bipolar cautery was used to stop bleeding that was not stopped by compression (Force FX coagulation system, Valleylab, Colorado, USA).

We measured the microcirculation in the following groups:

Controls (*n*=22).
UCLP patients before lip repair (*n*=29).
UCLP patients at the end of lip repair (*n*=27).
UCLP patients in the late post-operative period (*n*=33).

BCLP patients before lip repair (*n*=11).
BCLP patients at the end of lip repair (*n*=10).
BCLP patients in the late post-operative period (*n*=20).

We measured the arterial vascular flow in the following groups:

UCLP patients before lip repair (*n*=13).
UCLP patients at the end of lip repair (*n*=11).
UCLP patients in the late post-operative period (*n*=12).

We defined "late post-operative" as more than 6 months after lip repair. All patients measured at the end of lip repair had also been measured before surgery.

Microcirculation flow, oxygenation, and hemoglobin

Peripheral tissue perfusion was assessed using the O2C-1212 measuring system (LEA Medizintechnik, Giessen, Germany).[11] This system combines white-light tissue spectrometry and laser Doppler flowmetry and makes the following measurements simultaneously:

(1) The microcirculatory flow in vessels less than 100 µm in diameter, expressed in arbitrary units.

(2) The post-capillary oxygenated hemoglobin, expressed as a percentage of the total hemoglobin.

(3) The relative hemoglobin level in vessels less than 100 µm in diameter, expressed in arbitrary units.

We used an LSx-8c probe to make measurements on the columella; for all other points we used an LF-2 probe (tip diameter 4 mm and 12 mm, respectively). The approximate detection depths of the probes were 0.88 mm (LSx-8c) and 1.41 mm (LF-2). To avoid pressure-induced flow artifacts, we fixed the probes to the skin with double-sided adhesive tape (LTDT-001).

To allow for comparisons between the groups, we set the measuring points on the keratinized skin at corresponding anatomical landmarks (Fig. 1, *circles*): lateral lip, cupid, philtrum or prolabium, columella and ala. In UCLP patients we classified the symmetrical points as cleft- (C) or noncleft (NC)-sided, whereas in BCLP patients and controls we classified them as right- and left-sided.

Arterial flow velocity

The arterial blood flow velocity was assessed using a continuous-wave Doppler device (EZ-Dop, Compumedics, Singen, Germany) employing an 8-MHz probe with a tip diameter of 4 mm (HD.CW0800.06, MTB Medizintechnik Basler, Regensdorf, Switzerland). The probe angulation was chosen to maximize the signal, and the mean and maximum velocity (in cm/s) values were recorded.

We measured the blood flow velocity in the superior labial artery at the height of the labial commissure, cupid point and labial tubercle. At the alar base and columella we measured the blood flow velocity in the detected artery, regardless of the origin of its arterial main supply (Fig. 1, *rectangles*).

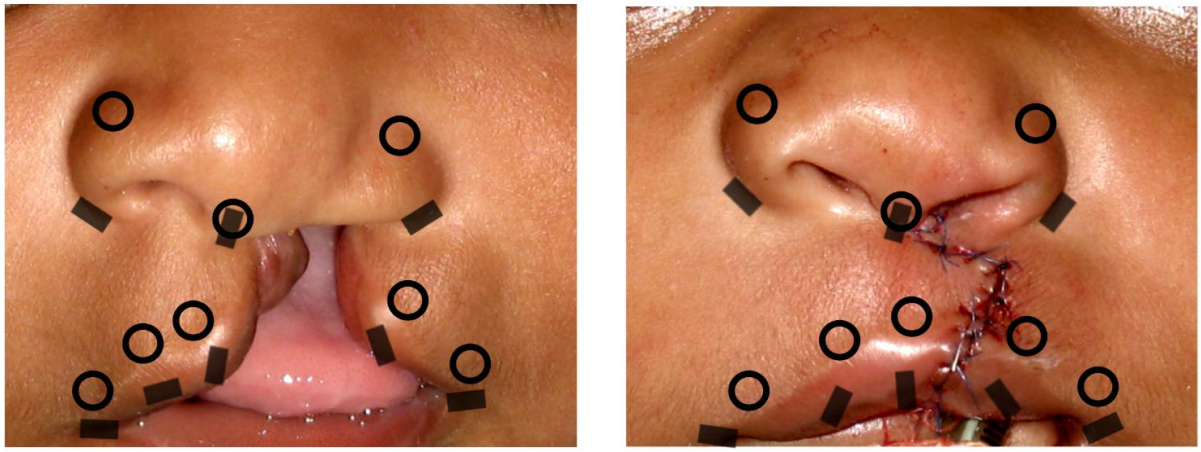

Fig. 1. Sites where the blood circulation was measured. At the points marked by *open circles*, we measured the microcirculatory flow, oxygen saturation and hemoglobin levels with an optical probe through the keratinized skin. At the points marked by *filled rectangles*, we measured the flow velocity in the underlying artery with an 8-MHz sound Doppler device. Corresponding sites were measured before and after surgery in unilateral and bilateral cleft lips, and in healthy controls.

Statistics

We performed a one-way analysis of variance (ANOVA) of data from identical or symmetrical measuring points. We used Tukey's multiple-comparison test to detect differences between groups, and between cleft and noncleft sides.

Anatomical dissections

The facial arterial network was dissected in 12 adult, formalin-fixed cadavers with the aid of a surgical microscope and microsurgical instruments (S&T, Neuhausen, Switzerland).

Results

Arterial blood flow velocity

No significant differences in vascular blood flow velocities were found at corresponding measuring points between the cleft and noncleft side, and between the different groups (p=0.05, ANOVA with Tukey's post-hoc comparisons) (Table 1).

Microcirculatory flow

The microcirculatory flow was significantly higher in the prolabium of BCLP patients before lip repair than in the philtrum of UCLP patients and controls. The microcirculatory flow was significantly lower at the end of lip repair than before lip repair at two points: in UCLP patients at the noncleft side ala, and in BCLP patients at the left ala. In addition, the microcirculatory flows at corresponding points was similar on the cleft and noncleft sides in UCLP patients, BCLP patients and controls, and before, after and later after lip repair (p=0.05, ANOVA with Tukey's post-hoc comparisons) (Table 2).

Post-capillary hemoglobin oxygenation

The hemoglobin oxygen saturation in the cleft-side lateral lip in UCLP patients was significantly lower at the end of lip repair than before lip repair. In addition, the microcirculatory oxygen saturation was similar on the cleft and noncleft sides in UCLP patients, BCLP patients and controls, and before, after and later after lip repair (p=0.05, ANOVA with Tukey's post-hoc comparisons) (Table 3).

Microcirculatory hemoglobin level

The relative hemoglobin level in the columella of UCLP patients was significantly lower before lip repair in UCLP patients that in BCLP patients and controls, while it was significantly higher in UCLP patients at the end of lip repair than before lip repair or in the late post-operative period. Furthermore, the relative hemoglobin level in the late post-operative period was similar on the cleft and noncleft sides in UCLP patients, BCLP patients and controls, and compared to before lip repair (p=0.05, ANOVA with Tukey's post-hoc comparisons) (Table 4).

Vascular anatomy in cadaver dissections

The following findings were encountered consistently in the dissections. The thickness of the superior labial artery was asymmetric in most cases (Fig. 2, *left* and *lower right*). The dominant side connected to a branch to the labial tubercle and to at least two philtral branches (Fig. 2, *left* and *lower centre*): a thick branch deep to the muscle and a thinner one superficial to it. The deep philtral branch ran around the lip muscle in front of the anterior nasal spine and connected to the columellar branch (Fig. 2, *left* and *lower centre*), while the superficial philtral branch ramified to the caudal nasal septum (Fig. 2, *upper centre*). The superior and inferior alar arteries connected either caudally to the superior labial artery (Fig. 2, *left*) or laterally to the facial artery (Fig. 2, *lower right*).

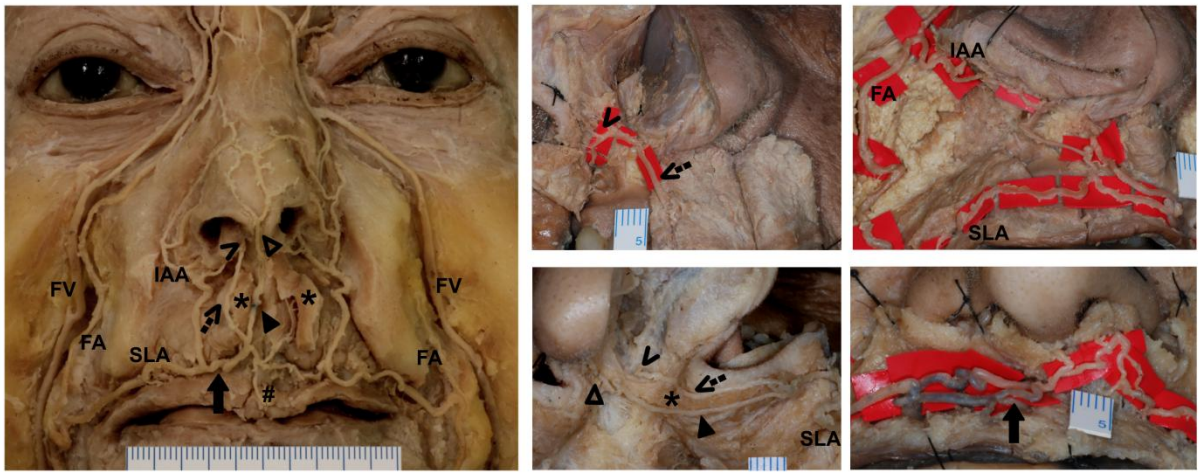

Fig. 2. Characteristics of the normal vascular anatomy. The superior labial artery (*SLA*) is dominant on one side (➔). The superficial (- - >) and deep (▲) philtral artery, and the labial tubercle branch (**#**) is mainly supplied by the dominant side. The deep philtral artery runs posteriorly to the labial muscle (*****) and connects to the columellar artery (**Δ**); whereas the superficial philtral artery ramifies (**Λ**) to the caudal nasal septum. The arterial trunk of the ala and inferior alar artery (*IAA*) connects either caudally to the superior labial artery (*Left, cadaver's both sides*) or laterally to the facial artery (FA) (*Upper right, cadaver's right side*). Scale increments indicate millimeters. FV, facial vein.

Vascular anatomy in cleft lip repair

On the noncleft side we frequently encountered an artery of 1–2 mm in diameter in the submucosa of the lip band, running along the posterior side of the lip muscle (Fig. 3). During the subperiostal approach to the caudal septal part, this artery was laterally shifted and preserved. On the cleft side we encountered a submucosal lip artery more rarely.

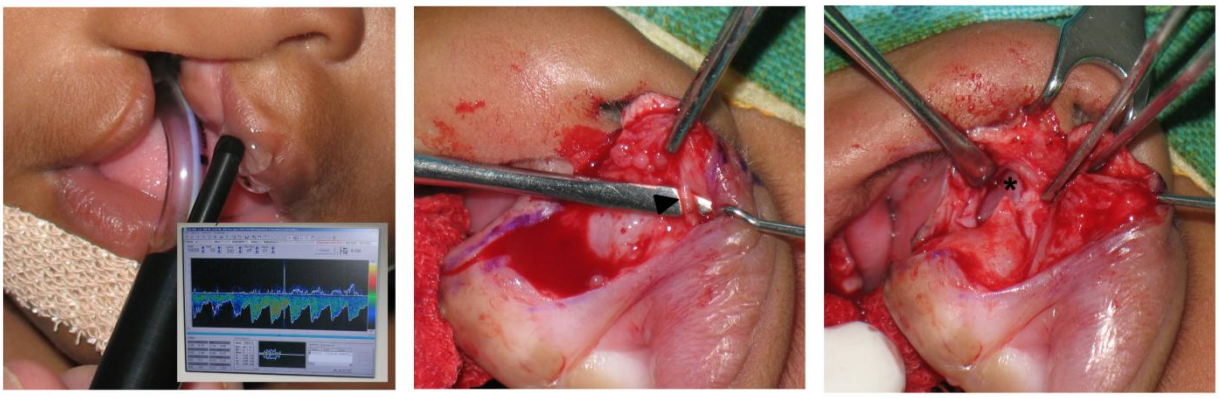

Fig. 3. Superior labial artery on the noncleft side. (*Left*) Frequently, the artery proceeds to the labial tubercle and can be localized before surgery by its strong Doppler signal. (*Centre*) During surgery, the artery (▲) is encountered in the submucosa of the lip band and can be frequently preserved. (Right) The caudal septum (*****) is approached from the cleft side and does not interfere with the artery.

On the cleft side, we consistently encountered an artery of 1–2 mm diameter in the subcutaneous plane of the hairy nostril skin – the artery perforated out of the inferior head of the transverse nasalis muscle (Fig. 4). The bleeding that occurred when the artery was accidentally cut always required coagulation. This artery was equally present in UCLP and BCLP patients.

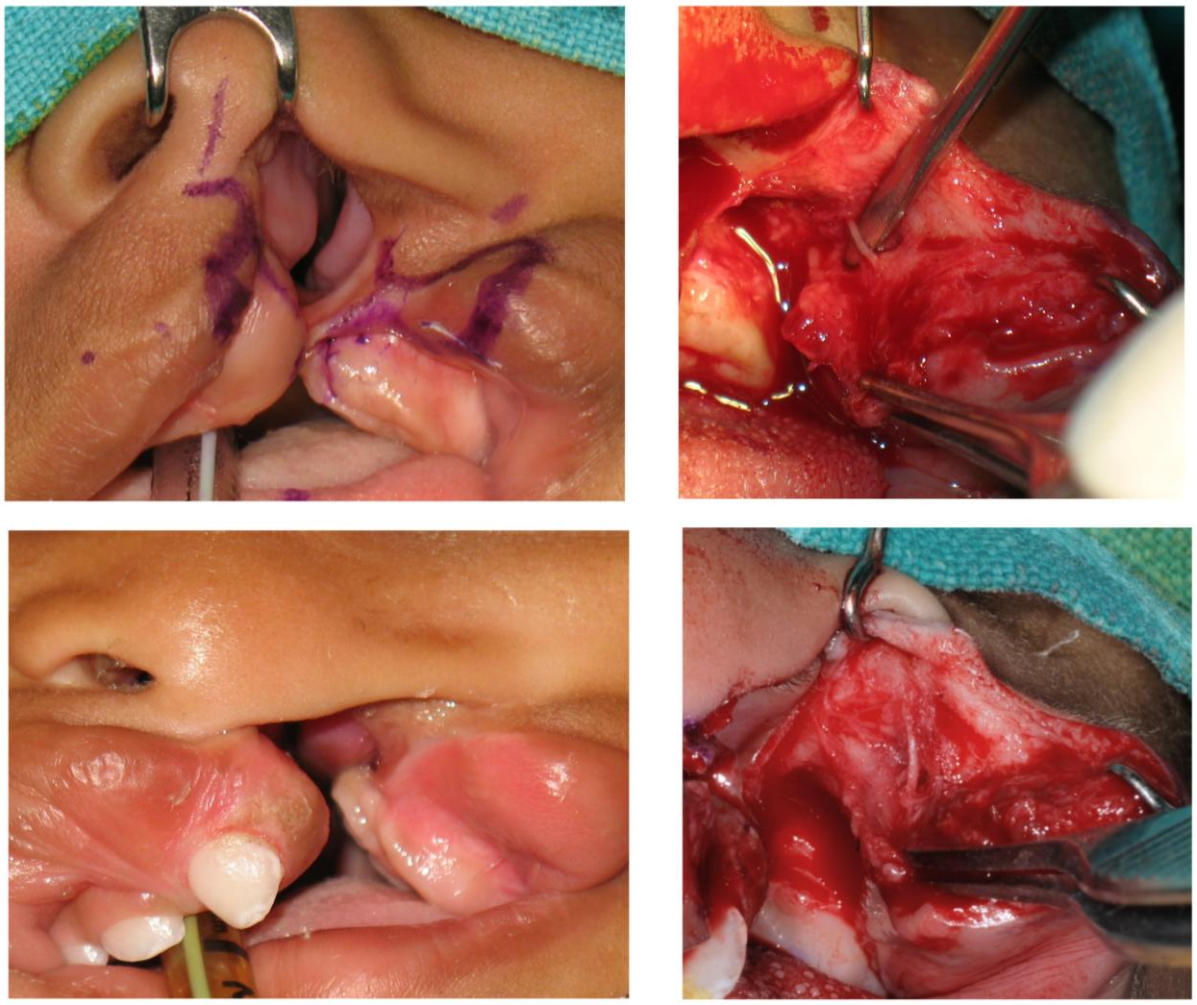

Fig. 4. The perforating artery of the transverse nasalis muscle on the cleft side. (*Upper left* and *right*) In the subcutaneous plane of the hairy nostril skin, a 1- to 2-mm-thick artery is consistently found during dissection of the transverse nasalis muscle, which is held in the forceps. (*Upper right* and *lower right*) The localization and course of this artery is constant, which makes its surgical preservation easier.

In the prolabium of BCLP patients we regularly encountered two arteries (Fig. 5) running on the right and left sides of the prolabium, being most superficial at the midway point between the columella–prolabial junction and the planned cupid points.

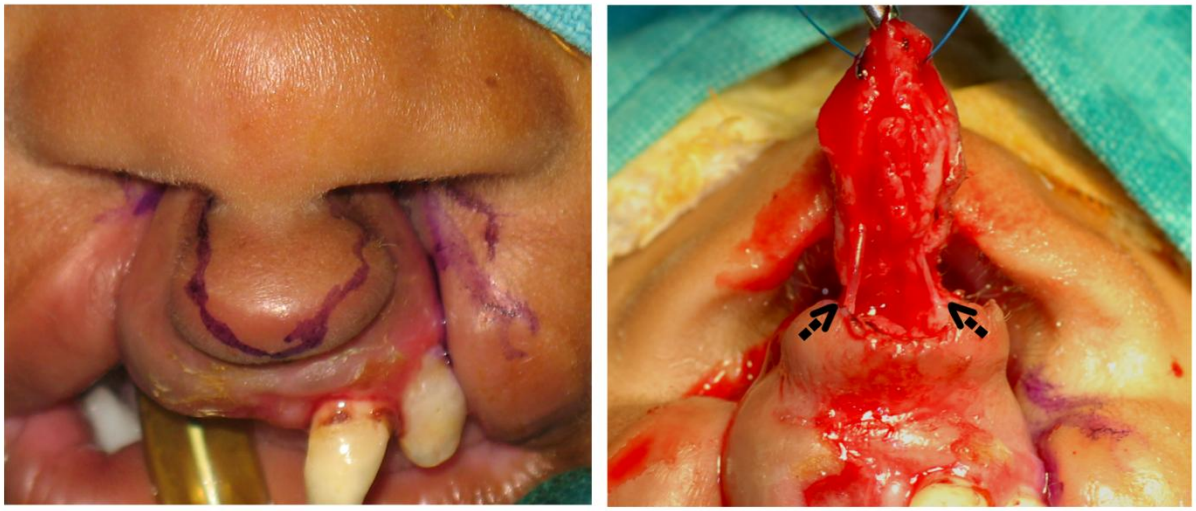

Fig. 5. The prolabium arteries. (*Left*) In the middle of the prolabium, between the columella base and the planned cupid points, a pair of prolabium arteries (- - >) is frequently observed running subcutaneously. (*Right*) While dissecting the prolabium from distal to proximal under constant tension, these arteries can be identified and preserved independently of the skin incision design.

Discussion

Circulation before lip repair

Before surgery, the arterial flow velocities and microcirculation values were symmetric on the cleft and noncleft sides, as well as among cleft groups and controls. Despite the aberrant cleft vascular anatomy, a symmetric and normal blood supply was found. Cleft repair should aim to preserve this function.

Anatomical studies have demonstrated that the superior labial artery is thicker on the cleft side than on the noncleft side and a fibrosis of the unoperated cleft muscles has been presumed.[5,12] However, no corresponding circulatory discrepancies have been found, which underlies the need to complement morphological descriptions with appropriate functional measurements.

While the blood supply on the cleft and noncleft sides did not differ from controls, there was a difference in the midline blood supply. The prolabium of BCLP patients had a significantly increased microcirculatory flow and the columella of UCLP patients had a significantly decreased hemoglobin level.

In a circulatory steady state the hemoglobin level reflects the microvascular density in the tissue. The columella in UCLP patients lacks the connection of an inferior alar artery on the cleft side, and thus its hemodynamic force to the columella.[13,14] Since the hemodynamic force is a key stimulus for the development and maturation of the vascular bed, this might partially explain the decreased relative hemoglobin level in the UCLP columella.

In the prolabium of BCLP patients we attribute the increased microcirculation flow to a strong hemodynamic need in this territory. The nutritive needs of the premaxilla might be

not fully met by the posterior septal artery, thus requiring a complementary supply from the prolabium circulation.[8]

Circulation at the end of lip repair

The microcirculation flow in the prolabium of BCLP patients remained higher than in controls and UCLP patients, both directly at the end of lip repair and in the late post-operative period. We attribute this to the intraoperative vessel preservation in the prolabium (Fig. 5). The surgical repair in BCLP did not reduce the high microcirculation flow in the prolabium, and thus we assume that the microcirculatory requirements were maintained.

A venous stasis in the microcirculation can be detected by an increase in hemoglobin level together with a decrease in flow. We encountered this at the end of lip repair in the columella of UCLP patients. The venous drainage through the columella runs in normal anatomy to the philtral plexus and to the septal mucosa plexus.[15,16] Thus, release of the caudal septum and the medial rotational incision might have interfered temporarily with the venous drainage – even though the medial rotation incision did not cross the midline. A sharp downturned back-cut was performed as necessary in the midline to allow for rotation (Fig. 4, *upper left*).

The blood circulation at the end of lip repair is influenced by multiple factors, including surgically induced vasodilatation, vascular coagulation, change in tissue tension, and systemic hemodynamic conditions. Thus, assumptions about cause-and-effect relationships must be made with caution. However, the cumulative effect of these factors appears to have led to an arterial blood flow velocity and microvascular flow in the lip and philtrum at the end of surgery that did not differ from the corresponding preoperative data. It is thus understandable that the evolution from staged to synchronous bilateral cleft lip repair proceeded without healing problems.

Circulation in the late post-operative period and in normal controls

In the late post-operative period, the arterial blood flow velocity and microcirculation in UCLP and BCLP patients was the same as in controls and the same as before surgery. We conclude that cleft lip repair without a surgical anastomosis of the lip artery allowed for normal circulatory development. This is consistent with the blood supply observed in normal faces, wherein the circulation is balanced on each side of the face independently and does not rely on a superior labial artery anastomosis.[17-20] The microcirculation measurement depth of 1.41 mm corresponds to the deep reticular network of the skin. This network is fed by collaterals of the superior labial artery and is therefore related to the circulation in the lip muscle.[21,22]

Hemodynamic forces are key elements for the remodeling of the vascular network after acute arterial occlusion. In arteriogenesis, shear stress induces the recruitment of arteriolar collaterals, which in turn differentiate into new arteries.[23,24] The circulation was completely restored after carotid ligation in growing animals, but there was a reduction in facial muscle weight.[25] Thus, the recovery of blood circulation observed in the present study may not represent the recovery of growth potential.

Intraoperative arteries

The cleft interrupts the normal course of the superior labial artery. Published schematic illustrations show that the superior labial artery on the cleft side runs to the alar base and joins the facial artery.[6,26,27] Conversely, photographs of arteriograms[5,28,29] and illustrations derived therefrom[9,30] show a superior labial artery that thins out at the inner side of the cleft nostril, whereas the superior alar branches connect laterally to the facial artery. Our intraoperative findings concur well with the arteriogram pattern.

We consistently found an arterial branch at the inner side of the cleft nostril. The artery emerged on top of the dissected transverse nasalis muscle and was easily preserved after its course had been estimated. We speculate that preserving this artery weakens post-operative cleft nostril contraction.

The facial vascular pattern does not develop as a stepwise branching from proximal to distal. Rather, preferred routes in a territory are formed consequent to its hemodynamic need and are connected to supplying trunks from distal to proximal. The cleft might reduce the available options for alar connections to supplying trunks, which leads to a more uniform vascular pattern on the cleft side. This might explain why we consistently found an artery on the inner side of the cleft nostril.

Summary and clinical interpretation

We studied the arterial flow velocity and microcirculation in cleft lip. Contrary to the asymmetric vascular anatomy in cleft lips, we found no functional asymmetry. Our results support the current standard of cleft-lip repair that does not involve surgical anastomosis of the lip artery.

While the blood circulation did not differ from that of controls in the late-postoperative period, we cannot assume that vascular function was normal in all aspects, especially with regard to its ability to support growth potential. We therefore advocate strict vascular preservation in cleft surgery. The complex relationship between vascular anatomy and physiology necessitates further in-vivo circulatory measurements to assess the optimal blood circulation for cleft surgery.

The surgical vascular anatomy exhibited a high degree of uniformity, which simplifies its consideration in current cleft surgical techniques. This finding has been confirmed for the paramedian prolabial arteries and the transverse nasalis perforating artery.

Conclusion

The blood circulation in UCLP and BCLP patients remains balanced before and after surgery, and is comparable to the normal situation, with no sign of an intrinsic circulatory deficit or a need for surgical arterial anastomosis. The increased flow in the prolabium of BCLP patients indicates a strong hemodynamic need in this territory, compelling its vascular preservation. We consistently found a perforating artery on the superficial side of the transverse nasalis muscle. Whether or not the surgical preservation of this artery has any long-term benefit should be addressed in future studies.

Acknowledgements

We acknowledge the Swiss National Science Foundation for a research grant to A.A.M., and the European Association for Cranio-Maxillo-Facial Surgery (EACMFS) for accreditation of the EACMFS-cleft surgery fellowship, during which the study was performed.

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Tables

Table 1. Arterial Blood Flow Velocity in Unilateral Complete Cleft Lip and Palate Patients

Measuring point	Before lip repair (n = 13)[1]		End of lip repair* (n = 11)[2]		Late postoperative (n = 12)[3]		ANOVA of mean values
	Maximum	Mean	Maximum	Mean	Maximum	Mean	
Superior labial artery							
Commissur, C	304 (85)	118 (48)$_{a,b}$	113 (32)	39 (13)$_{a}$	242 (122)	84 (56)$_{a,b}$	F (5, 30)= 2.537, p<.05
Commissur, NC	193 (88)	79 (35)$_{a,b}$	192 (108)	94 (59)$_{a,b}$	296 (143)	129 (55)$_{b}$	
Cupid, C	150 (107)	73 (63)	103 (63)	48 (24)	101 (41)	56 (37)	F (5, 46)= 1.689, ns
Cupid, NC	163 (65)	93 (51)	107 (48)	34 (18)	164 (98)	81 (53)	
Tuberculum	146 (51)	88 (33)	111 (50)	50 (36)	145 (74)	80 (53)	F (2, 23)= 1.792, ns
Columella branch	138 (58)	67 (39)	90 (23)	21 (12)	122 (54)	66 (47)	F (2, 23)= 2.272, ns
Lateral nasal artery							
Ala base, C	166 (61)	75 (50)	136 (59)	57 (24)	92 (31)	35 (12)	F (5, 50)= 0.737, ns
Ala base, NC	128 (79)	54 (40)	156 (88)	72 (70)	65 (22)	21 (7)	

Data are mean (SD) values in cm/s. A continuous-wave Doppler device employing an 8-MHz probe was used. Within rows of symmetric or the same measuring points, means with different subscripts differ at $p<0.05$, according to Tukey's multiple-comparisons test.

* Same individuals as measured before lip repair.

[1-3] Sample sex distribution and mean age (male/female, month, SD); [1](8/5, 13, 15), [2](6/5, 13, 16), [3](11/1, 16, 9)

[3] Months after lip repair (mean, SD); (8, 4)

C, cleft side; NC, non-cleft side

Table 2. Microcirculatory Flow in Unilateral and Bilateral Complete Cleft Lip and Palate Patients and Controls

Measuring point	Control	Before lip repair		End of lip repair*		Late postoperative		ANOVA
		UCLP	BCLP	UCLP	BCLP	UCLP	BCLP	
	(n = 22)[1]	(n = 29)[2]	(n = 11)[3]	(n = 27)[4]	(n = 10)[5]	(n = 33)[6]	(n = 20)[7]	
Lateral lip, C / left	95 (33)$_{a,b,c,d}$	89 (35)$_{a,b,c,d}$	113 (59)$_{a,b,c,d}$	67 (69)$_{a,b}$	54 (52)$_{a,b,c}$	112 (77)$_{b,c,d}$	130 (99)$_{c,d}$	F (13, 288)= 3.868, p< .001
Lateral lip, NC / right	95 (51)$_{a,b,c,d}$	92 (60)$_{a,b,c,d}$	98 (58)$_{a,b,c,d}$	47 (35)$_{a}$	64 (35)$_{a,b,c,d}$	90 (58)$_{a,b,c,d}$	143 (80)$_{d}$	
Cupid, C / left	104 (43)$_{a,b,c,d}$	93 (68)$_{b,d}$	184 (81)$_{c}$	60 (54)$_{a,d}$	72 (47)$_{a,b,d}$	117 (76)$_{a,b,c,d}$	98 (70)$_{a,b,c,d}$	F (13, 247)= 3.679, p<.001
Cupid, NC / right	128 (52)$_{b,c}$	89 (58)$_{a,b,d}$	128 (40)$_{a,b,c,}$	62 (46)$_{a,d}$	75 (48)$_{a,b,d}$	111 (40)$_{a,b,c,d}$	108 (75)$_{a,b,c,d}$	
Philtrum / Prolabium	119 (52)$_{a,b}$	116 (44)$_{a,b}$	216 (87)$_{d}$	78 (55)$_{b,c}$	172 (87)$_{a,b,d}$	120 (50)$_{a,b}$	169 (83)$_{a,d}$	F (6, 125)= 6.596, p<.001
Columella	122 (38)	119 (59)	130 (36)	95 (65)	129 (50)	122 (66)	119 (52)	F (6, 145)= 1.148, ns
Ala, C / left	104 (47)$_{a}$	98 (38)$_{a}$	88 (11)$_{ab}$	70 (29)$_{a,b}$	81 (68)$_{a,b}$	72 (39)$_{a,b}$	86 (55)$_{a,b}$	F (13, 238)= 2.589, p<0.01
Ala, NC / right	77 (24)$_{a,b}$	97 (43)$_{a}$	100 (44)$_{a,b}$	51 (21)$_{b}$	91 (23)$_{a,b}$	83 (45)$_{a,b}$	70 (38)$_{a,b}$	

Data are mean (SD) values in arbitrary units. The approximate optical measurement depth was 0.88 mm on the columella and 1.41 mm at all other points. Within rows of symmetric or the same measuring points, means with different subscripts differ at $p<0.05$, according to Tukey's multiple-comparisons test.

*Same individuals as measured before lip repair.

[1–7] Sample sex distribution and mean age (male/female, month, SD); [1](15/7, 62, 36), [2](18/11, 9, 6), [3](9/2, 14, 20), [4](17/10, 9, 6), [5](8/2, 14, 21), [6](22/11, 46, 47), [7](14/6, 41, 36)

[6,7] Months after lip repair (mean, SD); [6](28, 33), [7](31, 33)

C, cleft side; NC, non-cleft side; UCLP, unilateral complete cleft lip and palate; BCLP, bilateral complete cleft lip and palate

Table 3. Post-capillary Haemoglobin Oxygenation in Unilateral and Bilateral Complete Cleft Lip and Palate Patients and Controls

Measuring point	Control	Before lip repair		End of lip repair*		Late postoperative		ANOVA
		UCLP	BCLP	UCLP	BCLP	UCLP	BCLP	
	(n = 22)[1]	(n = 29)[2]	(n = 11)[3]	(n = 27)[4]	(n = 10)[5]	(n = 33)[6]	(n = 20)[7]	
Lateral lip, C / left	67 (11)$_{a,c}$	59 (11)$_{a,c}$	61 (9)$_{a,b,c}$	48 (21)$_{b}$	53 (22)$_{b,c}$	66 (17)$_{a,c}$	65 (14)$_{a,c}$	F (13, 288)= 6.777, p<.001
Lateral lip, NC / right	66 (16)$_{a,c}$	54 (12)$_{b,c}$	56 (6)$_{a,b,c}$	44 (15)$_{b}$	39 (23)$_{b}$	63 (17)$_{a,c}$	68 (12)$_{a,c}$	
Cupid, C / left	54 (18)$_{a,b}$	54 (14)$_{a,b}$	71 (12)$_{a}$	50 (22)$_{a,b}$	58 (18)$_{a,b}$	63 (15)$_{a,b}$	65 (16)$_{a,b}$	F (13, 245)= 2.845, p<.001
Cupid, NC / right	62 (12)$_{a,b}$	47 (12)$_{b}$	63 (10)$_{a,b}$	48 (25)$_{a,b}$	57 (24)$_{a,b}$	57 (12)$_{a,b}$	62 (14)$_{a,b}$	
Philtrum / Prolabium	63 (12)	56 (15)	61 (20)	52 (21)	69 (18)	61 (13)	60 (15)	F (6, 125)= 1.176, ns
Columella	44 (12)$_{a,b}$	43 (20)$_{a,b}$	51 (14)$_{a,b}$	43 (19)$_{a,b}$	65 (22)$_{a}$	41 (17)$_{b}$	45 (13)$_{a,b}$	F (6, 142)= 2.101, ns
Ala, C / left	56 (15)	53 (13)	62 (9)	50 (17)	42 (11)	47 (15)	51 (15)	F (13, 237)= 1.111, ns
Ala, NC / right	51 (14)	50 (14)	55 (10)	46 (20)	51 (17)	47 (17)	52 (16)	

Data are mean (SD) values expressed as a percentage of the total haemoglobin. The approximate optical measurement depth was 0.88 mm on the columella and 1.41 mm at all other points. Within rows of symmetric or the same measuring points, means with different subscripts differ at $p<0.05$, according to Tukey's multiple-comparisons test.

*Same individuals as measured before lip repair.

[1–7] Sample sex distribution and mean age (male/female, month, SD); [1](15/7, 62, 36), [2](18/11, 9, 6), [3](9/2, 14, 20), [4](17/10, 9, 6), [5](8/2, 14, 21), [6](22/11, 46, 47), [7](14/6, 41, 36)

C, cleft side; NC, non-cleft side; UCLP, unilateral complete cleft lip and palate; BCLP, bilateral complete cleft lip and palate

Table 4. Microcirculatory Haemoglobin Level in Unilateral and Bilateral Complete Cleft Lip and Palate Patients and Controls

Measuring point	Control	Before lip repair		End of lip repair*		Late postoperative		ANOVA
		UCLP	BCLP	UCLP	BCLP	UCLP	BCLP	
	(n = 22)[1]	(n = 29)[2]	(n = 11)[3]	(n = 27)[4]	(n = 10)[5]	(n = 33)[6]	(n = 20)[7]	
Lateral lip, C / left	76 (4)$_{d}$	74 (6)$_{b,c,d}$	72 (9)$_{a,b,c,d}$	72 (8)$_{a,b,c,d}$	66 (10)$_{a}$	76 (8)$_{d}$	78 (6)$_{d}$	F(13, 288)= 5.748, p<.001
Lateral lip, NC / right	77 (5)$_{d}$	72 (6)$_{a,b,c,d}$	71 (6)$_{a,b,c,d}$	69 (5)$_{a,b,c}$	68 (8)$_{a,b}$	75 (7)$_{c,d}$	79 (5)$_{d}$	
Cupid, C / left	75 (6)	72 (6)	77 (4)	77 (8)	73 (7)	75 (9)	76 (5)	F(13, 247)= 1.299, ns
Cupid, NC / right	77 (6)	73 (8)	71 (9)	72 (10)	76 (8)	75 (6)	75 (7)	
Philtrum / Prolabium	77 (6)$_{a,b}$	73 (8)$_{a}$	78 (9)$_{a,b}$	74 (11)$_{a}$	86 (9)$_{b}$	76 (7)$_{a,b}$	77 (5)$_{a,b}$	F (6, 125)= 2.510, p<.05
Columella	58 (8)$_{a,b}$	51 (7)$_{c}$	61 (8)$_{a,b}$	60 (9)$_{a}$	62 (9)$_{a,b}$	53 (9)$_{b,c}$	57 (7)$_{a,b,c}$	F (6, 144)= 5.006, p<.001
Ala, C / left	75 (5)	71 (7)	72 (4)	73 (7)	67 (5)	73 (6)	75 (4)	F (13, 238)= 1.592, ns
Ala, NC / right	74 (5)	71 (7)	71 (9)	69 (6)	72 (4)	73 (6)	73 (6)	

Data are mean (SD) values in arbitrary units. The approximate optical measurement depth was 0.88 mm on the columella and 1.41 mm at all other points. Within rows of symmetric or the same measuring points, means with different subscripts differ at $p<0.05$, according to Tukey's multiple-comparisons test.

*Same individuals as measured before lip repair.

[1–7] Sample sex distribution and mean age (male/female, month, SD); [1](15/7, 62, 36), [2](18/11, 9, 6), [3](9/2, 14, 20), [4](17/10, 9, 6), [5](8/2, 14, 21), [6](22/11, 46, 47), [7](14/6, 41, 36)

C, cleft side; NC, non-cleft side; UCLP, unilateral complete cleft lip and palate; BCLP, bilateral complete cleft lip and palate

5. Fourth study:

One-stage cleft repair outcome at age 6- to 18-years – a comparison to the Eurocleft study data

Study design:	A. A. Mueller
Financing:	Project funding to applicant A. A. Mueller SSO (Swiss Dentist Association): CHF 20'000 FAG (Voluntary Academic Association Basel): CHF 60'000
Project Leaders:	A. A. Mueller
Publication:	First authorship, British Journal of Oral and Maxillofacial Surgery, Impact Factor 2.717 (2012), Ranking 29/198 (Q1) Surgery

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Abstract

Optimising the relation of outcome quality and burden of care is a challenge in cleft lip and palate treatment. We analyse the long-term outcome after one-stage cleft repair to assess its benefits and limitations as an alternative cleft care model.

Lip repair, two-flap palatoplasty and cortico-cancellous alveolar bone graft was performed at 6 month of age. Thirty-three patients from 6–18 years were divided into three age groups (6-10.5y, 12-13.5y, 15-17.6y) and compared to mean outcome data from the Eurocleft centers and to Cephalometric Standards of healthy individuals. Fifteen of 33 patients complied for nasalance measurements.

The maxillary protrusion (SNA) and intermaxillary relation (ANB) in the one-stage groups differed significantly from healthy individuals but not from the corresponding Eurocleft means. The Bergland Score for alveolar ossification was grade I or II in 61%, and grade III in 15%; 24% underwent secondary alveolar bone grafting. No palatal fistulae occurred and the nasalance did not differ significantly from that of healthy controls. On an average, each patient got the primary surgery and one secondary procedure, which was half as much compared to multi-stage procedures.

The one-stage procedure led to significant growth disturbance however the degree of growth disturbance was similar to the mean values of multi-stage procedures obtained in the Eurocleft study. Primary alveolar bone grafting led to inconsistent alveolar ossification and was suspected to interfere with anterior maxillary growth – it has been abandoned. The patients benefited from half the number of surgical steps compared to multi-stage procedures.

Introduction

Optimizing the relation between outcome quality and burden of care is a continuous challenge in cleft lip and palate treatment. Facing this challenge, all unilateral complete cleft lip and palate (UCLP) patients received a one-stage cleft repair at 6 months of age, combined with primary alveolar bone grafting, between 1991 and 2002.[1]

There are several ways to close a unilateral cleft in one procedure. The main differences are (1) single[2,3] or double layer[1] hard palate closure, (2) hard palate repair with two-flap palatoplasty[1] or V-Y pushback,[4] or lateral releasing incisions,[2] (3) cranial pedicled[1,3] or caudally pedicled[2,5] vomer flap, (4) alveoplasty[4] or primary alveolar bone graft,[1] and (5) whether the one-stage repair is applied in all cleft patients[1,5] or only in those with narrow clefts.[3] Each of these variations might influence the outcome of growth, occlusion, alveolar ossification, nasalance, and number of subsidiary procedures divergently. We therefore assessed all these outcome parameters jointly.

Alveolar bone grafting at the time of lip repair and early two-layer hard-palate closure have been reported to result in unfavourable growth effects.[2] Therefore, in our one-stage repair evident growth retardation must be feared.

In addition to growth and speech, the third aspect under which a cleft protocol should be approached is the burden of care; whereof the number of surgeries is one aspect.[6,7] We assessed the long-term benefits and limitations of one-stage repair combined with primary alveolar bone grafting in all patients between 1991 and 2002 who had had unilateral complete cleft lip and palate (UCLP) repair at 6 month of age.[1]

Patients and Methods

Patients

From 1991 until 2002 when the surgeon retired, 53 nonsyndromic complete UCLP patients were operated upon. Of these, 33 participated in the study (age range 6-18 years (Table 1), 8 had moved away, and 12 did not participate because of the extra effort required.

Photographs, cephalograms, orthopantomograms, and dental casts were taken, and 15 patients also participated in a nasalance assessment. All patients gave written informed consent, and the study was approved by the local ethics committee (EK 256/06).

Surgery

All patients had a palatal obturator after birth to keep the alveolar segments in place and to facilitate feeding. All patients were operated by the same surgeon using a one-stage cleft repair comprising two-flap palatoplasty and a cortico-cancellous alveolar bone graft from the rib. A vestibular mucoperiosteal flap from the lesser maxillary segment was advanced to cover the alveolar bone graft.[1]

Methods

The classification of three age groups (6-10.5y, 12-13.5y, 15-17.6y) and the cephalometric parameters for hard- and soft-tissue analysis were chosen to allow comparability to the Eurocleft Study.[8] The cephalograms were analysed digitally using OnyxCeph Software (Image Instruments, Chemnitz, Germany). Landmark identification was conducted jointly by two raters. Ten randomly selected radiographs were analysed again by the same team after a time interval of at least 1 month. Means and standard deviations of the differences between both skeletal ratings were calculated.

The cephalometric mean values from all centers which completed the Eurocleft study (centers A, B, D, E, F) were used as comparative values of a variety of multi-stage procedures.[8] The comparative values of healthy individuals are based on the Cephalometric Standards.[9] An analysis of variance with Games-Howell post-hoc comparisons was performed to detect differences between three groups. Student's t-tests or Welch-tests were performed to detect differences between two groups.

The EUROCRAN Index was used to quantify an occlusal rating on dental casts.[10] The presence of permanent lateral incisors on the dental casts and orthopantomograms was noted. Alveolar bone ossification was evaluated using the Bergland Score.[11] Four sentences without nasal consonants[12] were recorded with the Nasometer II 64000 (Kay Elemetrics Corporation, Lincoln Park, New Jersey, USA). The same Nasometer analysis was repeated in a matched control group of healthy individuals.

The number and type of subsidiary operations were retrieved from the patients' documentation and confirmed by the parents. Comparative data from multi-stage procedures were used from the Eurocleft Study.[13]

Results

The 33 patients were divided into three age groups of 15, 7, and 11 patients each (Table 1).

Cephalometric skeletal values are listed in Table 2. The maxillary protrusion (SNA) and intermaxillary relation (ANB) in the intermediate and oldest one-stage groups differed significantly from healthy Cephalometric Standards but did not differ significantly from the corresponding Eurocleft means. The maxillary plane angle and the midface-to-total-height proportion differed significantly between the one-stage group and the Eurocleft mean in the youngest age group, but not in the intermediate and oldest age groups. The mean and standard deviation of the differences between two skeletal ratings are listed in the legend of Table 2.

The soft-tissue maxillomandibular relationships in all one-stage groups did not differ significantly from the corresponding Eurocleft mean (Table 3). In all one-stage groups, the midface-height-to-total-facial-height ratio was significantly higher than the Eurocleft mean, thus closer to normal values.

The lateral incisors were absent on the cleft and noncleft sides in 66.7% and 45.5% of cases, respectively (Table 4). The dental arch relationship, according to the EUROCRAN Index, in the youngest and intermediate age groups had a similar distribution: about 55

% had grade 1 or 2 (class I apical base relationship), and about 45 % had grade 3 or 4 (edge-to-edge or class III apical base relationship). In the oldest age group, 36.5% had grade 1, none had grade 2, and 63.5% had grade 3 or 4 (Table 4)

An alveolar cleft ossification (Bergland Score) of grade I or II was found in 60.6%, and of grade III in 15.2%; secondary alveolar bone graft was necessary in 24.2% of the total sample using an autologous cancellous bone graft from the iliac crest.

The mean nasalance values in the one-stage group (M = 12.13, SD = 8.68) did not differ significantly from an age- and sex matched healthy control group (M = 11.27, SD = 5.05): unpaired *t* test with Welch's correction for unequal variances, t(94) = 0.67, p>.05.

From the total sample of 33 patients, 20 (61%) underwent a subsidiary procedure (Table 5). The total number of surgeries in the one-stage cohort (2.1) was about half of that from centers with multi-stage procedures (3.5 to 4.8). No palatal fistulae occurred in the cohort, no orthognathic surgery was performed.

Discussion

Comparison with multi-stage procedures

In 72% from 201 European cleft centers the teams use 2 surgeries to close the lip, and hard and soft palates.[7] Two-thirds of the teams complete this treatment within the patient's first year of life. It seems reasonable to consider combining both surgeries.

The maxillary protrusion (SNA, ANB) and the vertical facial development in all one-stage age groups did not significantly differ from the mean values of the Eurocleft centers. A detailed comparison with the different centers of the Eurocleft Study revealed that the SNA lies between the Eurocleft values for the youngest age group, equal to the best value for the intermediate age group, and 0.4º inferior to the lowest value for the oldest age group.[8]

The mean Eurocleft SNA value is about the same as that determined for delayed hard-palate closure samples at an intermediate age (75.3° vs 75.5°)[8,14,15] and oldest age (74.4° vs 74.3°)[8,14] and is therefore quite representative for a variety of multi-stage procedures. Further the SNA value is robust against the error of landmark identification,[12] with the mean difference between two ratings of -0.4° (SD 1.4) in our sample (Table 2).

However, growth generalisation cannot be based solely on the SNA value.[15] Some cephalometric changes in cleft patients are due to the malformation itself, some occur after any kind of repair, and some are truly related to the protocol and surgeon. Unwanted protocol-dependent growth patterns are (1) reduced anterior maxillary height (2) reduced posterior maxillary height with clockwise rotation of the maxilla, and (3) midface retrusion due mainly to reduced maxillary length. The oldest one-stage group did not differ significantly from the Eurocleft mean in terms of these protocol-dependent growth patterns, but a nonsignificant maxillary shortening was noted. One contributing factor might be the high rate of missing lateral incisors in our sample.[16]

However, since maxillary length can be close to normal if the alveolar nasal floor has not been repaired at all,[15] the primary alveolar bone grafting needs to be judged critically.

Furthermore, it is suspected that primary alveolar bone grafting interferes with anterior maxillary growth, since of the nine patients who exhibited the best occlusal relationships (EUROCRAN grade 1) six of them experienced severe resorption of the graft after surgery. Thus, contrary to the original assumption, it seems that minimal and not maximal stabilisation of the alveolar segment leads to favourable occlusion. We thus abandoned obturators to keep the alveolar segments in place before surgery and abandoned primary alveolar bone grafting in 2003. In addition, the favourable occlusion in these cases suggests, that the surgical trauma per se does not necessarily compromise growth zones.

The primary alveolar bone graft was also abandoned following a regraft rate of 24%. In other protocols, the primary alveolar bone graft is placed in a separate operation after lip repair, when the alveolar cleft has narrowed.[17] However, this can be associated likewise with a regraft rate of 25% and additionally with a fistula rate of 26.8%.[17] In contrast, we did not encounter palatal fistulae, despite a wide mean anterior cleft width of 16.8 mm at the time of cleft repair. We assume that the primary alveolar bone graft together with two-layer palatal closure prevented the formation of anterior palatal fistulae.

The orthognathic surgery rate in UCLP samples from the literature ranges widely from about 20% to 45%.[17-19] The rates are difficult to compare, since they reflect not only growth outcome but also differences in the patients' demands, surgical cut-offs, and health-care funding. In the oldest group, we expect a 45% orthognathic surgery rate, while in the youngest group, a 20% orthognathic surgery rate is highly probable, since 20% have an SNA below –1° at 6 years.[20] Thus, we expect an orthognathic surgery rate that is in the upper range of that for other multi-stage procedures. Figure 1 and 2 shows the same patient, as preoperatively shown in the initial publication of the surgical technique from 1996.[1]

Fig. 1, 2. Facial profile and occlusion at 18 years 8 months of the same patient who was shown in the initial publication about the one-stage surgical technique in 1996.[1] He had velopharyngoplasty as a subsidiary operation at the age of 8 years 9 months and will have orthognathic surgery to complete the treatment (photograph published with the patient's consent).

Comparison with other one-stage procedures

De Mey et al. reported on a one-stage procedure with a cranial-pedicled vomer flap.[3] They excluded 48% of patients from the procedure due to their posterior cleft width being >11 mm at the time of surgery. Brusati also restricted the one-stage procedure to those with a maximal 10-mm posterior cleft width (keynote lecture, 9th European Craniofacial Congress 2011). In the study of De Mey et al., the growth results (SNA and ANB) at 10 and 15 years of age were greater than those found in the present study.[3] Fifty-two percent of our sample had a posterior cleft width of >11 mm at the time of surgery, which might largely explain the different growth outcomes. A palatal cleft width at surgery of 10% or less of the surrounding palatal surface has been suggested as a favourable growth predictor.[21] In turn, De Mey et al. encountered a fistula rate of 42%, of which two-thirds needed a subsidiary operation.[3] This diminishes the benefit of a reduced number of surgeries.

Fudalej et al.[5] and Kulewicz et al.[2] reported on one-stage repair in an unrestricted sample using a caudal-pedicled vomer flap and lateral releasing incisions. They encountered as well a fistula rate of 46% (poster, 9th European Craniofacial Congress 2011). Their sample of 9.5- to 14.7-year-old patients yielded SNA (75.3° vs 76.3°) and ANB (0.8° vs –0.2°) values similar to those reported herein, as well as a similar mean EUROCRAN dental arch relationship grade (2.58 vs 2.57).[5] Thus, despite alveolar bone grafting and double-layer palatal closure in our sample, the growth outcome was comparable to that reported for this less extensive procedure. We assume that limiting the one-stage procedure to cases with a narrow cleft width influences the growth outcome more strongly than does the variation in surgical procedure.

The speech quality allowed all patients to engage in normal schooling with healthy peers. The velopharyngeal insufficiency (VPI) correction rate was 12%, which is close to that reported for early combined hard- and soft-palate repair in multi-stage procedure (6–11.0%).[22]

A main objective of the one-stage concept was to reduce the number of surgeries, as they are considered to be a stressor for the family and to make a successful coping more difficult.[1] Recent evidences encourage this effort, since the number of subsidiary operations was found to negatively correlate with the child's physical well-being.[23] Further, multiple anesthetics before age 4 years, but not single anesthetics, were found to be a significant risk factor for development of learning disabilities.[24] Despite some patients will undergo additional secondary procedures in the future, we expect to finish the treatment below a mean number of 3 anaesthesias per patient.

Conclusion

The one-stage procedure led to significant growth disturbance however the degree of growth disturbance was similar to the mean values of multi-stage procedures obtained in the Eurocleft study. Primary alveolar bone grafting led to inconsistent alveolar ossification and was suspected to interfere with anterior maxillary growth – it has been abandoned. The patients did ultimately benefit from a reduced number of surgical steps, since the total number of surgeries was halved compared to multi-stage procedures.

Acknowledgements

I (A.A.M.) acknowledge the received research grants from the Swiss Dental Association (SSO) and the Basel Science Society (FAG). The sponsors did not influence the study. We thank Susanne Codoni, Iris Indri, and Ines Holzman for data gathering.

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Tables

Table 1. Unilateral complete cleft lip and palate study groups after one-stage repair

Variable	Total (n = 33)	G*roup 1* *(n = 15*)	Group 2 (n = 7)	Group 3 (n = 11)
Male / female	22 / 11	11 / 4	3 / 4	8 / 3
Cleft right / left	14 / 19	6 / 9	4 / 3	4 / 7
Mean (SD) age at study (years)	13 (4)	9 (2)	13(1)	16 (1)
Mean (SD) age at repair (months)	7 (2)	6 (1)	6 (1)	7 (2)
Soft tissue lip band	7	3	1	3
Mean (SD) anterior width of cleft (mm)[a]	17 (4)	18 (5)	15 (4)	17 (3)
Mean (SD) posterior width of cleft (mm)[b]	13 (4)	14 (4)	14 (4)	10 (4)

Data are mean (SD) and rounded up to whole numbers.

[a] Distance at cleft nostril at time of one-stage repair.

[b] Distance at posterior nasal spine at time of one-stage repair.

Table 2. One-stage cephalometric dentoskeletal values compared with the mean value from all centres that completed the Eurocleft study8 (centres A, B, D, E, and F) and normals (male and female values based on cephalometric standards9). Data are mean (SD). Angles are measured in degrees.

Variable	Group 1			*p*-value	Group 2			*p*-value	Group 3			*p*-value
	One-stage (*n* = 15)	Eurocleft (*n* = 25)	Normal (*n* = 62)		One-stage (*n* = 7)	Eurocleft (*n* = 25)	Normal (*n* = 71)		One-stage (*n* = 11)	Eurocleft (*n* = 25)	Normal (*n* = 32)	
Age (years)	9 (1)	10 (1)	6 (0)		13 (1)	13 (1)	12 (0)		16 (1)	17 (1)	16 (0)	
Maxilla												
S-N-A	76 (4)[a]	77 (4)[a]	81 (3)[b]	<0.001	76 (4)[a]	75 (4)[a]	81 (4)[b]	<0.001	73 (6)[a]	74 (4)[a]	82 (4)[b]	<0.001
S-N/ANS-PNS	14 (4)[a]	10 (4)[b]	6 (3)[c]	<0.001	10 (3)	9 (4)	7 (3)	<0.05	8 (2)[a,b]	9 (4)[b]	8 (3)[a]	<0.01
Mandible												
S-N-Pog	75 (4)	75 (4)	76 (3)	<0.05	79 (5)[a,b]	76 (4)[b]	78 (4)[a]	<0.05	79 (3)	78 (4)	80 (4)	0.081
S-N/Go-Gn	35 (4)	38 (5)	35 (5)	0.095	30 (7)[a]	37 (5)[b]	34 (5)[b]	<0.05	32 (5)	36 (6)	32 (4)	0.082
Maxillomandibular												
A-N-B	3 (3)	3 (3)	5 (2)	<0.05	–0.2 (3)[a]	0.9 (3)[a]	4 (2)[b]	<0.001	–3 (4)[a]	–1 (3)[a]	3 (2)[b]	<0.001
ANS – PNS/ILs	103 (15)	99 (9)	101 (9)	0.166	106 (3)[a]	97 (6)[b]	112 (6)[c]	<0.001	110 (6)[a]	94 (7)[b]	112 (6)[a]	<0.001
ILs/ILi	154 (12)[a]	145 (16)[a,b]	141 (14)[b]	<0.05	149 (6.4)[a]	141 (10)[b]	126 (10)[c]	<0.001	142 (8)[a]	137 (10)[a]	130 (12)[b]	<0.001
Vertical												
N-ANS:N-Gn %	41 (2)[a]	42 (2)[b]	43 (5)[b]	0.071	43 (3)[a]	43 (2)[a]	45 (6)[b]	0.092	41 (2)[a]	42 (2)[a]	44 (7)[b]	<0.05

Within each row of an age-group, means with different superscripts differ at the 0.05 level of significance according to Games-Howell post-hoc comparisons. Cephalometric dentoskeletal points used in the study:[8] N, nasion (most anterior point of the frontonasal suture); S, sella, (centre of the sella turcica); A,

subspinale (deepest point on the anterior contour of the upper alveolar arch); B, supramentale (deepest point on the anterior contour of the lower anterior process); ANS, anterior nasal spine (apex of the anterior nasal spine); PNS, posterior nasal spine (intersection of nasal floor and posterior contour of the maxilla); pogonion (most anterior point on the mandibular symphysis); Go, gonion tangent point (point of intersection between the mandibular and ramus lines); Gn, gnathion (most inferior point on the mandibular symphysis); ILs, incisor line superior (axis of the upper incisors); ILi, incisor line inferior (axis of the lower incisors). Data are mean (SD) and rounded up to whole numbers.

Table 3. One-stage cephalometric values of soft tissue profile compared with the mean value from all centres that completed the Eurocleft study (centres A, B, D, E, and F)8. Data are mean (SD). Angles are measured in degrees.

Variable	Group 1		*p*-value	Group 2		*p*-value	Group 3		*p*-value
	One-stage (*n* = 15)	Eurocleft (*n* = 25)		One-stage (*n* = 7)	Eurocleft (*n* = 25)		One-stage (*n* = 11)	Eurocleft (*n* = 25)	
Age (years)	9 (1)	10 (1)		13 (1)	13 (1)		16 (1)	17 (1)	
Maxillomandibular									
ss-ns-sms	6 (3)	5 (3)	0.54	4 (3)	4 (3)	0.54	2 (3)	3 (3)	0.38
sss-ns-pgs	5 (3)	4 (3)	0.45	1.6 (3.1)	3 (3)	0.64	0.3 (3)	1 (3)	0.54
gs-sn-pgs	187 (7)	187 (6)	0.90	181 (7)	185 (6)	0.42	177 (5)	182 (8)	0.76
Nasal profile									
gs-prn-pgs	150 (5)	151 (5)	0.40	146 (6)	149 (5)	0.094	149 (4)	147 (6)	0.83
ns-unt/N-S	107 (4)	105 (4)	1.60	112 (5)	106 (4)	0.008	109 (6)	108 (5)	0.67
ns-prn-sn	105 (6)	104 (6)	0.41	93 (8)	102 (5)	0.005	93 (4)	98 (5)	0.06
nst-sn-ls	102 (11)	102 (12)	0.75	102 (8)	97 (15)	0.31	95 (12)	105 (13)	0.10
Vertical									
ns-sn : ns-gns %	43 (2)	39 (2)	0.004	45 (2)	40 (2)	0.000	43 (2)	39(2)	0.000

Cephalometric soft tissue profile points used in the study.[8] gs, soft tissue glabella (most anterior point on soft tissue glabella); ns, soft tissue nasion (deepest point on frontonasal curvature); unt, upper nasal tangent point from ns; prn, pronasale (most prominent point on tip of the nose); nst, nasal septum tangent

point (anterior tangent point to tangent to the nasal septum through sn); sn, subnasale (deepest point in nasolabial curvature); sss, soft tissue subspinale (point of greatest concavity or convexity in midline of the upper lip); ls, labrale superius (most prominent point on prolabium of upper lip); sms, soft tissue supramentale (point of greatest concavity in midline of the lower lip); pgs, soft tissue pogonion (most prominent point on the chin); gns, soft tissue gnathion (soft tissue point overlying gn).

Table 4. Occlusal rating, lateral incisors status, and alveolar bone graft ossification after one-stage repair

Variable	Total (*n*=33)	Group 1 (*n*=15)	Group 2 (*n*=7)	Group 3 (*n*=11)
Mean (SD) age (years)	13 (4)	8.5 (1.5)	12.5 (0.8)	16 (1)
Occlusal EUROCRAN grade[a]				
1	27	20	29	37
2	21	33	29	0
3	24	33	0	27
4	27.3	13	43	37
Mean (SD)	2.5 (1.2)	2.4 (1.0)	2.6 (1.3)	2.6 (1.3)
Missing lateral incisor				
Cleft side	67	73	43	73
Non-cleft side	46	40	43	55
Bilateral	36	33	29	46
Primary alveolar bone graft				
With revision operation	24	13	43	27
Without revision	76	87	57	73
Ossification grade[b]				
I	42	33	43	55
II	18	33	0	9
III	15	20	14	9

[a] EUROCRAN index of dental arch relation.10 Grade 1: apical base relation is class I or II. Both central incisors have positive overjet and overbite or there is considerably increased overjet with no overbite (note: it is grade 2 if there are obvious dental compensations). Grade 2: apical base relation is class I. Non-cleft incisor is in positive overjet and overbite. Tilting or derotation of the cleft-side incisor would achieve stable overjet and overbite (note: it is grade 3 if there is moderate open bite). Grade 3: apical base relation is edge-to-edge or mild skeletal class III. One or both central incisors are edge-to-edge or in close anterior cross-bite. Tilting or derotation would not achieve stable overjet and overbite (note: it is grade 4 if there is severe open bite or if edge-to-edge position of incisor in class III is achieved by dental compensation). Grade 4: apical base relation is class III. Both central incisors are in anterior crossbite or one is in anterior crossbite with the other edge-to-edge.

[b] Ossification grade (Bergland score) of the alveolar bone graft measured on the panoramic radiograph. Two reference lines are drawn between the teeth on either side of the cleft, connecting the root tips and the

amelocemental junctions respectively. The distance between both lines defines the expected normal ossification height in the cleft area.11 Type I = normal height of interalveolar septum; type II = height of the interalveolar septum is at least 75% of the normal height; type III = interalveolar septum is less than 75% of the normal height.

6. Fifth study:

Serum-free culture of Wharton's-jelly-derived mesenchymal stromal cells and their potential for osteoblastic differentiation for the treatment of cleft lip and palate

Study design:	Dr. Dr. A. Mueller
Financing:	One Year Research Grant of Medizinische Abteilung der Margarete und Walter Lichtenstein-Stiftung: CHF 63'000 (subsidiary to SNF grant PBBSP3-128279)
Laboratories:	Cell Therapy Institute, Lyon, France Tissue Engineering University Hospital Basel, Basel, Switzerland
Project Leaders:	Prof. C. McGuckin, Dr. N. Forraz (Lyon) Prof. I. Martin, PD Dr. A. Scherberich (Basel)
Publication:	Submitted and under review

Abstract

Cleft lip and palate are increasingly being detected by prenatal ultrasound, which has resulted in the requirement of a future surgical bone repair often being known before the baby is born. This raises the opportunity of using the patient's own osteogenicity from umbilical cord mesenchymal cells, rather than discarding the umbilical cord at birth. However, the main limitation of this procedure is the growth of the cells under a fully defined and regulated protocol. The aim of this study was thus to address this limitation, whilst simultaneously evaluating the potential hurdles to therapy.

Wharton's-jelly (WJ)-derived mesenchymal stromal cells (WJMSCs) were isolated and expanded as a monolayer with defined serum-free medium. Osteoblastic differentiation was tested in WJMSCs as well as in entire WJ biopsy specimens. Serum-free cultured WJMSCs were included in hydroxyapatite granule–fibrin constructs, and without predifferentiation subcutaneously implanted into immune-incompetent mice.

The isolation and expansion of WJMSCs was successful under serum-free conditions if the umbilical cords were processed within 48 h after birth (*n*=47). They expressed standard MSC markers but were negative for hematopoietic markers. Osteogenically differentiated WJMSCs and WJ biopsy specimens produced a mineralized extracellular matrix. The

expression of genes of osteoblastic lineage in these cells increased significantly (*Hox-A10* and *Runx2*) and downstream osteogenic genes were up-regulated (*OSX*, *OCN*, *ALPL*, and *BSP2*). Although the WJMSCs formed a dense matrix with signs of osteoblastic differentiation adjacent to the granules in vivo, no mature bone tissue was found after 8 weeks. Whilst WJMSCs have osteogenic differentiation potential, the stumbling block to their clinical use is their transplantation, highlighting the difference between the in vitro and in vivo settings. Nevertheless, we have designed a fully defined osteogenic differentiation protocol, bringing a clinical protocol closer to regulatory approval.

Introduction

Together with cardiac malformations, cleft lip and palate are the most frequent congenital malformation, and they require surgical repair within the first months of life. One in every 500–700 newborns is affected by this condition, the severity of which varies widely.

Reconstruction of the cleft in the maxilla (alveolar bone) commonly involves the use of autologous bone from the pelvic bone. This method has important shortcomings, including donor site morbidity and the reconstruction of the alveolar graft needing to be delayed to preteen age, since earlier grafting leads to growth inhibition and graft resorption. Consequently, an open gum ridge persists with adverse effects on speech and the teeth alignment. Thus, an autologous, tissue-engineered material with no donor site morbidity and that can be used early in life and complies with craniofacial bone growth and characteristics would provide multiple benefits to the patient.

The choice of a distinct stem cell source, its osteogenic potential, the envisioned clinical use, and the laboratory protocol used to engineer tissue construct must be optimized to enable translation into clinical routine. Since cleft lip and palate malformations are most frequently diagnosed by a prenatal ultrasound [1], the autologous umbilical cord could serve as a noninvasive donor site for reconstruction of the cleft maxilla. Wharton's jelly (WJ)-derived mesenchymal stromal cells (WJMSCs) reportedly exhibit a stronger osteogenic differentiation and greater bone-formation capacity compared to adipose-derived stem cells [2]. It was assumed that mesenchymal stromal cells (MSCs) from neonatal tissue are more naïve [3] and have an improved proliferation capacity [4], lifespan, and greater differentiation potential relative to bone marrow MSCs, which are derived from adult tissue. This makes WJMSCs a promising cell source for the correction of bone defects related to congenital malformations.

However, one limitation is the absence of a method for growing the cells under a fully defined and regulated protocol. The present study was designed to address this limitation, while evaluating the potential hurdles to the therapeutic use of WJMSCs. The use of animal-derived products considerably limits the feasibility to progress a validated cell culture protocol to clinical standards [5]. Regenerative concepts that work under fully defined conditions should thus be targeted.

The challenge of rendering it clinically possible to use WJMSCs for autologous osteogenic tissue engineering for cleft lip and palate was addressed in the present study by evaluating the following three factors:

1. Efficacy of the process of isolation, expansion, and storage of WJMSCs under fully defined conditions.
2. In vitro osteoblastic differentiation of WJMSCs subsequent to cultivation under fully defined conditions or inside biopsy specimens.
3. In vivo survival and tissue formation by naïve WJMSCs seeded in three-dimensional constructs, made under fully defined conditions.

Material and Methods

Serum-free isolation, expansion, and cryopreservation of WJMSCs

Human umbilical cords taken from consenting parents were stored at room temperature (RT) in phosphate-buffered saline (PBS) with penicillin-streptomycin-amphotericin B (100 U/ml, 100 µg/ml, and 0.25 µg/ml, respectively) and processed within 48 h after delivery. Adherent cells were isolated using the explant method [6]. Briefly, 2-mm-thick slices (Fig. 1A) were cut from the cord and 2.5-mm diameter biopsy specimens were taken from these slices, in between the two umbilical arteries and the vein (Fig. 1B, arrow heads). Isolation dishes (35 mm in diameter) were coated with a 4:1 mixture of PBS and 200 µg/ml collagen I–III from human placenta (ABCell-Bio, Paris, France), replaced with serum-free medium SPE-IV/EBM (ABCell-Bio) and four WJ biopsy specimens, and maintained in a humidified atmosphere with 5%CO_2 and at 37°C. The medium contained clinical-grade human albumin and recombinant human growth factors. The cells were first passaged to a surface ratio of 1:3 to 1:5 after approximately 2 weeks using a cell-dissociation reagent that is free of animal- and human-derived components (TrypLE Select, GIBCO, Invitrogen, Carlsbad, CA, USA).

A 5100 Cryo 1°C freezing container was used for cryopreservation (Nalgene, Rochester, NY, USA). Cryopreservation medium that was free of human and animal components (Cryo3 Ref. 5617, Stem Alpha, St. Genis L'Argentière, France) was mixed with 10% dimethyl sulfoxide (DMSO; Ref. 8418, Sigma-Aldrich, St. Louis, MO, USA). WJMSCs in a confluent T25 flask, or alternatively three WJ biopsy specimens were suspended in 1.8 ml of cryopreservation-DMSO solution. The box was stored at –80°C. After thawing, the WJ biopsy specimens and WJMSCs were cultured as described above.

Osteogenic differentiation medium

The osteogenic medium contained high-glucose Dulbecco Modified Eagle's Medium (Ref. GIBCO 41966, Invitrogen) supplemented with 10% low-IgG fetal bovine serum (Ref. DE14-870E, Lonza, Basel, Switzerland) and the following osteogenic factors: 10 mM β-glycerol phosphate (Ref. G9422, Sigma-Aldrich), 50 µg/ml ascorbic acid (Ref. A4403, Sigma-Aldrich), and 10 nM dexamethasone (Ref. D2915, Sigma-Aldrich). The osteogenic control medium was free of any osteogenic factors. The complete media were stored in the dark at +4°C and used within 1 month. The osteogenic differentiation was initiated at a culture confluency of 80%, and the medium was changed twice per week for 3 weeks.

Constructs of WJMSCs, fibrin, and hydroxyapatite and in vivo test

Serum-free isolated and expanded WJMSCS at passages 2 and 3 were combined with fibrin sealant (TISSEEL Kit, Baxter Innovations, Wien, Austria) and porous silicate-substituted hydroxyapatite (Actifuse Microgranules, ApaTech, Hertfordshire, UK). WJMSCs (3×10^6) were suspended in 30 µl of fibrinogen (40 mg/ml) and mixed with hydroxyapatite microgranules (60 mm^3) that had been washed with serum-free medium. Coagulation was triggered by adding 30 µl of thrombin (12 IU/ml) and the preparation was incubated for 15 minutes at 37°C and 5% CO_2. Constructs without cells served as controls. For in vivo experiments, the constructs were implanted subcutaneously in the back of five female, 6-week-old immune-

incompetent mice (CD-Nu/NU; Charles River, Wilmington, MA, USA). The experiment was conducted in duplicate and the mice were euthanized 8 weeks after implantation (animal permit No. 1797).

Fluorescence-activated cell sorting

Serum-free isolated and expanded WJMSCs were incubated for 30 minutes at 4°C with fluorochrome-conjugated antibodies raised against the indicated proteins or an isotype control (Table 1). The cells were analyzed using fluorescence-activated cell sorting with the aid of a FACSCanto flow cytometer and software, by collecting 10,000 events (BD Biosciences, San Jose, CA, USA).

Quantitative real-time polymerase chain reaction

Serum-free isolated and expanded WJMSCS were expanded for 3 weeks in osteogenic medium and in control medium. Total RNA (4 µg) was used in the reverse transcription to synthesize 4 µg of cDNA (high-capacity cDNA reverse transcription kit, Invitrogen). The reaction was carried out in a thermal cycler TC-512 (Techne, Minneapolis, MN, USA). The template was 100 ng of cDNA in the presence of 0.25 µl of forward and reverse gene-specific primers (Table 2), and 10 µl of EXPRESS SYBR GreenER qPCR Supermix Universal (Invitrogen). The amplifications were performed on Mastercycler ep realplex^2 (Eppendorf, Hamburg, Germany). The relative gene expression levels were calculated as $2^{-\Delta\Delta Ct}$ using four samples for every *t*-test, and three housekeeping genes in replicates [7].

Labeling and staining

The viability of the cells was checked with a live-dead assay (Ref. L3224, Invitrogen); viable cells exhibit calcein green cytoplasmic fluorescence and the nuclei of dead cells exhibit red fluorescence. WJMSCs were fixed with 4% paraformaldehyde (Ref. HT5011, Sigma-Aldrich) for 10 minutes. Alizarin red stain was prepared fresh (20 mg/ml), filtered, and then left for 2 minutes on the cells. The OsteoImage mineralization assay was used for specific green-fluorescence labeling of the hydroxyapatite (Ref. PA-1503, Lonza).

In-vitro-differentiated WJMSC samples were fixed in alcohol-formol acetic acid for 24 h at RT, decalcified, and then dehydrated in acetone and xylene baths to prepare them for paraffin embedding; they were then sliced at 5 µm. The sections were stained with a solution of hematoxylin of Harris, eosin, and safranin. Von Kossa staining was performed in 5% silver nitrate under ultraviolet light with 2% sodium thiosulfate, followed by counterstaining with hematoxylin of Mayer.

The implanted constructs were excised as a single piece, fixed in 1% paraformaldehyde, and decalcified in EDTA-based decalcification solution for 2 weeks. The decalcified samples were embedded in paraffin and then sectioned at 7 µm. After being subjected to a conventional dewaxing and rehydration sequence, the sections were stained with either hematoxylin and eosin or Masson's trichrome (Ref. 361350, Reactifs RAL, Martillac, France). Cells of human origin were identified in the large constructs implanted in rats by staining the sections as described previously for human-specific Alu sequences with the aid of a ZytoFast CMV chromogenic in situ hybridization kit (Zytovision, Bremerhaven, Germany) [8].

Immunohistochemistry

The slides were incubated overnight at 4°C with polyclonal anti-BSP2 antibodies (Ref. ALX-210-312, Enzo, Farmingdale, NY, USA), monoclonal anti-alpha smooth muscle actin (α-SMA; Ref. MCA1905HT, AbD Serotec, Oxfordshire, UK), or anti-collagen I or V antibodies (Refs. 20111 and 20511, Novotec, Lyon, France), diluted in PBS-BSA 3% to 1/100, 1/1000, 1/1000, and 1/500, respectively. Secondary antibodies were applied (Ref. K4002 and K4000, Dako, Baar, Switzerland) and precipitated by diaminobenzidine (Ref. K3468, Dako). Counterstaining was performed with Mayer's hematoxylin.

Results

Isolation, expansion, and storage of serum-free WJMSCs

Isolation of WJMSCs from biopsy specimens of human umbilical cord always succeeded (*n*=47) if processed within 48 h after delivery (Fig. 1A,B). Although most of the samples (*n*=31) were from spontaneous deliveries rather than from cesarean sections (*n*=16), no bacterial or fungal contamination occurred. After 1 week, about half of all WJ biopsy specimens had condensed and the emigration of spindle-shaped cells had commenced (Fig. 1C). After 2 weeks, a dense layer of regular spindle-shaped cells had formed (Fig. 1D).

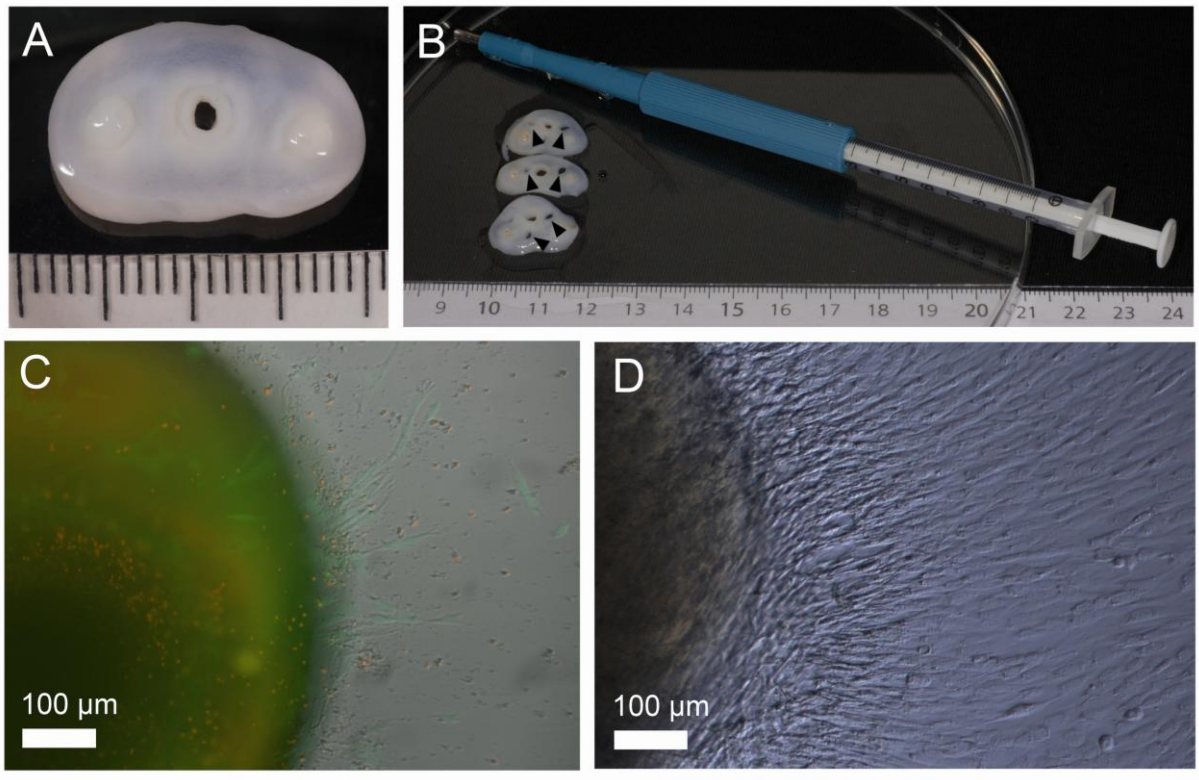

Fig 1. Isolation of WJMSCs. (A) A 2-mm-thick slice of human umbilical cord. (B) WJ biopsy specimens of 2.5 mm in diameter (arrow heads) are taken using a skin biopsy tool connected to a 1 ml syringe. (C) Viability assay of cell outgrowth from the WJ biopsy specimens after 1 week. (D) After 2 weeks, a dense cell layer surrounds the biopsy specimens. (A, B; increments on the scale are millimeters)

In vitro, the mean±SD number of WJMSCs was 121,600±72,519 per 35-mm diameter dish at the first passage and $1.1\pm0.6\times10^6$ per confluent T25 flask at the second passage. Vials containing WJMSCs at passage 1 or fresh WJ biopsy specimens were stored for more than 1 year at –80°C. After thawing, the WJMSCs and WJ biopsy specimens exhibited identical light-microscopic morphological appearances and expansion or isolation behaviors as their fresh counterparts (data not shown).

Surface markers of serum-free isolated WJMSCs

The surface marker expression of the WJMSCs was analyzed after isolation, cryopreservation for 1 month at passage 1, and re-expansion in serum-free medium to passages 3–5. The donors were the same as for the in vivo experiments. WJMSCs expressed standard MSC markers but were negative for hematopoietic markers (Fig. 2). WJMSCs expressed CD73, CD44, CD13, CD90, CD10, HLA-ABC, CD105, and CD166 but not or to a very low extent CD106, CD15, CD31, CD34, CD45, HLA-DR, and CD133. This spectrum of marker expression is typically known from MSCs derived from adipose tissue and bone-marrow (Table 3).

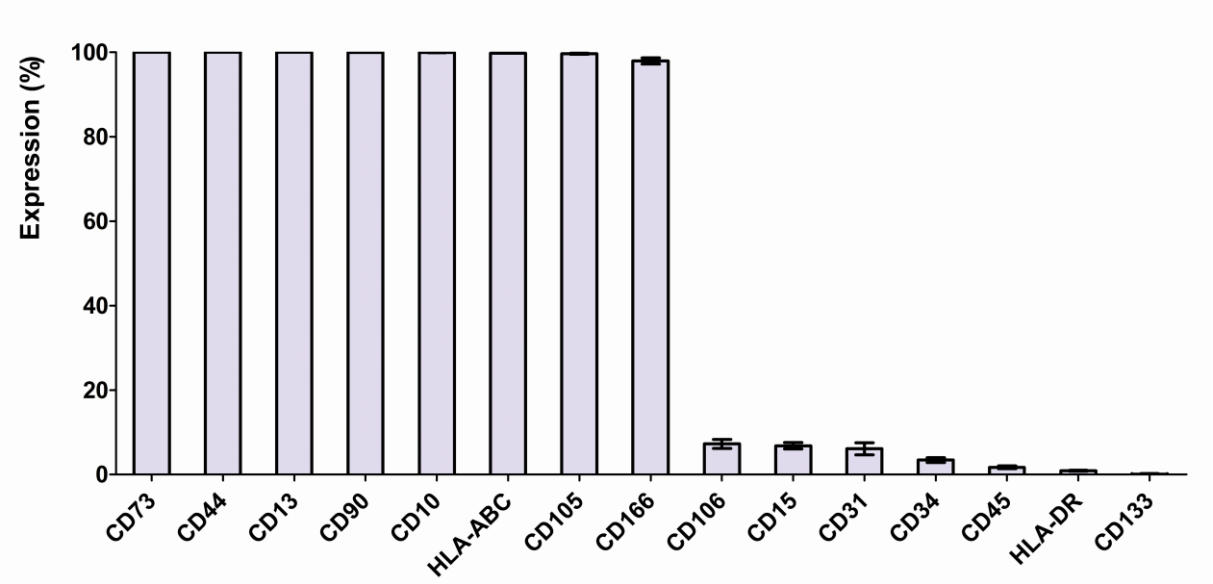

Fig. 2. Mean±SD values of flow cytometric expression of surface markers in WJMSCs after isolation, cryopreservation, and expansion in fully defined media (*n*=5; passages ≤5): CD73, 100±0%; CD44, 100±0%; CD13, 100±0%; CD90, 100±0%; CD10, 100±0.1%; HLA-ABC, 99.8±0.1%; CD105, 99.7±0.3%; CD166, 98.0±1.7%; CD106, 7.3±2.6%; CD15, 6.8±1.9%; CD31, 6.1±3.6%; C34, 3.4±1.4%; CD45, 1.7±0.8%; HLA-DR, 0.9±0.3%; and CD133, 0.1±0.2%.

In vitro monolayer osteogenic differentiation of WJMSCs

Serum-free isolated and expanded WJMSCs exhibited a high cell viability after 3 weeks of osteodifferentiation as well as in controls (Fig. 3A,D). In contrast to the controls (Fig. 3E,F), the osteodifferentiation samples exhibited mineralization, positive for hydroxyapatite staining with Alizarin red and OsteoImage (Fig. 3B,C).

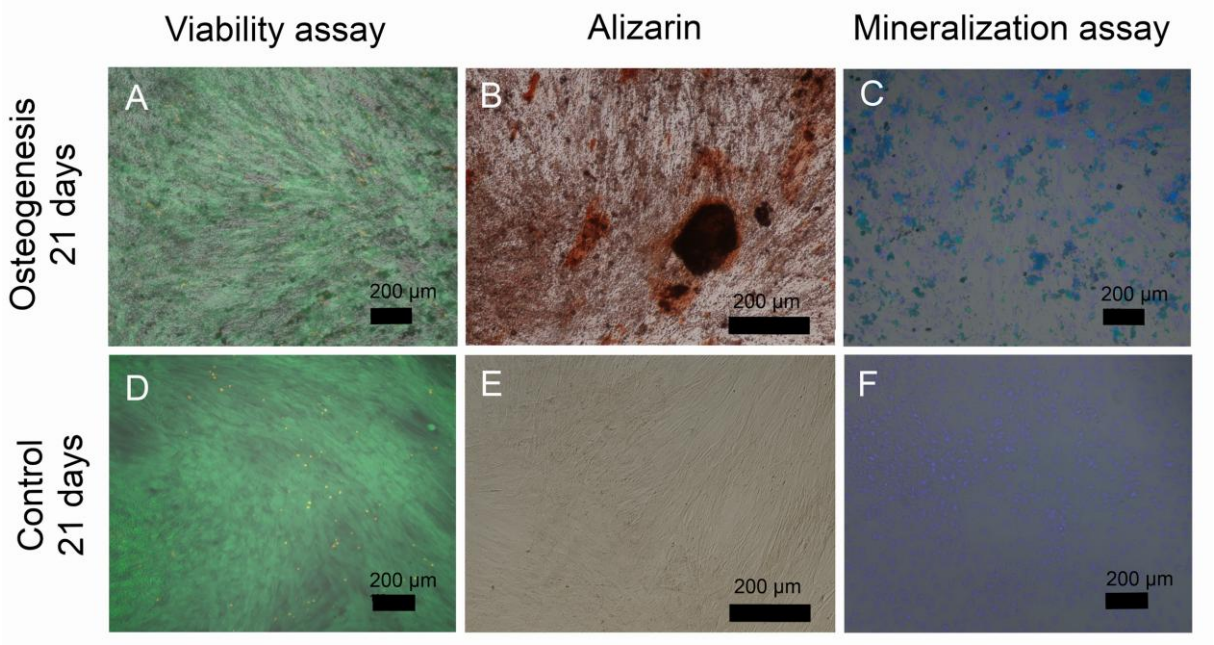

Fig. 3. Serum-free isolated and expanded WJMSCs after 21 days of differentiation with (A–C) and without (D–F) osteogenic factors, showing equally high viability (A). Positive staining for hydroxyapatite using Alizarin (B) and OsteoImage (C) in the differentiation group and negative staining in the control group (E, F).

Since the histology of the monolayer of osteodifferentiated WJMSCs exhibited osteogenic commitment, the osteogenic transcription profile was analyzed further. The expressions of osteogenic downstream genes increased after 3 weeks of osteodifferentiation. The increase was significant for the early downstream genes *RUNX2* and *Hoxa10,* and a non-significant trend was observed for *OSX*, *ALPL, OCN, and BSP2* (Fig. 4). The *STAT3* of the JAK-STAT pathway was significantly up-regulated and the WNT signaling cascade was inhibited by significant up-regulation of the WNT antagonist *DKK1* and a consecutive decrease in *CTNNB*, the downstream component of the canonical WNT signaling pathway (Fig. 4).

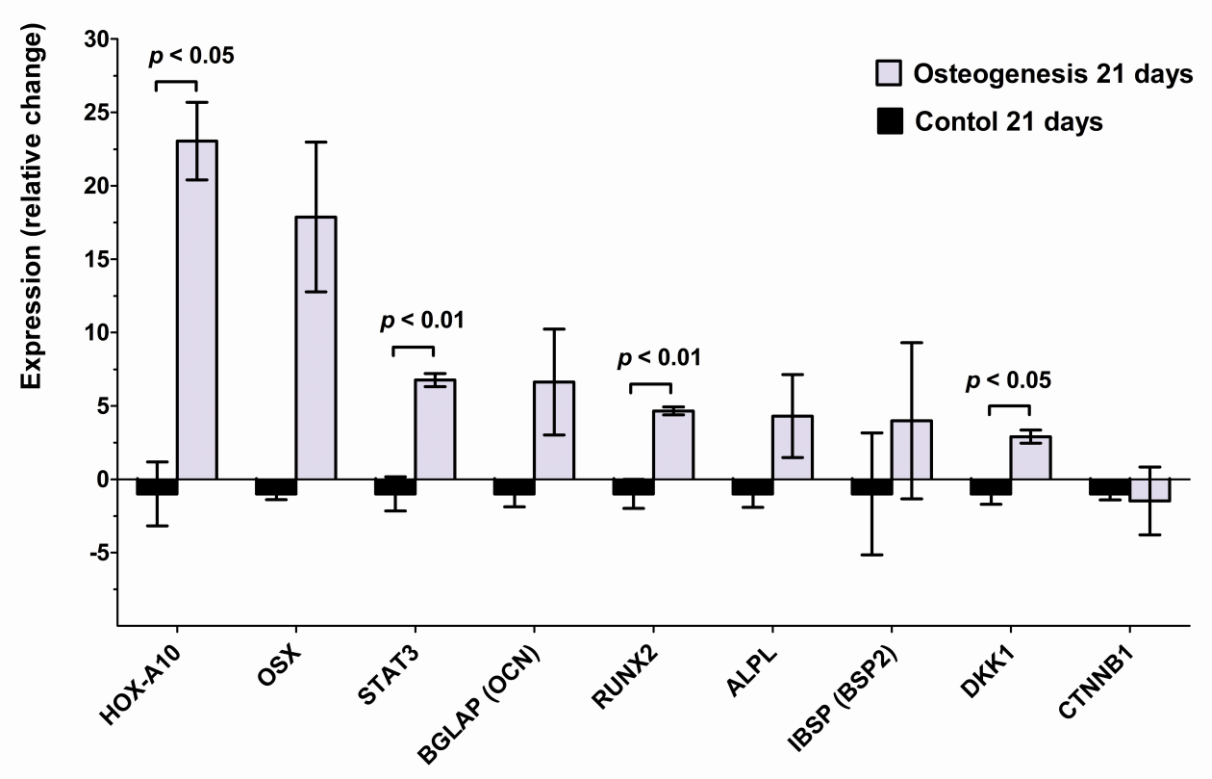

Fig. 4. Transcription profile of serum-free isolated and expanded WJMSCs after 21 days of differentiation with osteogenic factors (gray bars), presented as mean and SD values of the relative change in gene expression compared to the control condition without osteogenic factors (black bars). Transcription profile of controls are normalized to –1 (n=4; passages ≤5). Gene, relative change, p value: *HOX-A10*, 23.1, p<0.04; *OSX*, 17.9, p<0.16; *STAT3*, 6.8, p<0.005; *BGLAP*, 6.6, p<0.19; *RUNX2*, 4.7, p<0.005; *ALPL*, 4.3, p<0.21; *IBSP*, 4.0, p<0.58; *DKK1*, 2.9, p<0.01; and *CTNNB-1*, –1.5, p<0.65.

In vitro osteogenic differentiation of WJ biopsy specimens

Since WJ itself is composed of collagen I, as with bone, the direct use of WJ biopsy specimens itself as a scaffold for osteodifferentiation is appealing and was therefore tested for such. The osteodifferentiated WJ biopsy specimens (Fig. 5A), but not controls (Fig. 5D), exhibited peripheral hematoxylin-positive staining after 3 weeks, presumably corresponding to acidic mucopolysaccharides, like in bone matrix. At these sites hydroxyapatite deposits were confirmed by von Kossa (Fig. 5B) and OsteoImage staining, respectively (Fig. 5C), whereas it was absent in controls (Fig. 5E,F). In every donor, 80% of the differentiated WJ biopsy specimens exhibited such calcifications.

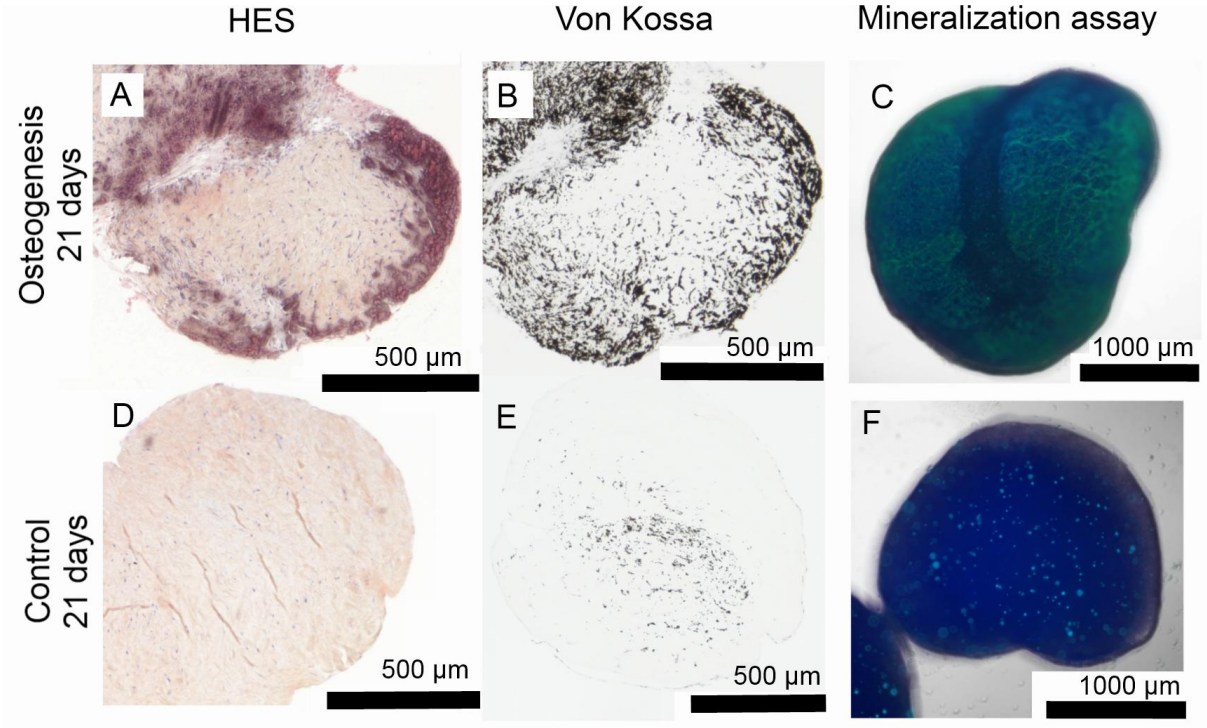

Fig. 5. WJ biopsy specimens after 21 days of in vitro differentiation with (A–C) or without osteogenic factors (D–F). (A) Basophilic granular staining (HES; Harris, eosin, and safranin) of the extracellular matrix in the periphery of differentiated samples, indicating calcifications. (B) Von Kossa stain confirming mineralization in the basophilic region. (C) Mineralization assay on native WJ biopsy specimens showing peripheral net-like hydroxyapatite staining on osteodifferentiated samples. Note the absence of contiguous mineralization zones in the control samples (D–F).

Staining for BSP2, which is a noncollagenous bone matrix protein produced by early committed osteogenic cells, was markedly increased in the periphery of osteogenic differentiated WJ biopsy specimens (Fig. 6A), with the intensity being maximal around the mineralization deposits (Fig. 6B). In control specimens BSP2 staining was negative (Fig. 6C,D).

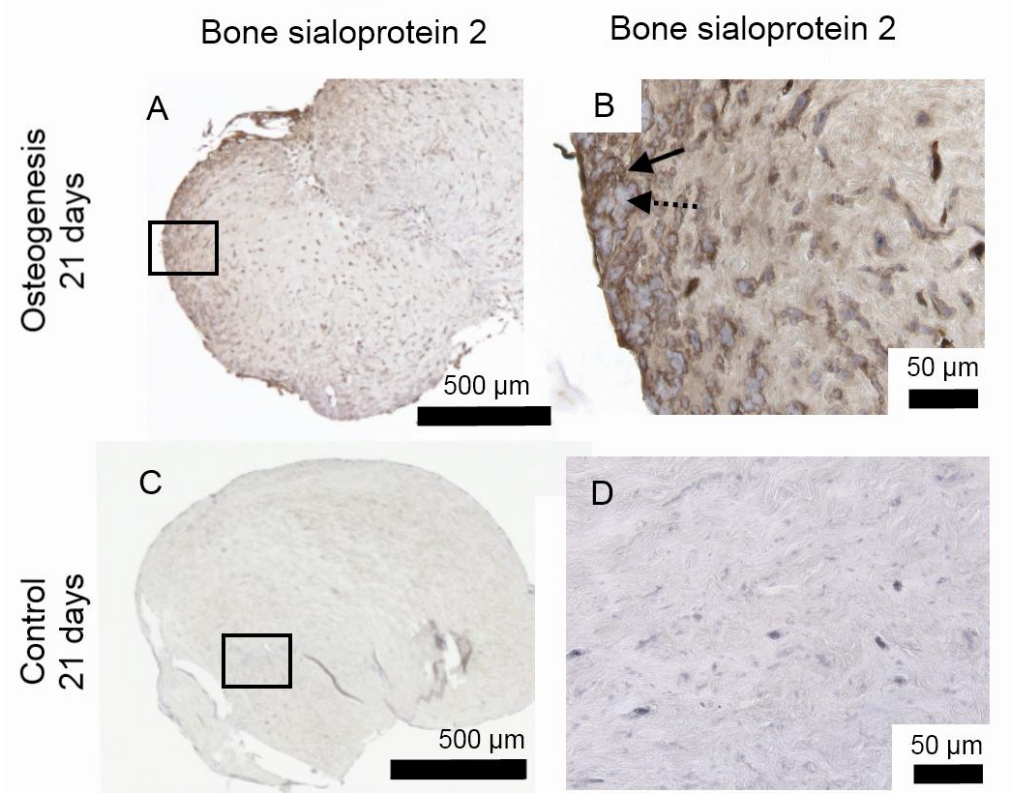

Fig. 6. BSP2-positive staining in the periphery of WJ biopsy specimens after 21 days of in vitro differentiation with osteogenic factors (A). The BSP2 staining intensity (B, solid arrow) was maximal around the bluish hydroxyapatite deposits (B, dashed arrow). Control samples without osteogenic factors were negative for BSP2 (C, D).

In vitro three-dimensional osteogenic differentiation of WJMSCs

For clinical application, WJMSCs need to be loaded into constructs that are adaptable to defects of variable shape. WJMSCs were included into constructs of hydroxyapatite granules (Fig. 7A) and fibrin. After 3 weeks of osteodifferentiation in vitro, the cells formed a dense cell layer around the hydroxyapatite granules (Fig. 7B,C), with a high rate of cellular survival (Fig. 7D).

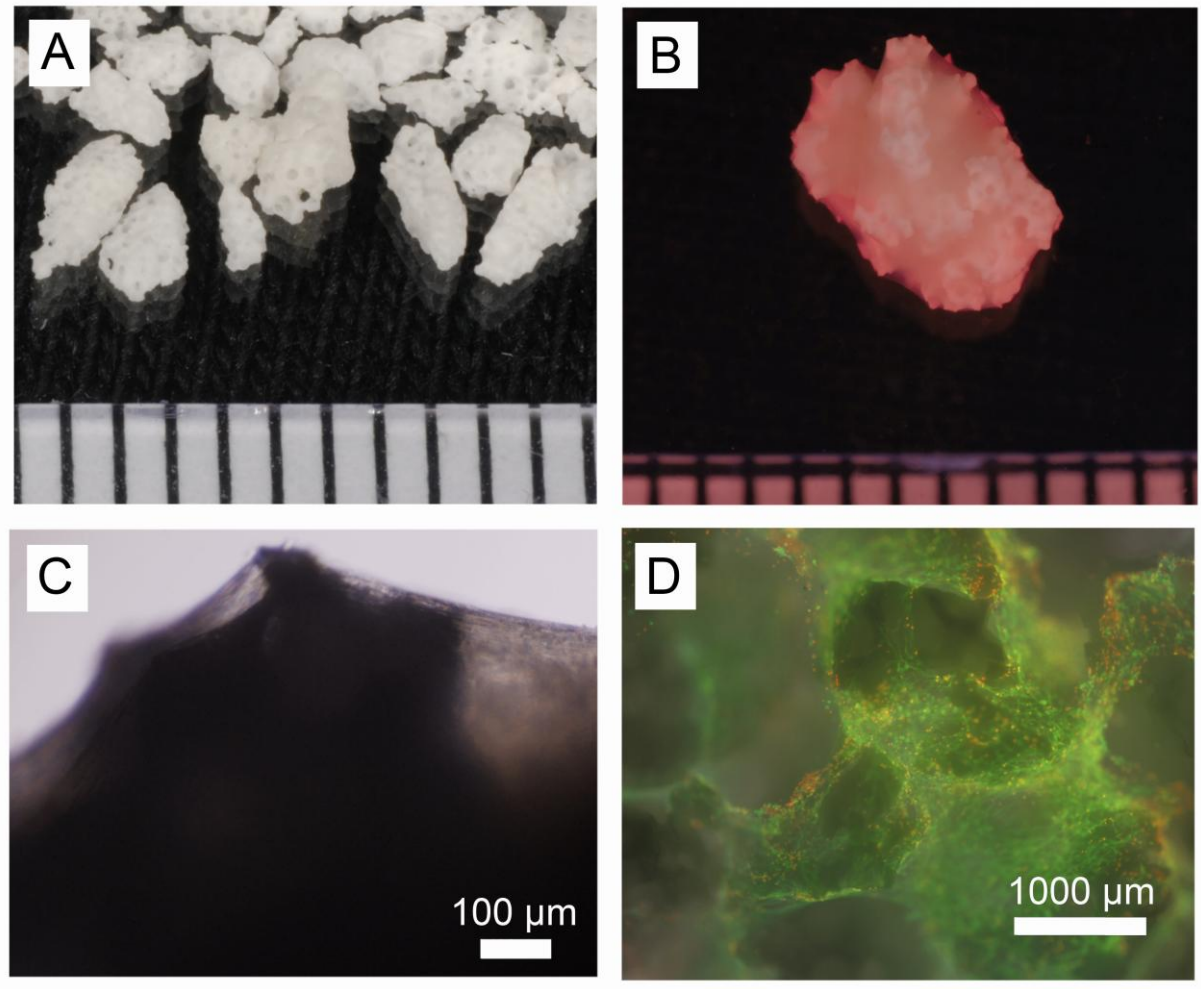

Fig. 7. Hydroxyapatite granules (A) were combined with WJMSCs and fibrin within osteogenic constructs (B). After 21 days of in vitro osteodifferentiation a dense cell layer formed around the hydroxyapatite granules (B, C), with high viability marked by green cytoplasmic fluorescence and a few dead cells marked by red nuclei fluorescence (D). (A, B; increments on the scale are millimeters)

Immunohistochemistry for collagens I and V was performed to further analyze the fibrillar matrix, and for BSP2 and α-SMA to identify osteoprogenitor cells and myofibroblasts. The WJMSCs within the fibrin-hydroxyapatite constructs synthesized abundant collagens I and V, in both the differentiation (Fig. 8A,B) and control samples (Fig. 8E,F). However, osteodifferentiated constructs (Fig. 8C,D) —but not controls (Fig. 8G,H)—exhibited layers of strongly BSP2- and α-SMA-stained cells toward the osteogenic medium and hydroxyapatite granules.

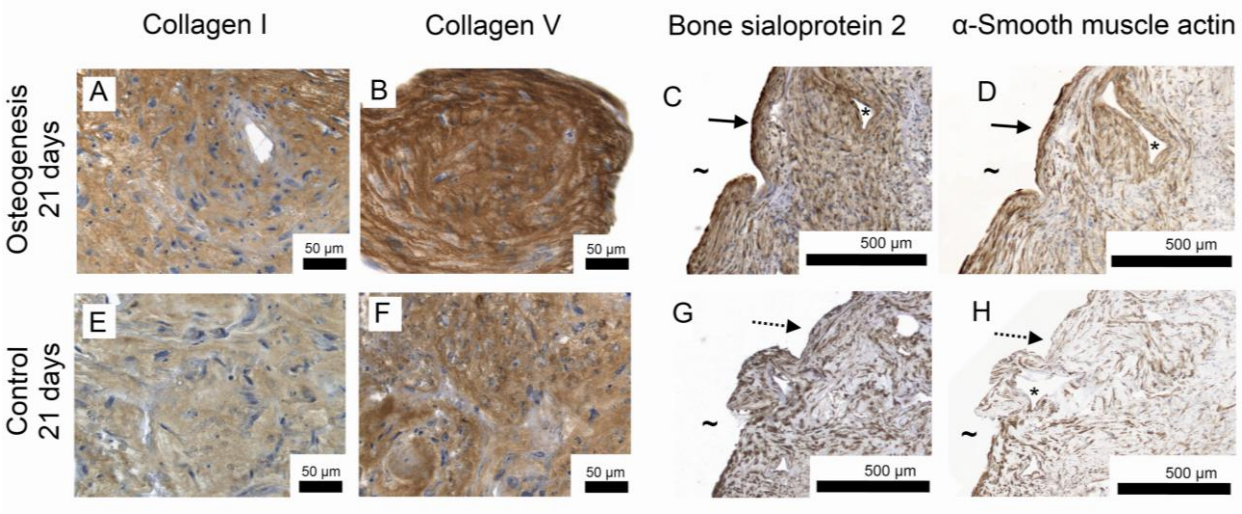

Fig. 8. WJMSC constructs with fibrin and hydroxyapatite synthesized abundant collagens I and V after 21 days of differentiation in the presence (A, B) and absence (E, F) of osteogenic factors. (C, D) Under the influence of osteogenic factors, BSP2 and α-SMA-positive cells accumulated in lamellar cell layers (solid arrow) toward the medium-containing area outside the construct (~) and hydroxyapatite granules inside the constructs (*). (G, H) In the control condition, BSP2- and α-SMA-positive cells were randomly distributed in the matrix (dashed arrow).

In vivo testing of osteogenic potential of serum-free cultured WJMSCs

The intrinsic bone-forming capacity of naïve human WJMSCs was investigated by implanting constructs of hydroxyapatite granules and fibrin with and without WJMSCs subcutaneously into immune-incompetent mice. After 8 weeks, the cell loaded constructs displayed a uniform tissue (Fig. 9A,B) of higher density than in acellular controls (Fig. 9E,F). The cell loaded constructs displayed a dense fibrillar extracellular matrix (Fig. 9C), and all of the granules were surrounded by a dense continuous cell layer (Fig. 9D). Control acellular constructs exhibited only loose connective tissue between the granules(Fig. 9G,H).

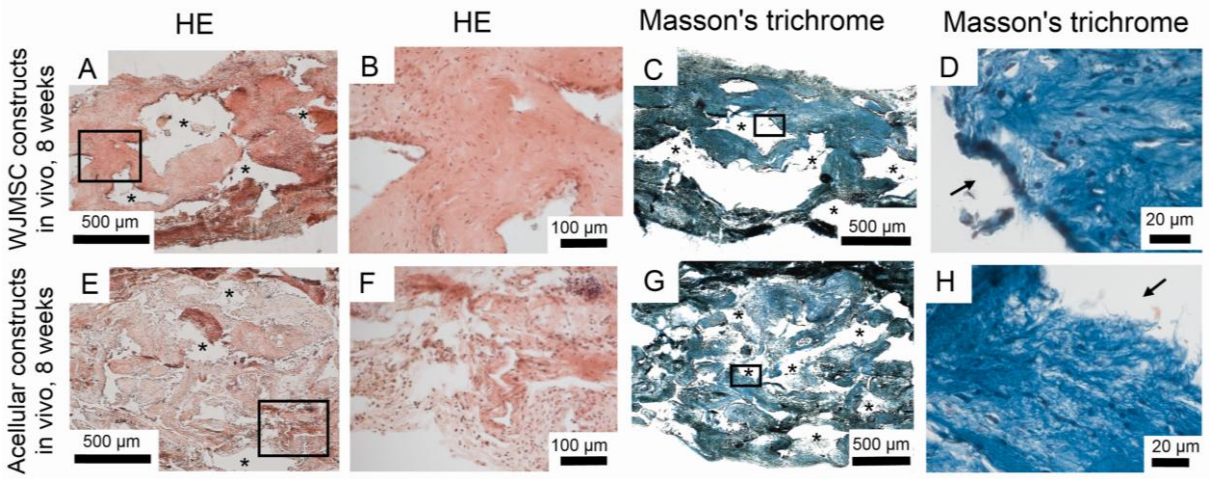

Fig. 9. Constructs of hydroxyapatite granules and fibrin with (A–D) and without (E–H) human WJMSCs after 8 weeks of subcutaneous implantation in immunoincompetent mice. WJMSC-containing constructs formed a dense uniform tissue (A, B) with fibrillar matrtix (C, D) and the hydroxyapatite granules were surrounded by continuous cell layers (D, arrow). In constructs

without WJMSCs, the tissue was irregular and loose (E, F, G), no cell layer formed around the granules (H, arrow). (*, Lacunae of hydroxyapatite granules; HE, Hematoxylin and eosin).

The staining for specific DNA human Alu sequences confirmed that the cells within the dense fibrillar matrix were derived from the human WJMSCs (Fig. 10A,B), whereas the staining was absent in acellular constructs (Fig. 10D-F). The human cells within the matrix displayed spherical large nuclei (Fig. 10C).

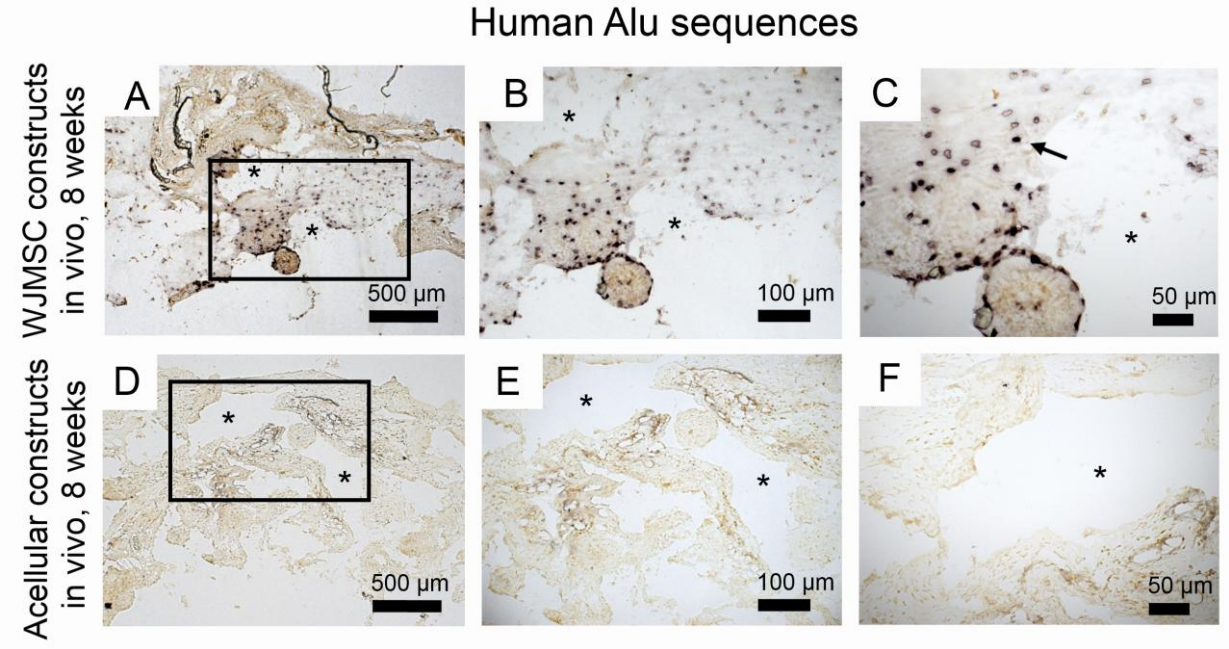

Fig. 10. Cells of human origin were identified by staining for human-specific Alu sequences. Constructs of hydroxyapatite granules and fibrin with (A-C) and without (D-F) human WJMSCs after 8 weeks of subcutaneous implantation in immunoincompetent mice. (A, B) Human WJMSCs are embedded within the dense lamellar matrices, as highlighted by the purple nucleus staining. (C, arrow) The WJMSCs showed spherical large nuclei. Constructs devoid of human WJMSCs were negative for human Alu sequence staining (D-F). (*, Lacunae of hydroxyapatite granules).

Furthermore, α-SMA staining revealed intense labeling of the cell layers adjacent to the hydroxyapatite granules (Fig. 11A,B). This finding was similar to that for the in vitro differentiated samples (Fig. 8D). In control acellular constructs, the granules were not surrounded by α-SMA-positive cells (Fig. 11C,D). The smooth muscle cells of the vessel walls (Fig. 11B,D, dashed arrows) allow the distinction of positive α-SMA staining from the background stain.

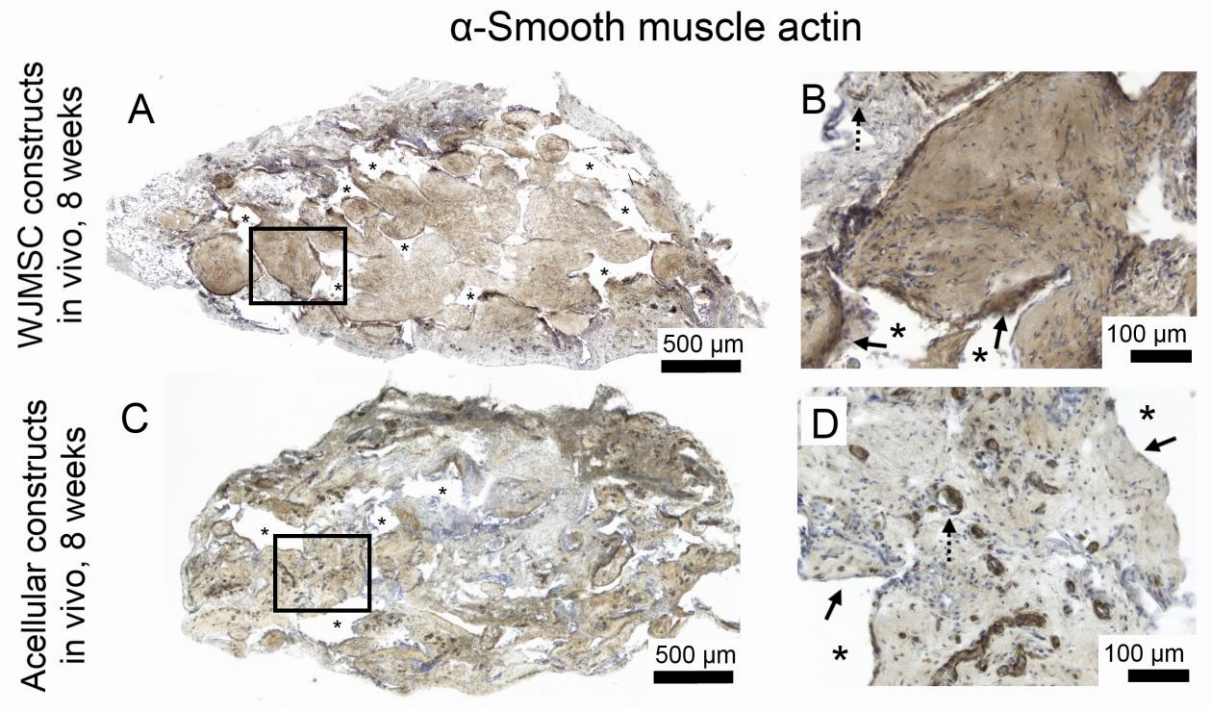

Fig. 11. Constructs of hydroxyapatite granules and fibrin with (A, B) and without (C, D) human WJMSCs after 8 weeks of subcutaneous implantation in immunoincompetent mice. (A) WJMSC-containing constructs formed a dense organized matrix around the hydroxyapatite granules. (B) The cells next to the granules stained strongly positive for α-SMA (B, solid arrow), as expected for osteoprogenitor cells. (C) In constructs devoid of human WJMSCs, the connective tissue was looser and (D) α-SMA-positive cells were absent around the granules (D, solid arrow). α-SMA staining of blood vessels acts as a positive control for α-SMA (B, D, dashed arrows). (*, Lacunae of hydroxyapatite granules).

Discussion

The stem cell type and donor site, its regenerative potential, and the intended therapeutic use must be closely aligned to increase the chance of successful clinical translation. In newborns, the umbilical cord is -in contrast to fat or bone marrow- an abundant MSC source without donor site morbidity. Since the cleft lip and palate is recognized at birth, the patient's own WJMSCs could be preserved for bone regeneration. Stem cell induced bone regeneration for cleft patients would be interesting beyond its use as an autologous bone graft. More important, bone regeneration which complies with craniofacial growth could be targeted. Since this is currently not achieved, almost 50% of cleft lip and palate patient show at the end of growth a short upper jaw with need of surgical correction to improve jaw relation and facial profile [9]. The clinical translation of a stem cell based concept critically depends upon whether it functions under fully defined conditions [10]. We therefore focused on this aspect while developing a protocol of autologous bone formation from WJMSCs.

WJMSCs are well protected in the umbilical cord and always survived for up to 48 h at RT in the present study. This time window eases the clinical handling of WJMSCs. The cord piece

can be quickly obtained even in the rush of a delivery, and can later be processed for cryopreservation or transferred into a designated laboratory. Even storage at –80°C for up to at least 1 year appears to be possible; it is therefore feasible to temporarily store WJMSCs in operating-room freezers, which are commonly available for autologous bone grafting. The isolation technique allowed the removal of 32×10^6 WJMSCs from a 10-cm cord piece at passage 1; this is sufficient for 10 constructs of 5-mm diameter, which allows the filling of cleft alveolar bone defects of any shape.

The WJMSC CD marker signature observed in the present study matched well the commonly described MSC phenotypes of adipose tissue and bone marrow, with the exception of immunostaining for CD106, which was negative in WJMSCs, whereas in bone marrow MSCs it is commonly expressed (Table 3). CD106 corresponds to vascular cell adhesion molecule 1, which mediates the adhesion of white blood cells and is not a mesenchymal-specific stem cell marker. Accordingly, in adipose-tissue-derived MSCs, CD106 is reportedly variably positive [10,11] or even negative [12]. Although defined CD marker signatures are commonly described for distinct MSC phenotypes, individual marker cannot predict the clinical performance of bone formation. Thus, regardless of the question as to whether WJMSCs are true MSCs [13], their ability to form bone remains to be established.

Osteodifferentiated WJMSCs exhibited characteristic mineralization in monolayers. The serum-free isolation, cryopreservation, and re-expansion did not inhibit its osteogenic differentiation capacity. In line with the histology, the expression of early and specific genes downstream to osteogenesis increased significantly under osteogenesis: the expression of *RUNX2* increased and up-regulated the transcription of *OSX* and *ALPL*, and the expression of *Hoxα10* increased, which is a functional element in osteoblast genes, including *OCN*, *BSP2*, and *ALPL* [14]. Accordingly, with respect to ongoing cell differentiation, the state of both of the cell signaling cascades, WNT and JAK-STAT, favored MSC migration [15] and osteogenic differentiation [16], respectively.

Osteodifferentiated WJ biopsy specimens exhibited hydroxyapatite mineralization in the WJ matrix, which we attribute to osteodifferentiation of WJMSCs within their natural niche. Bone sialoprotein, is a major noncollagenous glycoprotein of the bone matrix that promotes the initiation of mineralization. Accordingly, it was found in maximal concentrations around the hydroxyapatite particles. To our knowledge this is the first report on the osteogenic differentiation of native WJ tissue. The structural similarity between the extracellular matrix of WJ and bone, both comprising collagen I with coassembled collagen V [17], might be an advantage for bone tissue engineering based on WJ or WJMSCs. However, more studies, in particular in vivo implantation of WJ tissue in an ectopic or orthotopic model, will have to be performed to challenge the clinical suitability of this approach.

When isolated and reintroduced into compounds with fibrin and bone substitute material, WJMSCs continue to produce collagens I and V, just as in WJ, regardless of the presence of osteodifferentiation factors. However, cells producing BSP-2 accumulated toward the media and bone substitute compartments uniquely under the influence of osteogenic medium. This osteodifferentiation-medium-dependent distribution pattern of osteoprogenitor cells was confirmed by α-SMA immunostaining. Although α-SMA is mainly a marker of myofibroblasts, it is also expressed in cells that differentiate into osteoblasts [18,19] or are already engaged in bone formation [20]. Thus, we hypothesize, that the osteodifferentiation ability of WJMSCs within constructs withstand the serum-free cultivation method.

The in vivo bone-forming capacity of WJMSCs was tested in a standard in vivo model in which at the time of implantation, the compounds contained naïve WJMSCs without osteogenic predifferentiation; those compounds were implanted subcutaneously, thus excluding signaling from bone. Despite these two factors, the WJMSCs started to produce a dense lamellar matrix around the hydroxyapatite granules. The therein embedded WJMSCs displayed large spherical nuclei, resembling to osteoblastic lineage cells . Additionally, the WJMSCs next to the hydroxyapatite granules stained strongly positive for α-SMA, which potentially indicates a preserved osteodifferentiation capcity. The differentiation ability might be due to the biophysical stimulus of the hydroxyapatite and its porosity, which can direct stem cells toward the osteodifferentiation path, even in the absence of additional chemical stimuli. Although the serum-free cultured WJMSCs produced an unmineralized matrix similar to bone, the differentiation was not sufficient to produce woven or lamellar bone within an 8-week period.

The data in the literature regarding the ability of WJMSCs to produce ectopic bone after 12 weeks are inconsistent. While mature bone formation has been reported for predifferentiated WJMSCs on hydroxyapatite–polylactic-acid scaffolds [21], others have found a solely mineralized matrix, but no mature bone, when using predifferentiated polycaprolactone-tricalcium-phosphate-WJMSC constructs [2]. Nonetheless, predifferentiation of WJMSCs might critically enhance their ability to form bone in vivo. Predifferentiation has also been applied to tests of orthotopic bone formation by WJMSCs [22,23]. In vitro predifferentiation is commonly performed with bovine serum, which is difficult to replace with fully defined components. In the future, autoserum from the cord blood could be an option to satisfy autologous and fully defined conditions [24].

A striking finding of our work concerned the conversion of WJ tissue into mineralized tissue under osteogenetic differentiation. This raises new options for bone-regenerative techniques from the umbilical cord. The long survival time of WJMSCs after umbilical cord harvesting, combined with the 100% success rate of serum-free WJMSC expansion and storage, renders WJ an ideal donor tissue for autologous bone tissue engineering for cleft lip and palate. We present herein an in vivo protocol in which WJMSCs exhibit signs of osteogenic commitment and form a dense collagenous matrix under fully defined conditions, and which is compatible with clinical translation. However, more work is required to achieve mature bone from WJMSCs under fully defined conditions in vivo. The enhanced bone-forming capacity of WJMSCs might be possible with in vitro predifferentiation using autologous serum, longer in vivo differentiation times, the use of a modified bone substitute material, the addition of bone-signaling molecules, or orthotopic implantation.

Acknowledgment

The authors acknowledge the Margarete and Walter Lichtenstein-Foundation, Basel, for the research grant to A.A.M. We thank Hopital Natecia, Lyon, for assistance in collection and consenting of parents for human specimens.

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Tables

Table 1. Antibodies for fluorescence activated cell sorting

Marker	*Fluorochrome*	*Provider*	*Reference*
CD10	PE	BD Biosciences	555375
CD13	APC	BD Biosciences	557454
CD15	V450	BD Biosciences	642917
CD31	FITC	BD Biosciences	555445
CD34	APC	BD Biosciences	555824
CD44	APC-H7	BD Biosciences	560532
CD45	V500	BD Biosciences	560777
CD73	PE-Cy7	BD Biosciences	561258
CD90	FITC	BD Biosciences	555595
CD105	PE	BD Biosciences	560839
CD133	PE	Miltenyi Biotech	130-090-853
CD106	FITC	BD Biosciences	551146
CD166	PE	BD Biosciences	559263
HLA-ABC	V450	BD Biosciences	561346
HLA-DR	V500	BD Biosciences	561224

FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin; BD Biosciences, San Jose, CA, USA; Miltenyi Biotech GmbH, Bergisch Gladbach, Germany.

Table 2. Primers for quantitative real time polymerase chain reaction of gene expression

Gene	*Reference*	*BP*	*Forward primer (5' - 3')*	*Reverse primer (5' - 3')*
ALPL	NM_000478.4	132	TATAAGGCGGCGGGGGTGGT	TGTTCCAATCCTGCGCAGAGCAC
BGLAP (OCN)	NM_199173.4	159	CCTATTGGCCCTGGCCGCAC	ACTGGGGCTCCCAGCCATTGA
CTNNB1	NM_001904.3	260	TGGCCCAGAATGCAGTTCGCC	CATGCGGACCCCCTCCACAAA
DKK1	NM_012242.2	139	TTGACAACTACCAGCCGTACCCGT	CGCATGCAGCGTTTTCGGCG
HOX-A10	NM_018951.3	167	GAGCGAGCCCTCGATTCGCC	GAATTGCCCAGGGAATCCTTCTCCG
IBSP (BSP2)	NM_004967.3	76	CCAGAGGAAGCAATCACCAAA	TTGAGAAAGCACAGGCCATTC
OSX	NM_001173467.1	77	CACTCTCCCTGCCAGACCTCCAG	GCAGATGGAGAGAGCTGGGGGAAC
RUNX2	NM_001024630.3	128	ACAGAACCACAAGTGCGGTGCA	TGCTTGCAGCCTTAAATGACTCTGT
STAT3	NM_139276.2	129	CGGCTTGGCGCTGTCTCTCC	CAGGGGTCCCAACTGTTTCTCCG
GAPDH	NM_002046.3	170	GGCTGGGGCTCATTTGCAGGG	TGACCTTGGCCAGGGGTGCT
r18S	NM_022551.2	345	ACCAACATCGATGGGCGGCG	TCGGACACGAAGGCCCCAGA
PTK9	BT019691.1	190	CCAGACCGGCATCCAAGCAAGT	TGGTTGTTTGTCCTCCAACAGGGG

BP, Product size in base pairs.

Table 3. MSC marker expression in serum free cultured WJMSC compared to known expressions in BMMSC, ATMSC andWJMSC

	CD 73	CD 44	CD 13	CD90	CD10	HLA ABC (MHC-I)	CD105	CD166	CD106	CD15	CD31	CD34	CD45	HLA-DR (MHC-II)	CD 133
WJMSC	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○
BMMSC ‡	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○
ATMSC †	●	●	●	●	●	●	●	●	●	○	○	●	○	○	○
WJMSC *	●	●	●	●	●	●	●	●	●	NA	○	○	○	○	NA

MSC, mesenchymal stromal cell; WJMSC, Wharton's-jelly derived MSC; BMMSC, bone marrow derived MSC; ATMSC, adipose tissue derived MSC; NA, not applicable; ●, negative expression ; ●, positive expression; * Results compiled from [3,13,21,22,23,25,26,27]; † Results compiled from [10,11,12,28]; ‡ Results compiled from: [10,11,13,28].

7. Discussion and Outlook

The following chapter outlines, how the studies relate to current techniques of biomedical engineering and how they can contribute to the development of new surgical concepts. Based on this analysis, next promising research projects will be defined and subsequently targeted.

Computer assisted design and manufacturing for partial facial reconstruction

The morphable face model in the ***first study*** lead to realistic facial shape reconstruction of the missing nose-lip region, for which no contralateral side is available to act as a shape reference (Fig. 1).[1] The direct manufacture of the nose prosthesis from skin-colored polyamide by laser sintering fulfilled the requirements for temporary use. In a next step adjustable coloring and margin adjustments with a self-curing resin must be developed to reach the standard of permanent use.

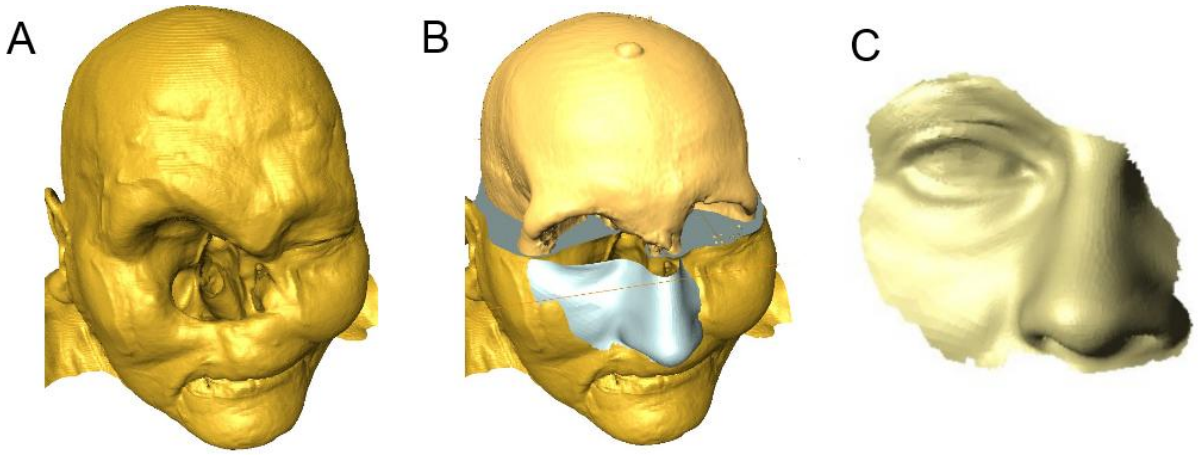

Fig. 1. Patient after nose and eye resection due to invasive skin cancer (A). The morphable face model leads to a natural shape reconstruction of the missing facial is part (B, white facial part). The modeled facial part contains complete shape information and can thus directly transferred into a scaffold for prosthetic or tissue engineering use (C).

The use of the morphable face model is not limited to prosthesis manufacturing. The shape designed could also be used for computer assisted manufacturing of patient specific scaffolds for tissue regeneration. Computer assisted manufacturing has been applied to bone substitute scaffold made from polycaprolactone, polyglycolic acid, and calcium phosphate cements.[2,3] Aside the osteogenic scaffolds, collagen could be molded into a cast of patient specific shape, to generate patient specific constructs from chondocytes.[4]

To engineer large tissue engineered constructs creates two difficulties. First, to seed the cells with a regular density throughout the constructs. Second, to ensure the blood supply for nutrition and oxygen delivery to the cells in the center of the scaffold. In vitro this problem can be solved by using a bioreactor, in which the scaffold is perfused for cell seeding and nutrition.[5] However, at the time of implantation of the large scaffolds, there is no vessel connected to the scaffold that can take over the perfusion function of the bioreactor.

Microsurgery to connect tissue or engineered constructs to the blood flow

Vascular microsurgery offers techniques to promote the vascularisation of implanted tissue engineered constructs. Mandibular reconstruction has been performed by implanting subtotal mandibular constructs made from hydroxyapatite, cancellous bone graft and BMP-7, temporarily into a cuff of latissmus dorsi muscle.[6] The muscle which contained the construct was transplanted in a second stage as a microvascular latissimus dorsi graft into the defect area. Similarly, a tissue engineered nasal framework could be implanted temporarily into a distant body site, to ensure vascularisation, maturation and soft tissue covering of the construct. Due to its bulkiness a muscle cuff would not allow natural shape contours of the nasal framework, but a vascularized periostal graft could be suitable. The periosteum in the supracondylar region of the distal femur could be an ideal donor site (Fig. 2). The periost vascularization in this region is constantly delivered by ramifications of the descending genicular artery and its accompanying veins.[7]

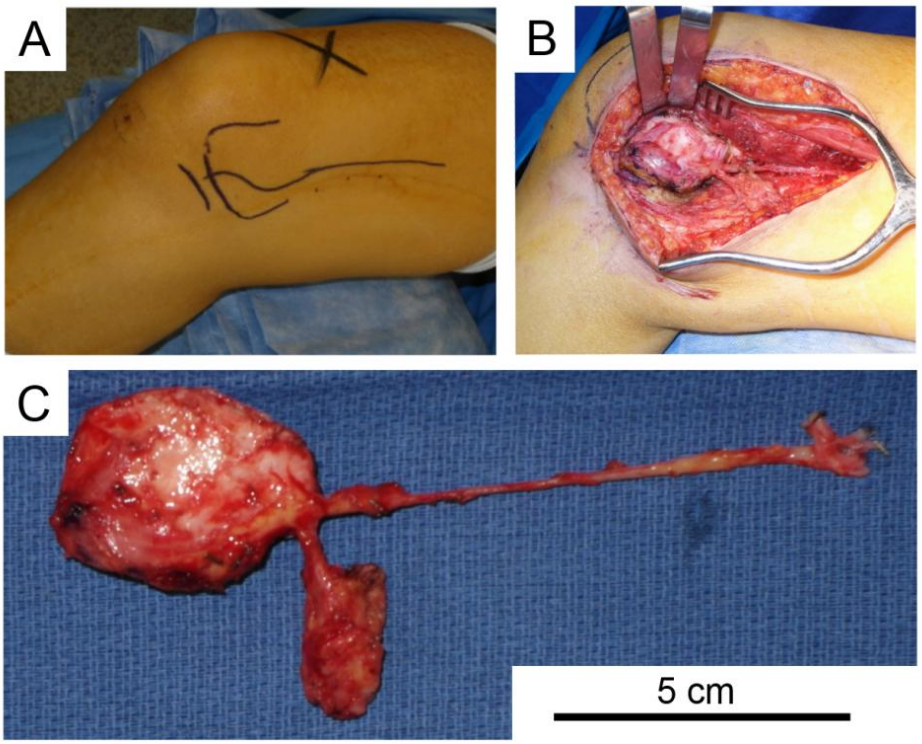

Fig. 2. Microvascular periostal or periostal-bone graft. Skin incision outlined from the medial femoral condyle ascending the thigh (A). The descending genicular artery which supplies the periost over the medial condyle is identified (B) and the periost, with or without underlying bone, can be harvested (C).

Surgical techniques and perioperative medical management of microsurgical reconstruction vary largely among surgical teams. In the ***second study*** we therefore assessed the current practice in microsurgical head and neck reconstruction in maxillofacial units of Germany, Switzerland and Austria.[8] It showed, that microsurgical techniques have become a routine technique for maxillofacial reconstructive surgery in the majority of the departments. The key to success of microsurgical intervention showed to be strongly related to the surgical technique and not to postoperative medication regimen. Therefore microsurgical techniques

can be considered as a valid surgical option to be combined within tissue engineering of large constructs in order to promote the blood and nutrition supply to the constructs.

The implantation of tissue engineered construct at a distant implant site and subsequent transplantation to the defect site has two drawbacks. It is a two-stage procedure and the vascularization is extrinsic, growing from the exterior surface to the center of the constructs. Therefore an alternative technique has been described, in which at the time of scaffold implantation a microvascular arterio-venous loop is positioned into the center of the constructs.[9] By this means an axial vascularization from inside the construct (intrinsic) can be achieved (Fig. 3).

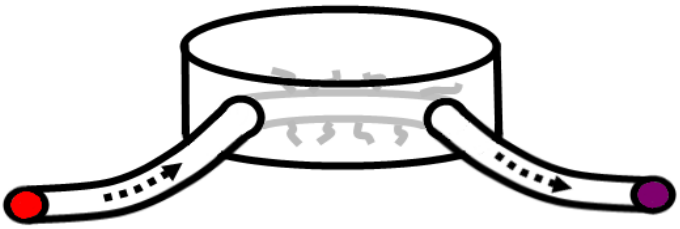

Fig. 3. Principle for axial vascularization of a tissue engineering construct. A vessel is inserted into the tissue engineering construct (cylinder) that leads to vascular outspread inside the construct (grey vessel portion).

The ***anatomical part of the third study*** reveals suitable vascular connection sites in the face to support a tissue-engineered construct in the nose region.[10] A tissue engineered construct, from the type of axial vascularization or vascularized periost, can be microsurgically anastomosed to the facial artery and vein, which has a prominent diameter at the height of the labial commissure (Fig. 4). The surgical access to the facial artery and vein, as well as the microsurical anastomosis, can be performed from intraorally, with no need for a visible surgical incision in the face .[11]

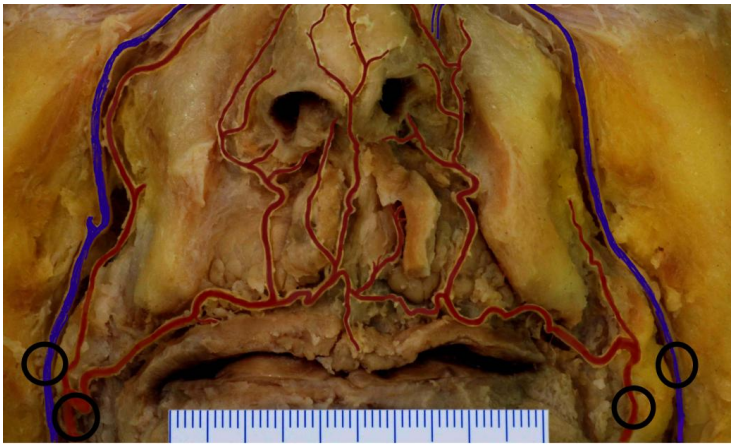

Fig. 4. Possible arterial and venous anastomosis sites (circles) for vascularized nasal tissue grafts. The facial artery (red) and vein (blue) can be accessed from intraorally.

To date, no small caliber vascular substitutes exists that can be used as implants in clinical routine. However, the use of bacterial synthetized cellulose allowed to produce 10 mm long arterial substitutes of 3 mm internal diameter that show a 88% patency rate after 3 month as carotid interposition graft in pigs (Fig. 5).[12] The bacterial cellulose synthesis is made from

glycan extrusion from *Gluconacetobacter xylinus* bacteria that aggregates into microfibrils. Currently, the endothelialisation and patency rate of 10 and 15 cm long small caliber grafts is studied.

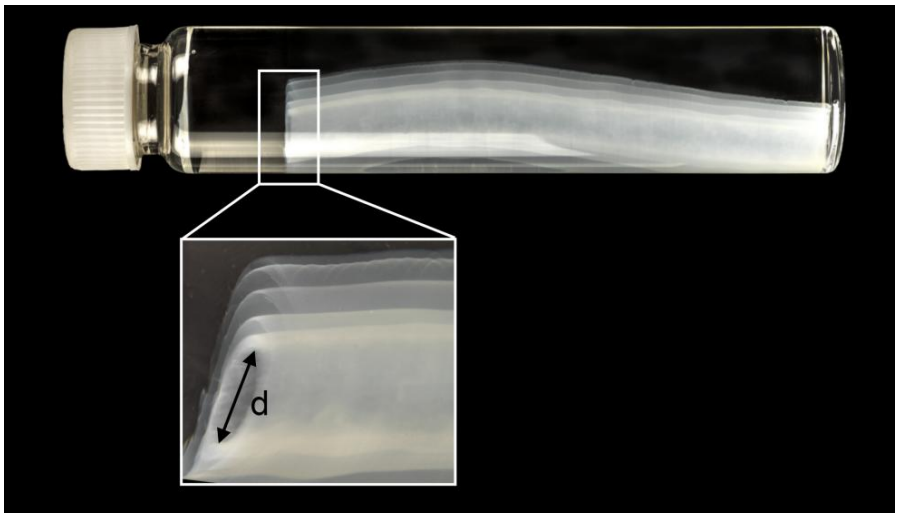

Fig. 5. Bacterial synthetized vascular graft with a minimal inner diameter of 3mm (d). At the end of production the graft is stored sterile in 0.9% NaCl. Specimen donated by Prof. Dieter Schumann, Polymet Jena, Germany.

Although small caliber vessels are engineered with the main clinical application for coronar vascular heart surgery, their potential for vascularization techniques of large tissue engineering constructs remain to be explored.

Normal vascular blood flow and microcirculation after cleft lip surgery

The importance of vascular development and blood flow applies for tissue engineering constructs as well as for the entire organism. The blood flow ensures the supply of oxygen, nutrients and defense cells to the tissue. As such it is a driving force of development and growth.

Despite its biological importance, the functional vascular anatomy remains unexplored in the cleft lip, which is one of the most frequent inborn malformations, that occurs in about every seven hundredth newborn. The cleft lip divides lip and nostril and interrupts the normal vascular anatomy of the lip-nose region. This prompted us to investigate the microcirculation and the intraoperative vascular anatomy in cleft lip and palate surgery, which forms the ***clinical part of the third study***.[10] In sum, the microcirculation measurements showed, that before and after lip repair the microcirculation in the lip-nose regions is equal to normal control and no evidence was found that would urge an microvascular reconstruction of the separated lip artery. In bilateral cleft lips, the prolabium, which is the island-like central lip part, showed a significant higher microcirculation than in normal, indicating a high hemodynamic and nutritional need in this region. If only a small soft tissue bridge is present on both sides, the preferential connection to the lip artery is established nonetheless (Fig. 6, A,B). Just, if the prolabium is completely separated from the lip, strong arterial connection forms to the dorsal nasal artery (Fig. 6 C,D).

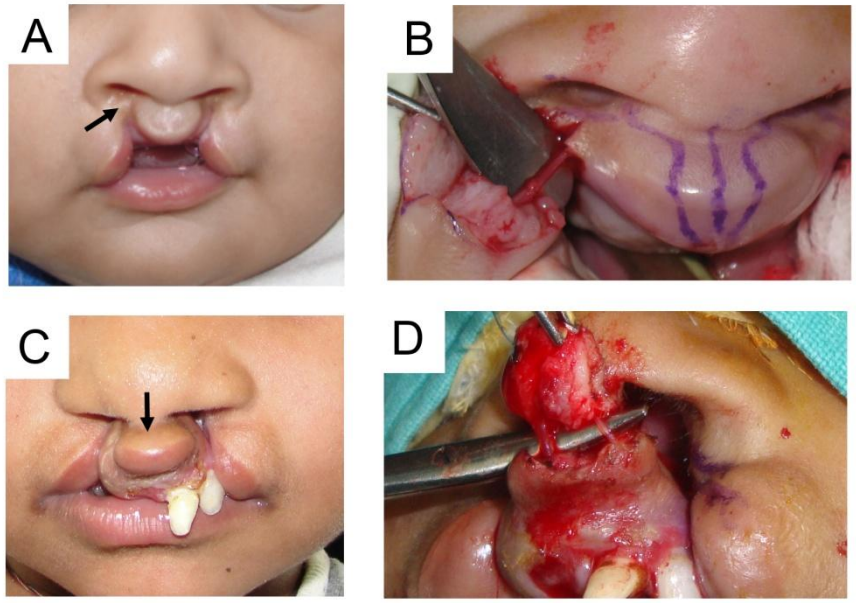

Fig. 6. Intraoperative vascular anatomy in bilateral cleft lip. If the cleft is incomplete (A) the central lip part still connects to the lateral lip part (B). In complete bilateral clefts (C) the prolabium must connect to the dorsal nasal artery (D).

The high hemodynamic and nutritional need in the central lip region might also reflect a functional hemodynamic contribution of the lip to the underlying bone. There is an intimate morphological connection between the microvascular network of the lip (Fig. 7A) and the anterior bony part of the maxilla (Fig. 7B), as visualized by anatomical corrosion casts.

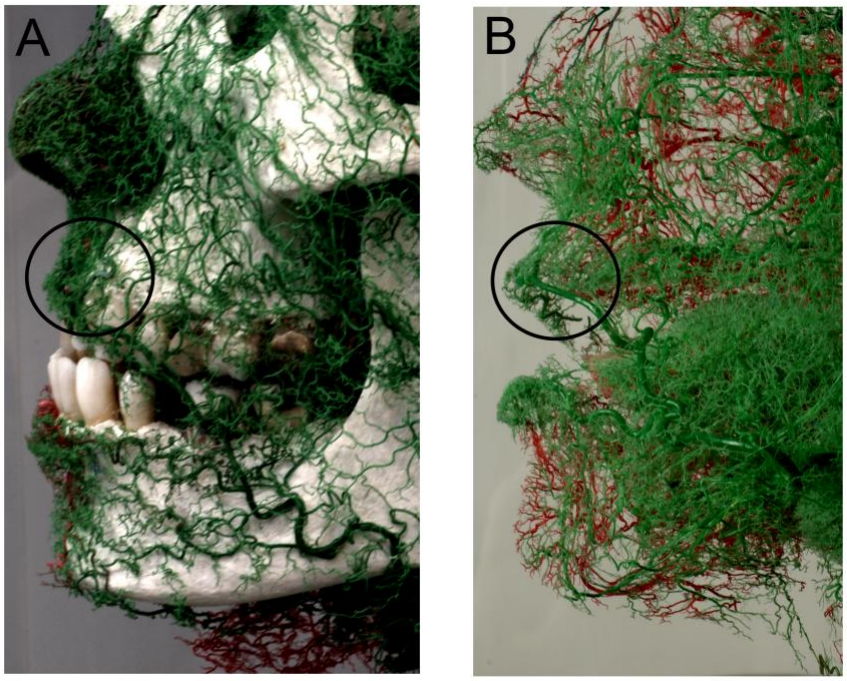

Fig. 7. The vascular network of the lip (A, circle) is intimately connected with that of the anterior maxillary bone (B, circle). Anatomical preparation by G.-R. Klaws, Kiel, Germany.

Due to the vascular morpho-functional relation between lip and underlying bone, it seems therefore advisable, that the cleft lip surgery technique should interfere as less as possible with this contiguous vascular territory. We therefore advance the lip from the cleft side to the midline without detachment or releasing incision on the alveolus.

Arteriogenesis, which is the recruitment of arterioles and enlargement of collateral vessels[13], is assumed to be the main mechanism for arterial flow restoration after cleft lip surgery. The conditions for this are favorable in the lip, due to the rich arterial network (Fig. 7A,B) and because arteriogenesis is known to be accelerated, the more dense the preexisting collateral network is.[14]

The need to recapitulate normal development in cleft lip and palate defects

Despite delicate surgical techniques, patients with a combined cleft of the lip and palate (Fig. 8), show at the end of growth frequently a short upper jaw. In literature frequencies of up to 48% of these patients are reported to undergo surgical maxillary advancement at end of growth.[15] In an attempt to reduce the maxillary growth deficiency, many different surgical techniques have been proposed and lead to a high variability of treatment plans to close a cleft lip and palate defect. Grossly, treatment plans vary on; 1) The number of surgeries used to close the lip, alveolar part of the maxilla, hard and soft palate (Fig. 9); 2) The sequence in which lip, hard- and soft palate is closed; 3) The age of the patient at the time of the surgeries.

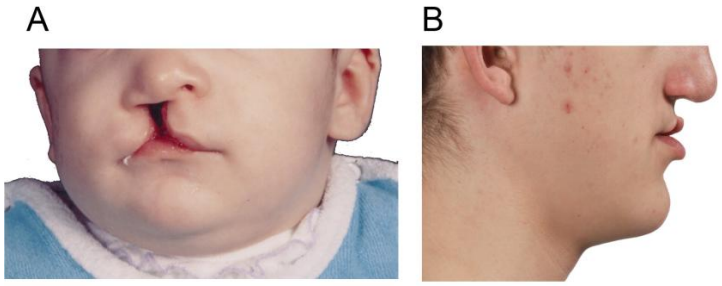

Fig. 8. Maxillary growth deficiency. (A) Patient at 5 month of age before one-stage cleft lip, alveolus, hard- and soft palate repair. (B) Patient at 17 years, without having undergone jaw corrective surgery, exhibit shortness of the upper jaw.

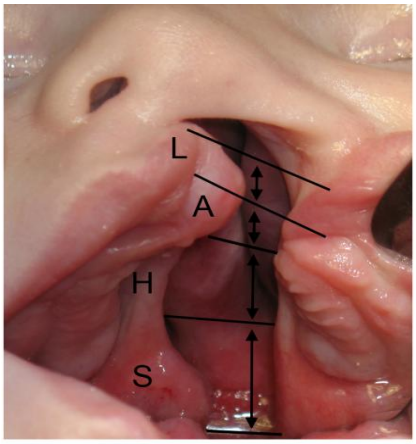

Fig. 9. Complete cleft lip and palate. The anatomical portions of the cleft comprise: lip (L); alveolar part of the maxilla where teeth will erupt (A); osseus hard palate (H); and muscular soft palate (S).

Due to variations in the sequence and number of operations to close a cleft lip and the patients age at the time of surgery, the treatment schedule vary widely in european countries .[16] Consequently, growth findings from a specific treatment plan have only limited validation for a diverging concepts. We assessed therefore the longterm growth results of the applied one-stage surgical concept of our clinic in the ***forth study***.[17]The surgical closure of the cleft lip, alveolus and palate in one surgical procedure at the early age of 6 month

showed comparable growth results as compared to staged procedures at a later stage. However, the results indicated, that the early interposition of a rib bone graft into the alveolar cleft might inhibit the growth. The osseus fusion might block the growth movement of the anterior maxilla. Early alveolar bone grafting has also shown negative growth effects in combination with multi-staged closure of cleft lip and palate defects. It has therefore become common sense, to postpone the osseous bridging of alveolar cleft to the late preteen age, when most of the craniofacial growth has taken place and when the permanent teeth need to be aligned in the cleft area.[17]

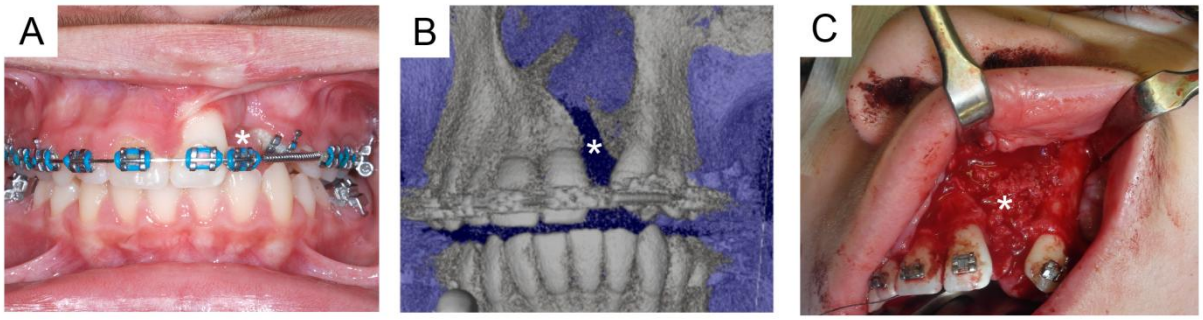

Fig. 10 Alveolar bone grafting at preteen age. Although ghe gum of the alveolar maxillary parts are in contact a broad gap in the dental arch remains (A, *). There width of the alveolar part of the maxilla is seen in the radiography (B, *). Cancellous graft from the patients pelvic bone is used to fill the alveolar cleft and to form a bony bridge between both maxillary parts (C, *).

Despite a large variations of treatment protocols, there are two clinical shortcomings common to all treatment protocols: the need to postpone alveolar bone graft to late preteen age and the tendency of maxillary growth deficiency. Both implies major clinical drawbacks for the patient. First, leaving the alveolar cleft open to preteen age is associated with frequent adverse effects as: tongue interposition with speech problems, insufficient teeth alignment with biting problems, incomplete separation of oral and nasal cavity leading to food and speech emission through the nose. Second, the retruded maxilla cannot be surgically corrected until the growth has ceased and thus leaving the patient with a functional and psychosocial burden all over the teenage years. Given the countless surgical variations in use and the persistently high percentage of growth deficiencies, it is doubtful that surgery alone can ever provide the solution.

Tissue engineering might allow for new treatment plans, being less traumatic and leading to real tissue regeneration as in normal development. The most obvious way to use an osteogenic cell construct would be as an alternative to the alveolar bone graft. However, this alone has limited advantage for the patient, since the cancellous bone graft is very efficient with minimal donor site morbidity. Therefore a more promising way to use osteogenic cells would be the realization of an midpalatal growth zone, by recapitulating the suttural bone formation - similar to what has been achieved for endochondral bone formation.[19]

Clinical translation of cord stem cells to treat inborn craniofacial defects

Stem cells, having the ability to develop towards mature cells of different tissues, are the basic material to engineer tissues. The stem cells that are able to differentiate into mature cells as from fat, cartilage and bone are called mesenchymal stem cells (MSCs). MSCs are

found in numerous tissues, and among others in the umbilical cord. Since the cleft malformation is frequently known before birth, due to prenatal ultrasound, the umbilical cord could serve as an autologous MSCs donor site without any harvesting morbidity for the cleft patient (Fig. 11).

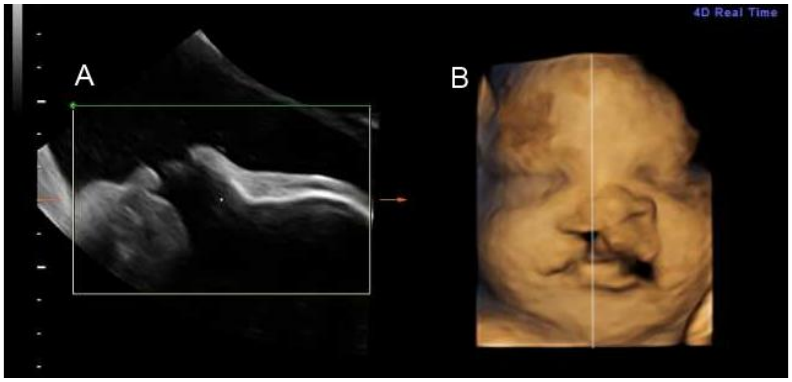

Fig. 11. Prenatal ultrasound at 37th week of pregnancy. Sagittal section (A) and three-dimensional surface image of the face showing a right sided complete cleft lip and palate (B).

This could offer the opportunity to use the patient's own umbilical cord to engineer bone tissue at the time, when it is needed for cleft treatment. A requirement for translation of a given tissue engineering protocol into clinical application is to dispense from potential infectious or immunogenic animal-derived products. In the ***fifths study*** we therefore studied umbilical cord MSCs for bone tissue engineering under conditions that would allow for clinical translation (Fig. 12).

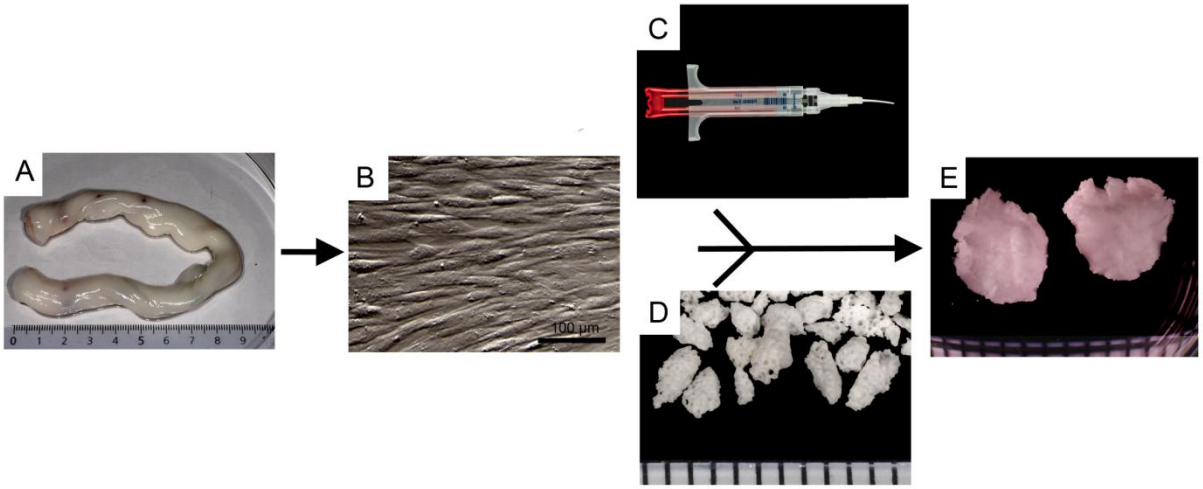

Fig. 12. From the umbilical cord to osteogenic constructs. From the Wharton's Jelly part of the umbilical cord (A) mesenchymal stromal cells (MSCs) are isolated (B). MSCs are combined with fibrin sealant (C) and synthetitc hydroxyapatite bone substitutes (D) to form osteogenic constructs (E) (Increments on the scale are millimeters).

Our protocol allowed for successful isolation and expansion of MSCs from the Wharton's jelly tissue of the umbilical cord. After 3 weeks of subcutaneous implantation into immunoincompetent mice the cells showed early stages of osteogenic differentiation but mature bone formation was absent. Mature bone formation could not be readily expected, since the implanted cells were naïve without in vitro predifferentiation towards osteoblasts

and the cells were implanted under the skin, where osteogenic stimulation is lacking. In a next step, the protocol will include the use of predifferentiated WJMSCs and implantation into a bone defect, which both should favor the bone forming capacity.

A bone tissue engineering approach could also be an interesting treatment option for malformations or defects of the cranial vault. In Craniosynostosis the bone plates of the cranial vault show a bony fusion and they lack the natural interposed growth zone (Fig. 13A,B). After birth the rapidly growing brain suffers therefore from being rigidly encased and recall surgical release of the cranial bone plates. In the course of growth after the surgical release broad gaps or osseus defect might form between the bone plates, demanding cranial bone substitution (Fig. 13C,D)

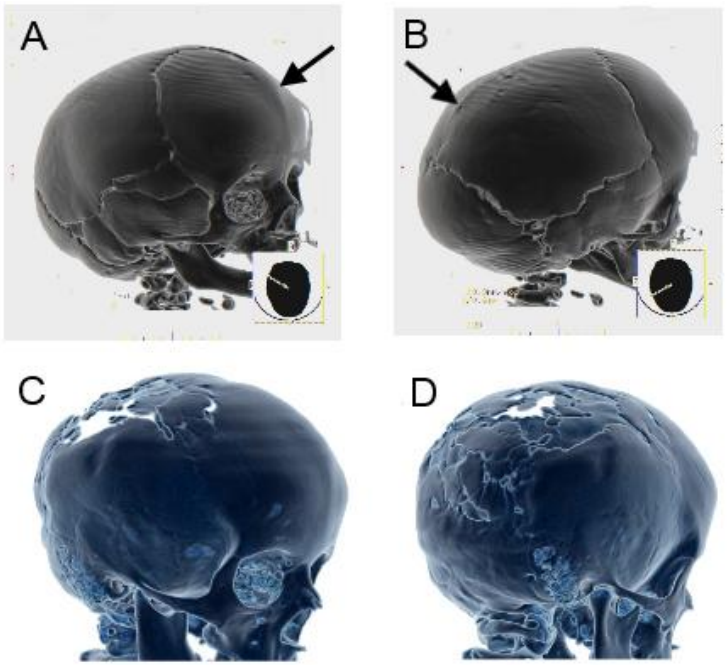

Fig. 13. Inborn fusion of the frontal (A, arrow) and parietal (B, arrow) bone plates with need for surgical release (A,B at 7 month of age). After release of the bony fusion extensive bony gaps in the cranial vault can persist (C,D at 8 years of age).

Large non healing cranial vault defects in the growing patient are difficult to treat. Synthetic bone substitutes lack osseus integration and become unfitting in the course of growth and have a risk of infection – while split bone grafts from healthy cranial vault parts necessitates a good bone thickness over a large cranial vault area, which is often lacking. A tissue engineering approach, that uses the patient's own mesenchymal stem cells for cranial vault regeneration could be advantageous for growing patients.

We have previously shown, as a proof of principle, that cranial defects that do not spontanaeusoly heal can be regenerated by use of guided bone regeneration - even in absence of osteogenic stem cells (Fig. 14).[20] For large non-healing cranial defects, the efficiency of this technique by adding osteogenic stem cells need to be explored. In the first two years of life the cerebral membrane has a natural ability to regenerate the cranial vault in humans. We reproduced this effect in the cranial defect model in rabbits.[21] Enhancing the bone forming capacity of the cerebral membrane, by use of stem cells, could be an alternative approach to treat non-healing cranial defects.

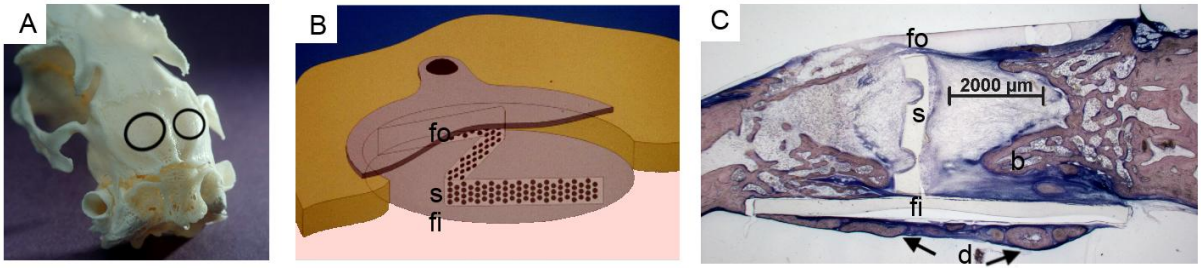

Fig. 14. Critical size cranial defect model. (A) Bilateral cranial defects of 8 mm diameter are made in rabbits since they do not spontaneously heal. (B) The principle of guided bone regeneration is applied using a polylactide foil on the inner (fi) and outer (fo) defect surface and a spacer in between (s). (C) After 8 weeks the bone edges (b) have grown towards the center of the defect and new bone forms within the dura (d, arrows). Mueller and Leiggener, AO Research Institute, Davos, Switzerland

Conclusion

We herein present biomedical engineering strategies to overcome particular surgical challenges to reconstruct lip-nose defects - both from traumatic and inborn origin. The used strategies comprised databank based facial planning, computer assisted manufacturing, microsurgery, microcirculation and stem cell based tissue engineering. Each strategy exhibited promising solutions to the addressed surgical challenges to reconstruct tissue shape, function, cell content, and nutrition. It seems that even greater benefit for surgical care can arise from complementary use of these techniques. It is therefore beneficial for surgeons to be familiar with a wide range of biomedical engineering strategies and to participate in the development of such strategies. This allows to closely align the biomedical methods to the intended surgical strategy.

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8. Acknowledgements

This work would not have been possible without tremendous support from many people. First of all, I wish to thank the patients that participated in the studies.

I would like to thank Professor Hans-Florian Zeilhofer, who encouraged me to enter into the PhD program, gave me a sabbatical from my clinical activity so that I could complete it and connected me with the scientific teams that I needed to realize the projects. I thank Professor Ivan Martin, whose critical feedback of my cellular experiments kept me on track, and whose prompt and kind replies to all my questions helped me to stay optimistic when experiments failed. Both of you gave me the crucial support I needed to seek the necessary project funding. My gratitude also goes to all members of the PhD board of the Department of Clinical Morphology and Biomedical Engineering, and in particular its head, Professor Bert Müller, who enabled me as a clinician the opportunity and the trust to run this PhD project.

I also thank Katja Schwenzer-Zimmerer, who has been teaching me about the practice and science of cleft surgery every day for almost 10 years. Discussing and interpreting the results with you always gave me new perspectives and insights into this field, and I continue to learn from you on a daily basis. Working with Dieter Schumann, whose question, “why do we not surgically reconstruct the lip arteries in clefts?” initiated a scientific journey that led us far away across the world to India, has being a thrilling adventure.

I also offer my thanks to Professor Magdalena Müller-Gerbl and the anatomical dissectors, Peter Zimmermann and Roger Kurz, who supported this work from a very early stage, at a time when the preliminary results were lacking and research questions were vague. Thank you for this great trust.

I have very much enjoyed learning from Professor Thomas Vetter and Pascal Paysan about the capability of facial computer graphics. Working with you both towards a facial prosthesis for tomorrow was a futuristic experience, and creative in the real sense of the word.

The year I spent with Gosla and Rajgopal Reddy in Hyderabad was the most intensive I have ever experienced. The new impressions and experiences are too numerous to summarize. Myself, my wife Regula and our son Oscar will treasure this period in our hearts. I would also like to thank Krishna and Kaliani Gupta for taking us into their family – your hospitality and warm kindness meant that we easily adapted to the Indian rhythm of life and its differences from our usual lives. It was so hard to leave you all.

I would like to acknowledge Professor Hermann-Franz Sailer, whose efforts result in thousands of babies with cleft palates benefitting from free surgery in India. The treatment of the study participants was only possible because of your generous funding. Your spontaneous and constructive input to the study outline and discussions were precious and highly motivating for me.

I am also grateful to Professor Colin McGuckin and Nico Forraz, whose team introduced me to the world of basic research. You allowed me to seek the necessary project funding and generously supported our common work. I admire your perseverance in performing basic research.

Finally, I thank Regula. Your love, optimism and pragmatism provide the grounding allowing me to pursue this work. You empower me and make my life worthwhile.

9. List of publications on PhD topic

Journal publications

Mueller AA, Forraz N, Gueven S, Atzeni G, Degoul O, Pagnon-Minot A, Pesquet A, Hartmann D, Martin I, Scherberich A, McGuckin C.
Serum-free culture of Wharton's-jelly-derived mesenchymal stromal cells and their potential for osteoblastic differentiation for the treatment of cleft lip and palate. Submitted and under review

Mueller AA, Schumann D, Reddy RR, Schwenzer-Zimmerer K, Mueller-Gerbl M, Zeilhofer HF, Sailer H, Reddy GS.
Intraoperative vascular anatomy, arterial blood flow velocity and microcircultion in uni- and bilateral cleft lip repair. Plast and Reconstr Surg. 2012;130 (5):1120-30.

Mueller AA, Zschokke I, Brand S, Hockenjos C, Zeilhofer HF, Schwenzer-Zimmerer.
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Mücke T*, Mueller AA*, Kansy K, Hallermann W, Kerkmann H, Schuck N, Zeilhofer HF, Hoffmann J, Hölzle F; DÖSAK collaborative group for Microsurgical Reconstruction.
Microsurgical reconstruction of the head and neck--current practice of maxillofacial units in Germany, Austria, and Switzerland. J Craniomaxillofac Surg. 2011 Sep;39(6):449-52. *equal contributions.

Mueller AA, Paysan P, Schumacher R, Zeilhofer HF, Berg-Boerner BI, Maurer J, Vetter T, Schkommodau E, Juergens P, Schwenzer-Zimmerer K.
Missing facial parts computed by a morphable model and transferred directly to a polyamide laser-sintered prosthesis: an innovation study. Br J Oral Maxillofac Surg. 2011;49(8):e67-71.

Proceedings

Brand S, Müller AA. 2012. Quality of life among children, adolescents and adults with orofacial clefts. In: JH Stone, M Blouin, editors. International Encyclopedia of Rehabilitation. Available online: http://cirrie.buffalo.edu/encyclopedia/en/article/294/

Oral presentations

24.05.2013	63. Annual Congress of the German Society of Oral- and Maxillofacial Surgery, Essen, Germany „Arterial intraoperative vascular anatomy and microcirculation in unilateral and bilateral cleft lips
13.04.2013	6. Symposium of the Austrian Cleft Palate Craniofacial Association, Salzburg “Strategy and Timing of upper airway obstruction in Pierre Robin sequence”
15.03.2013	Research day 2013 of the Department of Surgery, University hospital Basel “Serum-free culture of Wharton’s jelly mesenchymal stromal cells and their subsequent potential for osteoblastic differentiation to treat cleft lip and palate bone defects
14.09.2012	21. Congress of the European Association for Cranio-Maxillo-Facial Surgery EACMFS, Dubrovnik (accepted) “Characterization and osteogenic capacity of Wharton-Jelly mesenchymal stromal / stem cells”
15.06.2012	8. International Bernd Spiessl Symposium: Face and Identity – Light and Body Interaction in Arts and Medicine, Basel “Achieving normal and symmetrical microcirculation in unilateral cleft lip repair” The osteogenic capacity of umbilical cord mesenchymal stem cells.
01.06.2012	62. Annual Congress of the German Society of Oral- and Maxillofacial Surgery, Freiburg, Deutschland “6 to 18-years results of growth, speech and subsidiary operations after all-in-one cleft repair compared to data from the Eurocleft study”
30.3.2012	Research day 2012 of the Department of Surgery, University hospital Basel “One-stage cleft repair outcome at age 6- to 18 years compared to multi-stage cleft repair outcome in the Eurocleft study”
17.09.2011	9. European Craniofacial Congress, Salzburg “The 6 to 18-years results after one-stage closure of unilateral cleft lip and palate”
17.06.2011	7. International Bernd Spiessl Symposium: Face and Identity – 3d Navigation and Planning, Basel “Achieving normal and symmetrical microcirculation in unilateral cleft lip repair”
08.04.2011	Research day 2012 of the Department of Surgery and Anaesthesiology, University hospital Basel „Achieving normal and symmetrical microcirculation in unilateral cleft lip repair”
16.09.2010	20. Congress of the European Association for Cranio-Maxillo-Facial Surgery EACMFS, Bruges

	“Microcirculation of the naso-labial part of the facial artery angiosome in unilateral clefts compared to normal”
19.06.2010	6. International Bernd Spiessl Symposium: Face and Identity – Spirit of Innovation “Microcirculatory parameters in the soft tissue of cleft lips compared to normal”

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10.5.2007	Voluntary academic society Basel (FAG), research grant
15.06.2007	Swiss Society for Oral Medicine (SSO), research grant
2001-2003	Margarete and Walter Lichtenstein-Foundation, Basel
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